Investigation into signalling mechanisms of BDNF in neurological disorders

A THESIS SUBMITTED IN TOTAL FULFILMENT OF THE REQUIREMENTS OF DOCTOR OF PHILOSOPHY

Yoon Lim

MSc in Biochemistry

BSc in Genetic Engineering



University of South Australia

Neuroregeneration Laboratory

School of Pharmacy and Medical Sciences

Division of Health Sciences

University of South Australia

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ABBREVIATIONS

aa	amino acid
18M	18 months follow-up
AchEI	acetylcholinesterase inhibitor
AD	Alzheimer's disease
AHI1	Abelson helper integration site 1
AIBL	Australian Imaging, Biomarker and Lifestyle
aMCI	amnestic mild cognitive impairment
ANOVA	Analysis of Variance
APP	amyloid precursor protein
AP-2	adaptor protein complex 2
AR	androgen receptor
Aβ	amyloid beta
BACE1	β-Site amyloid precursor protein cleaving enzyme 1
BBB	blood-brain barrier
BCA	bicinchoninic acid
BDNF	brain derived nerve growth factor
BDNF-B	BDNF-biotin
BL	baseline
BPD	bipolar disease
BrdU	bromodeoxyuridine
BSA	Bovine serum albumin
CaMKII	calcium-calmodulin dependent kinase
CCP	clathrin coated pits
CCV	clathrin-coated vesicle

cDNA	complimentary deoxyribonucleicacid
CEI	cholinesterase inhibitors
CFP	cyan fluorescent protein
CHO	chinese hamster ovary
CLC	clathrin light chain
CME	clathrin-medicated endocytosis
CNBr	cyanogen bromide
CNS	central nervous system
Co-IP	co-immunoprecipitation
CREB	cAMP-calcium response element binding protein
CSF	cerebrospinal fluid
CSIRO	Commonwealth Scientific Industrial and Research Organisation
CV	coefficient of variation
DAG	diacyl-glycerol
DF	dilution factor
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleicacid
DRG	dorsal root ganglion
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EEA1	Early Endosomal Autoantigen 1
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
exo-BDNF	exogenous BDNF
FBS	fetal bovine serum

FRET	Fluorescence resonance energy transfer
Gab1	Grb-associated binder 1
$GABA_AR$	γ-amino butyric acid type A receptor
GFP	green fluorescent protein
Grb2	growth factor receptor-bound protein 2
GSK-3β	glycogen synthase kinase 3 beta
GST	glutathione S-transferase
HA	Hemagglutinin
HAP1	Huntingtin-associated protein 1
HC	healthy controls
HD	Huntington's disease
HEK293T	Human embryonic kidney 293T cells
HET	heterozygote
HIP1	huntingtin interacting protein 1
HRP	horseradish peroxidase
HRSD-21	the21-itemHamiltonRating Scale forDepression
HSD	honestly significant differences
htt	Huntingtin
mHuntingtin	mutant huntingtin
ICD-10	InternationalStatisticalClassification ofDiseasesand RelatedHealthProblems10thRevision
$InsP_3R1$	inositol 1,4,5-trisphosphate receptor type 1
IP3	inositol trisphosphate
IRS1/2	insulin receptor substrates 1/2

JNK	c-Jun N-terminal kinase
KLC	kinesin light chain
KO	knock out
LOD	limit of detection
LTD	long-term depression
LTP	long term potentiation
mBDNF	mature brain-derived neurotrophic factor
MCI	mild cognition impairment
MDD	major depressive disorder
MEK	MAP/ERK kinase
Met	methionine
MMP-3	metalloproteinases-3
MMP-7	matrix metalloproteinase 7
MMPs	matrix metalloproteinases
MMSE	Mini–mental state examination
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MVB	multivesicular bodies
NADE	$p75^{NTR}$-associated cell death executor
NGF	nerve growth factor
NRAGE	neurotrophin receptor-interacting MAGE homologue
NRIF	neurotrophin receptor-interacting factor
NSC	neural stem cell
NT3 (NT-3)	neurotrophin 3
NT4 (NT-4)	neurotrophin 4
OD	optical density

$p75^{NTR}$	p75 neurotrophin receptor
PBS	phosphate buffered saline
PBS-CM	Phosphate-buffered saline with calcium chloride and magnesium chloride
PBST	phosphate buffered saline Tween-20
PC5/6-B	proprotein convertase 5/6-B
PCR	polymerase chain reaction
PD	Parkinson's disease
PET	positron-emission tomography
PiB	Pittsburgh compound B
PiB-PET	positron emission tomography with $^{[11]}$C-Pittsburgh compound B
PI3K	phosphoinositide3-kinase
PLCγ-1	phospholipase-C-γ-1
PNS	peripheral nervous system
preBDNF	prodomain of proBDNF
proBDNF	precursor of BDNF
PC1/3	proprotein convertases 1/3
PtdIns(4,5)P2	phosphatidylinositol 4,5-bisphosphate
Rab5	Ras-related protein 5
Raf	Ras associated factor
RFP	red fluorescent protein
RIPA	radioimmunoprecipitation assay
RPM	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation

SEM	standard error of the mean
Shc	src homology domain containing
SNP	single nucleotide polymorphism
SOS	son of sevenless
SPSS	Statistical Package for Social Sciences
SUVR	standardized uptake value ratio
TBP	TATA-binding protein
TBS	Tris-buffered saline
TMB	tetramethylbenzidine
tPA	tissue plasminogen activator
TrkA	tyrosine receptor kinase A
TrkB	tyrosine receptor kinase B
TrkB-FL	full length TrkB
TrkB-T1	truncated isoform of TrkB
TrkC	tyrosine receptor kinase C
Val	valine
Vs	versus
YFP	yellow fluorescent protein
WT	wild type

THESIS SUMMARY

While mature brain-derived neurotrophic factor (mBDNF) plays critical roles in neuronal development and survival, proBDNF, its precursor protein, has opposite functions to BDNF such as neuronal apoptosis and long-term depression. BDNF endocytosis through TrkB is essential for neuronal activity such as survival and plasticity. In addition, a number of studies have reported that BDNF levels are altered in many neurodegenerative and psychiatric disorders. In this thesis, we intended to decipher the huntingtin associated protein 1 (HAP1)'s role in BDNF and its receptor endocytosis and its down-stream signalling pathways in neurons. We also aimed to develop mBDNF - specific ELISA and investigate the levels of mBDNF, proBDNF and their receptors in major depression disorder (MDD) and analyse how mBDNF serum levels are correlated with cognitive function in Alzheimer's disorder (AD).

We have found that HAP1 interacts with BDNF and its receptors, $p75^{NTR}$ and TrkB, and highly co-localises with endocytosed BDNF. The internalisation of BDNF and TrkB and TrkB down-stream signalling pathways are abolished in cultured cortical neurons of HAP1 KO mice. Furthermore, *in vivo* and *in vitro* study showed the proliferation of cerebellar granule cell was impaired in HAP1 KO mice.

We have developed a new mBDNF sandwich ELISA which showed no cross-reactivity with other growth factors (NT-3, NT-4, and NGF) and negligible cross-reactivity for proBDNF. The assay was validated through the measurement of mBDNF levels in the brain of $BACE1^{-/-}$ and WT mice and compared to Western blot results. In addition, we have also demonstrated that the mBDNF/proBDNF ratio varies in different rat brain regions.

We have found that the blood proBDNF, sortilin and $p75^{NTR}$ levels are significantly higher whereas the levels of mBDNF and TrkB are significantly lower in MDD compared to healthy subjects. Likewise, mRNA levels of sortilin and $p75^{NTR}$ were significantly higher while mRNA levels of BDNF and TrkB were significantly lower in MDD patients than healthy subjects. We have found that the levels of proBDNF/sortilin/$p75^{NTR}$ are positively correlated with the severity of the MDD while mBDNF/TrkB levels are negatively correlated with the severity of the MDD. We have also shown that there is a negative correlation between serum mBDNF and proBDNF levels in MDD patients.

In a separate study we investigated the levels of serum mBDNF levels using patient samples from AIBL cohort and correlated our findings with the patients' cognitive function. We have found that serum mBDNF levels are correlated with cognitive decline, however there was no significant mBDNF serum level difference among healthyHC, MCI and AD at BL and 18M. We also did not find a correlation between serum mBDNF and amyloid burden. Moreover, we found there was a technical error during the assay with Melbourne site samples.

Taken together, we have presented that HAP1 is a critical molecule in BDNF and its receptor endocytosis and down-stream signalling pathways in neurons. We have also developed a mBDNF-specific ELISA and demonstrated that the levels of mBDNF/TrkB and proBDNF/$p75^{NTR}$/sortilin are correlated with the severity of MDD and serum mBDNF levels are correlated with cognitive function in the AIBL cohort.

DECLARATION

I declare that:

a. this thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university;

b. to the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, is clearly acknowledged;

c. the author acknowledges that copyright of published works contained within this thesis (as listed below) resides with the copyright holders of those works.

Lim, Y., Zhong, JH., Zhou, XF (2015). "Development of mature BDNF-specific sandwich ELISA." J Neurochem 134(1): 75-85.

Zhou, L., J. Xiong, **Lim, Y**., Ruan, Y., Huang, C., Zhu, Y., Zhong, J., Xiao, Z., Zhou XF (2013). "Upregulation of blood proBDNF and its receptors in major depression." J Affect Disord **150**(3): 776-784. **First equal co—author**

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Chapter 1: Introduction

1.1 Brain-derived neurotrophic factor (BDNF)

1.1.1 What are neurotrophins?

Neurotrophins are a group of proteins which have similar structure and functions (Leibrock, Lottspeich et al. 1989, Hallbook 1999) and play important roles in the development, survival, differentiation, synaptogenesis and synaptic plasticity in the central nervous system (CNS) and peripheral nervous system (PNS) (Levi-Montalcini 1987, Lewin and Barde 1996, Huang and Reichardt 2001). Four mammalian neurotrophins, brain-derived neurotrophic factor (BDNF) (Leibrock, Lottspeich et al. 1989), nerve growth factor (NGF) (Levi-Montalcini 1987), neurotrophin 3 (NT3) (Ernfors, Ibanez et al. 1990, Maisonpierre, Belluscio et al. 1990) and neurotrophin 4 (NT4) (Berkemeier, Winslow et al. 1991, Hallbook, Ibanez et al. 1991), have been identified in mammals. The first neurotrophin was isolated in 1954 by Cohen et al. (1954) from chick embryos and was named the nerve growth factor (NGF) as it boosted growth and survival of sensory and sympathetic neurons in ganglia of chick embryos during development (Cohen, Levi-Montalcini et al. 1954). About 30 years later, a ground-breaking discovery was made by Barde et al. (1982) who identified the second neurotrophin isolated from the pig brain which was named the brain-derived neurotrophic factor (BDNF) and was found to be present at very low levels (2 µg BDNF was purified from 3 kg of brain). This study reported that BDNF promoted survival of cultured sensory neurons from chick embryos similar to NGF (Barde, Edgar et al. 1982). Later, NT3 and NT4 neurotrophins were identified by molecular cloning techniques in early 1990's (Lewin and Barde 1996).

Neurotrophins are essential for the development of the peripheral nervous system and functional maintenance of the brain. Neurotrophins share a similar molecular structure with a high homology in amino acid sequence (Gotz, Raulf et al. 1992). They bind to two functionally distinctive transmembrane receptor classes, the p75 neurotrophin receptor ($p75^{NTR}$) and tyrosine - kinase (Trk) receptors including TrkA, TrkB and TrkC (Dechant and

Barde 2002, Huang and Reichardt 2003). NGF and NT3 bind to TrkA and TrkC respectively while BDNF and NT4 bind to TrkB (Dechant and Barde 2002, Huang and Reichardt 2003). Neurotrophins bind to their cognate Trk receptors, which activate subsequent signalling cascades. Trk receptors have a kinase domain which is activated on dimerization, which then phosphorylates other tyrosine residues in the cytoplasmic domain of the receptor. This results in signalling cascades including small G proteins such as Ras, Rap-1 and Cdc42-Rac-Rho family and down-stream signalling pathways including phospholipase-C-γ (PLCγ), phosphoinositide3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) pathways (Huang and Reichardt 2003). Besides Trks, all neurotrophins bind to a pan receptor, $p75^{NTR}$ (Chao and Hempstead 1995). It is well established that $p75^{NTR}$ and Trk receptors have relatively equal affinity to neurotrophins, but there is a higher binding affinity when $p75^{NTR}$ combines with Trk receptors (Esposito, Patel et al. 2001).

1.1.2 Introduction to BDNF: function, synthesis and secretion

BDNF is a key molecule that plays a role in many neuronal functions including neuronal survival, differentiation and proliferation in the CNS and PNS (Davies 1994).

Thus, it has been demonstrated that BDNF facilitates cellular survival, growth and proliferation in the various types of cells including embryonic neocortical neurons (Cheng, Wang et al. 2003), hippocampal progenitor cells (Vicario-Abejon, Johe et al. 1995, Choi, Li et al. 2009), cultured retinal ganglion cells (Meyer-Franke, Kaplan et al. 1995) and fibroblasts (Glass, Nye et al. 1991). BDNF also promotes maturation of hippocampal neuronal precursors (Vicario-Abejon, Johe et al. 1995). In addition, BDNF contributes to the CNS development by facilitating synapse formation (McAllister, Lo et al. 1995, Park and Poo 2013) through axon branching (Cabelli, Hohn et al. 1995, Cohen-Cory and Fraser 1995). BDNF is also an important molecule for learning and memory; it does so through

facilitation of long term potentiation (LTP) (Chao 2003). As BDNF is the main focus of this work, the BDNF synthesis, secretion and BDNF receptor signalling pathways will be further discussed in order to elucidate how BDNF transmits its neurotrophic signals and performs its various neuronal functions.

Similar to other secreted proteins including neurotrophins, BDNF is originally generated in the endoplasmic reticulum as a precursor protein, preproBDNF. Then, it is converted to proBDNF by removal of the signal peptide followed by transport to the Golgi apparatus to undergo one of two different secretion pathways, the constitutive or regulated secretion pathways (Fig. 1-1). In the constitutive secretion, BDNF is stored in small diameter vesicles/granules (50 – 100 nm) and secreted by fusion with plasma membrane in a Ca^{2+}-independent manner without any stimulation. In the regulated secretion BDNF is stored in larger diameter vesicles/granules (300 nm) and secreted by fusion with plasma membrane in a Ca^{2+} - dependent manner (Mowla, Pareek et al. 1999, Lessmann, Gottmann et al. 2003) in response to a stimulus. In addition, soma and proximal processes are usual sites for constitutive secretion of BDNF, while distal processes are the normal places for the regulated secretion in neurons (Brigadski, Hartmann et al. 2005).

ProBDNF is processed to mature BDNF (mBDNF) intracellularly or extracellularly although it is controversial where this processing occurs exactly. Matsumoto et al. (2008) used BDNF-myc knock-in mice to analyse location of proBDNF and mature BDNF *in vivo* and *in vitro*. The results demonstrated that proBDNF is speedily processed to BDNF intracellularly rather than extracellularly (Matsumoto, Rauskolb et al. 2008). On the other hand, Yang et al. (2009) used BDNF-HA knock-in mice and showed that significant amount of proBDNF is found within the newborn mice neurons and in the hippocampal neuron culture medium supernatant. These authors also showed that proBDNF is present in the postnatal mice brain (Yang, Siao et al. 2009) suggesting that proBDNF is present not only

during development but also during adulthood. Dieini et al.(2012) showed that the prodomain peptides and mature BDNF are colocalized in secretory granules in presynaptic axon terminals using immunohistochemistry and electron microscopy with specific antibodies against prodomain of proBDNF and mature BDNF (Dieni, Matsumoto et al. 2012), proposing that the secretory granules may be the site where the conversion of proBDNF to mature BDNF takes place. Intracellular processing of proBDNF is performed by subtilisin/kexin-like convertase family including furin, proprotein convertases 1,2, 4, 5,7 and furin/paired basic amino-acid-cleaving enzyme 4 (Seidah and Chretien 1999, Mowla, Farhadi et al. 2001). Extracellular processing of proBDNF is conducted by plasmin and matrix metalloproteases which convert proBDNF to mature BDNF (mBDNF) (Pang, Teng et al. 2004, Nagappan, Zaitsev et al. 2009). Processing of proBDNF to mBDNF is essential for many neuronal functions, including long term plasticity as, for example, shown in mice hippocampal slices (Pang, Teng et al. 2004).

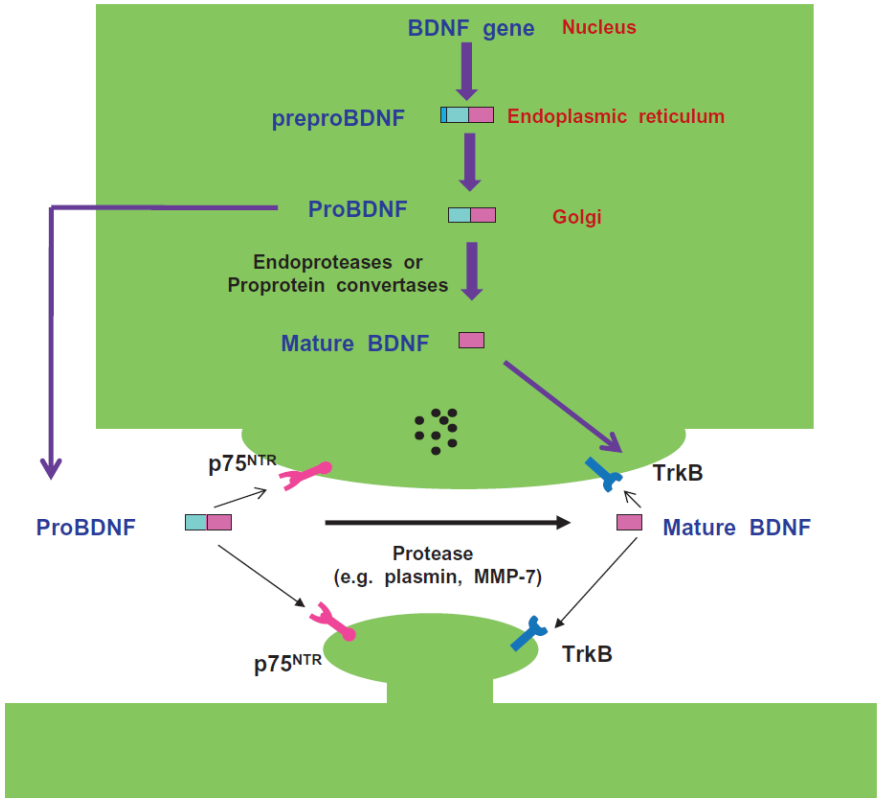


Figure 1-1 Synthesis of mature BDNF from proBDNF (Hashimoto 2010).
MMP-7: matrix metalloproteinase 7; $p75^{NTR}$: p75 neurotrophin receptor; TrkB: tyrosine-receptor kinase B; BNDF: brain-derived neurotrophic factor.

1.1.2.1 BDNF single nucleotide polymorphism (SNP, V66M) affects intracellular proBDNF trafficking and secretion

A single nucleotide polymorphism (SNP) in the gene which encodes BDNF leads to substitution from a valine (Val) to methionine (Met) at codon 66 and it is frequent in humans (Proschel, Saunders et al. 1992, Gratacos, Gonzalez et al. 2007, Pivac, Kim et al. 2009).

Egan et al. (2003) reported for the first time that the BDNF Val66Met polymorphism affects regulated secretion of BDNF (Egan, Kojima et al. 2003). Firstly, in cultured hippocampal neurons, BDNFval/val and BDNFmet/met showed distinct cellular distribution. BDNFval/val was mostly found in dendrites, however BDNFmet/met was dominantly

distributed in cell bodies. Secondly, under regulated secretion environment, secreted BDNFval/val level was much higher than BDNFmet/met. In contrast, in constitutive secretion test in neurons, both BDNFval/val and BDNFmet/met showed similar results, suggesting that the Val66Met polymorphism affects regulated secretion, but not the constitutive one. Thirdly, BDNFval/val and BDNFmet/met showed different subcellular distribution in hippocampal neurons. Some of BDNFval/val and BDNFmet/met were co-localized with each other and TGN38 Golgi marker protein. However, BDNFmet/met did not co-localize with the secretogranin II, the secretory vesicle marker. This suggests that BDNFmet/met is not targeted from Golgi to the secretory granules. This study also showed that the SNP causes functional and cognitive deficits in humans. In 2004, Chen et al. (2004) reported intracellular trafficking and regulated secretion of BDNF are damaged in wild type neurons and neurosecretory cells by BDNFmet/met, but not in smooth muscle and endothelial cells. This implies that the Val66Met polymorphism affects CNS specifically. Furthermore, co-expression of BDNFmet/met changed intracellular trafficking and regulated secretion of wild type BDNF in PC12 cells (Chen, Patel et al. 2004). In 2006, Chen et al. generated variant BDNFmet knock-in mice (Chen, Jing et al. 2006) and showed that the secreted endogenous BDNF level in the regulated secretion pathway was remarkably decreased in cultured embryonic hippocampal-cortical neurons obtained from the BDNFmet/met mice compared to wild type mice. Dysregulated BDNF secretion in BDNFmet/met neurons is an important finding as most of the released BDNF occurs via the regulated secretion in neurons. A number of clinical studies have been performed to find the linkages between the BDNF Val66Met polymorphism and neurological disorders, especially psychiatric disorders involving anxiety disorder, depression disorder, schizophrenia, bipolar disease although there have been controversial results generated from different studies(Hashimoto and Lewis 2006, Enoch, White et al. 2008, Louhivuori, Arvio et al. 2009, Montag, Basten et al. 2010, Zhou, Yan et al. 2010). In case of depression disorder,

some studies have reported no associations between the BDNF Val66Met polymorphism and depression disorder (Figueira, Malloy-Diniz et al. 2010, Yoshimura, Kishi et al. 2011, Zhou, Lu et al. 2011), whereas other studies have demonstrated that the BDNF Val66Met polymorphism is linked with depression (Ozan, Okur et al. 2010, Zou, Ye et al. 2010, Suchanek, Owczarek et al. 2011), suggesting that more studies are required to decipher the correlation between BDNF Val66Met polymorphism and depression. Taken together, it is suggested that BDNF Val66Met polymorphism could be critical for correct BDNF secretion so that brain functions can be maintained and can be also associated with various neurological disorders

1.1.2.2 BDNF signalling

As mentioned earlier, once BDNF binds TrkB, TrkB is activated by dimerization and phosphorylation. TrkB signalling activates three major pathways including extracellular signal-regulated kinase (ERK), PI-3 kinase (PI3K) and phospholipase-C-γ (PLCγ) pathways (Huang and Reichardt 2003) (Fig. 1-2). These three signalling pathways support neuronal survival and differentiation (Huang and Reichardt 2001, Patapoutian and Reichardt 2001) and will be discussed further in more detail.

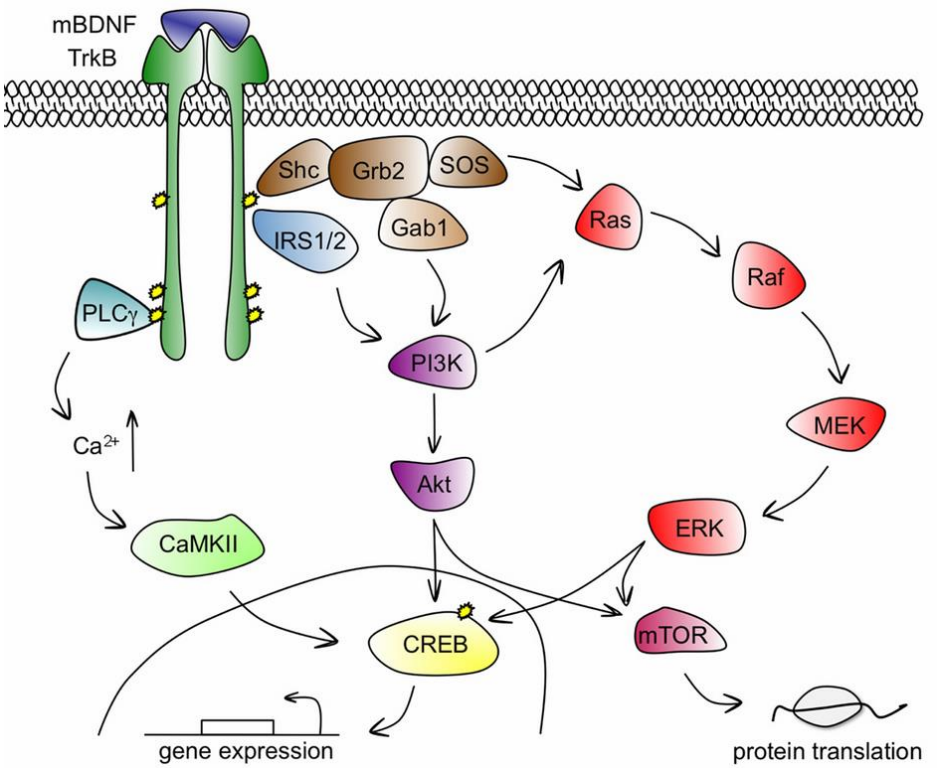


Figure 1-2 TrkB signalling pathway (Cunha, Brambilla et al. 2010).
Binding of BDNF to TrkB with high affinity leads to dimerization and autophosphorylation of TrkB. Phosphorylated tyrosine residues act as binding sites for various molecules which stimulate three major signalling pathways, PLCγ, PI3K and ERK cascades. Finally, CREB, a transcription factor, is activated by phosphorylation, followed by gene expressions which are critical for the differentiation and survival of neurons. ERK: extracellular signal-regulated kinase; CaMKII: calcium-calmodulin dependent kinase; PI3K: phosphatidylinositol 3-kinase; PLCγ: phospholipase Cγ; CREB: cAMP-calcium response element binding protein; mTOR: mammalian target of rapamycin; SOS: son of sevenless; Raf: Ras associated factor; Shc: src homology domain containing; Ras: GTP binding protein; Grb2: growth factor receptor-bound protein 2; Gab1: Grb-associated binder 1; IRS1/2: insulin receptor substrates 1/2; MEK: MAP/ERK kinase.

1.1.2.2.1 The TrkB- PI3K-Akt and TrkB-Ras-ERK signalling pathways

The PI3K-Akt and Ras-ERK-CREB (cAMP-response element binding) pathways are most important amongst many other intracellular signal pathways related to neuronal survival. It has been demonstrated that ERK stimulation is linked to BDNF-dependent neuronal survival (Hetman, Kanning et al. 1999). Almeida et al. (2005) showed that BDNF has neuroprotective function through the PI3K and Ras/ERK signalling pathways against the induced-cell death by glutamate in cultured hippocampal neurons (Almeida, Manadas et al.

2005). In this study, the protective effect of BDNF against neuronal death induced by glutamate was dramatically reduced by PI3K inhibition, demonstrating that the PI3K signalling pathway is critical for BDNF neuronal protective function in hippocampal neurons. The increases in BDNF expression and phosphorylated Trk by norepinephrine application to embryonic hippocampal neurons are prevented through inhibition of ERK and PI3K (Chen, Nguyen et al. 2007). This result suggests that BDNF expression induced by norepinephrine comes after a positive feedback loop as norepinephrine up-regulates CREB phosphorylation and ERK and the phosphorylation of CREB is down-regulated by PI3K and ERK inhibitors and these inhibitors also repress TrkB and CREB phosphorylation, respectively. A number of studies have found that the activation of PI3K pathway is linked to the cell survival (Kaplan and Miller 2000, Yuan and Yankner 2000, Patapoutian and Reichardt 2001, Weinreb, Amit et al. 2006, Zhang, Liu et al. 2011). Akt is a serine/threonine kinase and a downstream intermediator of PI3 kinase stimulation (Franke, Kaplan et al. 1997). PI3K/Akt signalling pathway is important for neuronal survival in CNS (Brunet, Datta et al. 2001). Endocytosis of Trk receptors is also important for neurotrophin- induced signalling pathway (Grimes, Zhou et al. 1996). Importantly, Zheng et al. (2008) showed that the activation of Akt but not ERK by BDNF is prevented in cultured CNS neurons by blocking of clathrin, dynamin and adaptor protein complex 2 (AP-2) (Zheng, Shen et al. 2008). The authors also showed that BDNF-mediated neuronal survival and dendrite growth requires clathrin-dependent endocytosis by using various inhibitors of Akt, clathrin and dynamin. These findings suggest that the clathrin and dynamin – dependent endocytosis is required for the response to BDNF stimulation. In summary, signalling, it is suggested that TrkB- PI3K-Akt and TrkB-Ras-ERK signalling pathways are critically linked to neuronal survival.

1.1.2.2.2 The TrkB-PLCγ-CaMKII pathway

Trk receptors are able to trigger the PLCγ pathway (Clapham 2003, Jia, Zhou et al. 2007). It is widely accepted that PLCγ activation elicited by BDNF leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) producing inositol trisphosphate (IP3) and diacyl-glycerol (DAG). IP3 is needed for intracellular Ca^{2+} channel stimulation (Huang and Reichardt 2003) and increased intracellular Ca^{2+}, as a result, up-regulates BDNF gene expression through calcium response elements (Tao, Finkbeiner et al. 1998, Shieh and Ghosh 1999, Hartmann, Heumann et al. 2001, Balkowiec and Katz 2002), suggesting that this pathway can contribute to neuronal functions.

1.1.3 proBDNF function and signalling

Since it was firstly reported that proNGF induced cell apoptosis through $p75^{NTR}$ *in vitro*, it has been shown that proNGF plays a role in developmental cell death and the progress of disease like Alzheimer's disease (Hempstead 2009).

Similar to NGF, the pro-form of BDNF (proBDNF) was initially regarded as a just precursor protein which did not have any biological function. Nonetheless, several studies revealed that proBDNF and proNGF are able to be secreted from neurons (Lee, Kermani et al. 2001, Pang, Teng et al. 2004, Woo, Teng et al. 2005, Bruno and Cuello 2006, Yang, Siao et al. 2009). However, it has been controversial in regards to the intracellular processing efficiency and its release into extracellular space (Matsumoto, Rauskolb et al. 2008, Yang, Siao et al. 2009). While neuronal apoptosis on injury is stimulated by proNGF, a major role of proBDNF could be to regulate the efficacy of synapse function during brain development (Woo, Teng et al. 2005, Yang, Siao et al. 2009) through long term depression which was studied in hippocampal slices from WT and $p75^{NTR}$ KO mice. Tissue plasminogen activator /plasmin protease cascade converts proBDNF to mature BDNF extracellularly and this conversion triggers late phase long-term potentiation which is TrkB-dependent (Pang, Teng

et al. 2004). In contrast, proBDNF application to $p75^{NTR}$ –expressing hippocampal slices stimulated long-term depression and these results indicate that proBDNF can regulate long-term depression as an endogenous ligand (Woo, Teng et al. 2005). These findings suggest that proBDNF and mature BDNF have opposite effects on synapse via activation of two different receptors, $p75^{NTR}$ and TrkB respectively. It has been shown that proBDNF increases apoptosis in superior cervical ganglion neurons and sympathetic neurons *in vitro* (Teng, Teng et al. 2005, Kenchappa, Zampieri et al. 2006). Koshimizu et al. (2009) also demonstrated that cleavage-resistant proBDNF increases apoptosis in cerebellar granule neurons in a low potassium-containing medium and inhibits neurite outgrowth of basal forebrain cholinergic neurons in *vitro* (Koshimizu, Kiyosue et al. 2009). Similarly, we have recently reported that proBDNF causes a collapse of neurite outgrowth by $p75^{NTR}$ mediated RhoA activation (Sun, Lim et al. 2012) (Fig. 1-3). These findings from our group and other groups suggest that proBDNF has an opposing role to mature BDNF (Sun, Lim et al. 2012) with respect to the neuronal survival and outgrowth.

The $p75^{NTR}$ is the nerve growth factor receptor identified for the first time in 1986 (Johnson, Lanahan et al. 1986, Radeke, Misko et al. 1987). Although it has been known that $p75^{NTR}$ is able to play a role of a co-receptor for various ligands which show different biological activities, its signalling mechanisms and biological functions remain unclear (Dechant and Barde 2002, Hempstead 2002, Roux and Barker 2002, Barker 2004, Gentry, Barker et al. 2004, Teng and Hempstead 2004).

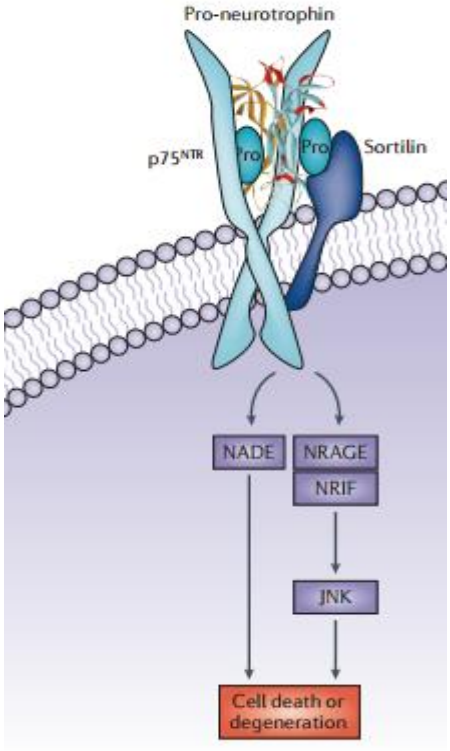


Figure 1-3 p75NTR signalling pathway (Longo and Massa 2013).
Proneurotrophin binds $p75^{NTR}$-Sortilin complex and activates cell-death pathways. NADE, p75NTR-associated cell death executor; NRAGE, neurotrophin receptor-interacting MAGE homologue; NRIF, neurotrophin receptor-interacting factor; JNK, c-Jun N-terminal kinase

Lee et al. (2001) showed that proneurotrophins such as proNGF and proBDNF induce neuronal apoptosis by means of binding to $p75^{NTR}$ while mature neurotrophins boost neuronal survival through triggering Trk receptors (Lee, Kermani et al. 2001). Several studies have revealed that proneurotrophins lead to cell death in different cell types including smooth muscle cells (Lee, Kermani et al. 2001) and retinal microglia (Srinivasan, Roque et al. 2004). After spinal cord injury, oligodendrocytes undergo apoptosis in wild type mice. However, in $p75^{NTR}$ KO mice, the number of oligodendrocytes which undergo apoptosis is reduced while the survival of oligodendrocytes increases (Beattie, Harrington et al. 2002). Nykjaer et al. (2004) showed that proNGF forms a complex with $p75^{NTR}$ and sortilin and it was the first study demonstrating that not only the $p75^{NTR}$ but also sortilin are required for apoptosis (Nykjaer, Lee et al. 2004). Another study demonstrated that proBDNF induces

apoptosis in cultured sympathetic neurons which express $p75^{NTR}$ and sortilin (a co-receptor of $p75^{NTR}$) while mature BDNF elicits TrkB phosphorylation, which promotes cell survival. The apoptosis is decreased in $p75^{NTR}$KO mice or by the antagonist of sortilin in dissected superior cervical ganglion neurons, suggesting that the sortilin/$p75^{NTR}$ complex is required for the cellular apoptosis (Teng, Teng et al. 2005).

Recently, we have demonstrated that the neurite outgrowth is collapsed in cultured dorsal root ganglion and cortical neurons by proBDNF via the $p75^{NTR}$-RhoA signalling pathway (Sun, Lim et al. 2012). The neurite outgrowth is not affected by proBDNF in $p75^{NTR}$KO mice and is stimulated by anti-proBDNF antibody treatment in WT mice, suggesting that the apoptotic signal by proBDNF secreted from neurons is mediated by $p75^{NTR}$ activation followed by RhoA activation (Sun, Lim et al. 2012).

In summary, BDNF and proBDNF play different roles through activation of different receptors. While BDNF facilitates neuronal survival, differentiation, and proliferation through binding to TrkB, proBDNF causes neuronal cell death and collapse of neurite outgrowth by binding to $p75^{NTR}$. Also, BDNF promotes long-term potentiation through TrkB-dependent manner, whereas proBDNF stimulates long-term depression through $p75^{NTR}$.

1.1.4 BDNF endocytosis and endosomal trafficking

1.1.4.1 Neurotrophin endocytosis and signalling endosome model

Neurons require the neurotrophin support during target cell innervation in the development of PNS and CNS. After neurotrophins are released from target cells and bind to innervating neurons, they undergo endocytosis through their receptors. Endocytosis is the process of molecule uptake through invagination of the cell surface membrane, which is an important cellular activity in all eukaryotic cells (Mellman 1996, Mukherjee, Ghosh et al. 1997). The

basic processes involved in neurotrophin endocytosis are depicted in Figure 1-4. Binding of neurotrophins to Trk receptors gathers PI3K, Ras/ERK and PLC-γ to trigger downstream signalling pathways (Fig. 1-4A). It seems that the activation of Trk receptors on their neurotrophin ligand binding determines specific endocytic pathways. For example, it has been shown that PI3K inhibition in the distal exon of neonatal sympathetic neurons reduces NGF/TrkA endosome accumulation in the cell bodies, suggesting retrograde signalling requires PI3K activation (Kuruvilla, Ye et al. 2000). There are two major types of endocytosis; clathrin-dependent and clathrin-independent (Fig. 1-4B). Clathrin-mediated endocytosis recruits clathrin, a triskelton-shaped cytosolic protein which is composed of three heavy and three light chains. Adaptor protein 2 recruits and elicits clathrin assembly on plasma membrane to shape clathrin lattice (Brodsky, Chen et al. 2001). Then, those clathrin lattices invaginate to form clathrin coated pits, followed by pinch off from plasma membrane to form clathrin coated vesicles. In this process, dynamin (a large GTPase protein) plays a critical role in the step of developing of clathrin coated vesicles, however the exact molecular mechanism of this process is not clear (Engqvist-Goldstein and Drubin 2003). It has been reported that NGF - activated TrkA complexes are internalized by clathrin-coated pits in PC12 cells (Bronfman, Tcherpakov et al. 2003). There are also clathrin-independent endocytosis mechanisms which generally are less understood (Doherty and McMahon 2009, Mooren, Galletta et al. 2012). Macropinocytosis is an example of clathrin-independent endocytosis and involves engulfing of extracellular fluid to form large vesicles (0.5 – 5.0 μm). Accumulated data have shown that dynamin and pincher are included in macropinocytosis, however it is still not clear at which stages these proteins are required for macropinocytosis (Engqvist-Goldstein and Drubin 2003, Weible and Hendry 2004).

The large Rab family members of small GTPases are important molecules which are involved in endocytosis regulation by controlling the endocytosis process of neurotrophin/receptor from beginning of internalization at plasma membrane to endosome's

trafficking (Horgan and McCaffrey 2011). For example, Rab5 is involved in early endosome formation while Rab7 is linked to late endosomes (Fig. 1-4C). However, it is not understood how different Rabs are located to the different endosomes containing special transport machineries in neuronal endosomal signalling. Endosomes can be transported along the microtubules anterogradely or retrogradely (Fig.1-4D).

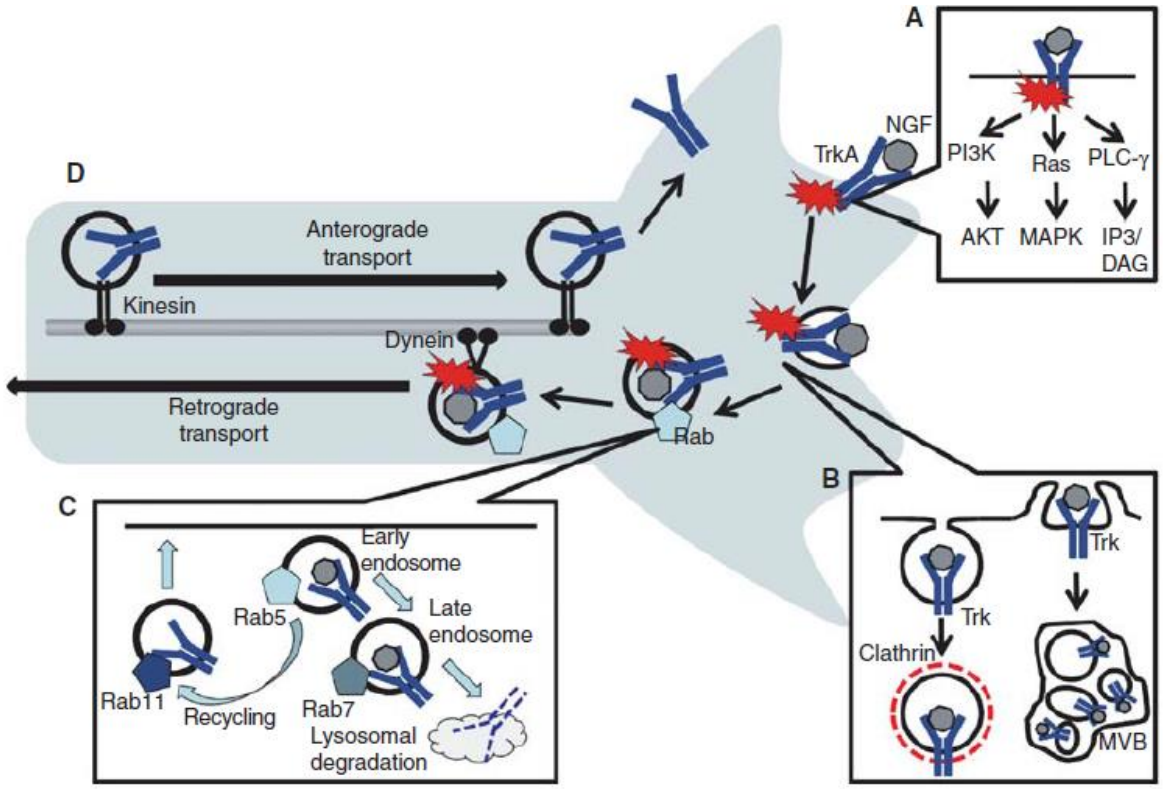


Figure 1-4 Neurotrophin endocytosis in neurons (Cosker and Segal 2014).
A) Once a neurotrophin binds Trk receptors, Trk receptors become activated and down-stream signalling pathways, PI3K/Akt, Ras/MAPK and PLC-γ/IP3/DAG, are initiated. B) Neurotrophin/Trk complex can be internalised by clathrin-dependent mechanisms to form early endosomes or clathrin-independent mechanisms such as micropinocytosis that leads multivesicular bodies (MVB). C) The endosomal trafficking contains maturation from Rab5-associated early endosomes to Rab7-associated late endosomes. Vesicles are to be either recycled back to the plasma membrane with Rab11 association or degraded in lysosomes. D) Endosomes are trafficked retrogradely to the cell body by dynein or anterogradely to the distal axon by kinesin along the axon.

Once neurotrophins are endocytosed, they are delivered to the cell body and sorting vesicles by means of retrograde trafficking. Concurrently, the triggered receptors start signalling cascades locally, which also intermediate transcriptional occurrence. This model is named *signalling endosome model* (Bartlett, Reynolds et al. 1998, Howe and Mobley 2004) (Fig. 1-5).

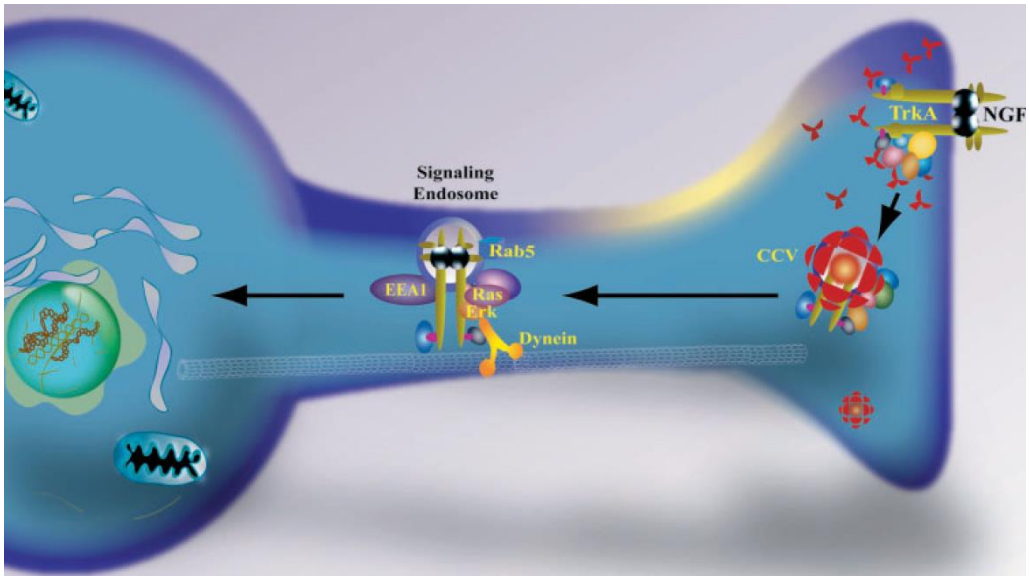


Figure 1-5 A schematic diagram of signalling endosome hypothesis (Howe and Mobley 2004).
Once NGF binds to TrkA, various elements relating clathrin-mediated endocytosis pathway are recruited to the NGF-TrkA complex. Following endocytosis, early endosomes containing NGF-TrkA complex and Ras and Erk are trafficked retrogradely from the axon end to the cell body of neuron. EEA1 is a marker of early endosome. Ras: GTP binding protein; CCV: clathrin-coated vesicle; Rab5: Ras-related protein 5; EEA1: Early Endosomal Autoantigen 1; NGF: neuronal growth factor; TrkA: tyrosine receptor kinase A

Delcroix et al. (2003) showed that NGF /TrkA complex is internalized and the signalling endosome includes activated components of ERK, PI3/Akt and PLCγ pathways in mature dorsal root ganglia neurons. The authors also demonstrated that the signalling endosome is retrogradely trafficked (Delcroix, Valletta et al. 2003). Recently, it was shown that $P75^{NTR}$ is embedded into clathrin coated pits (CCPs), which is followed by clathrin-medicated

endocytosis (CME) which is necessary for $p75^{NTR}$ to be transported retrogradely to cell body (Deinhardt, Reversi et al. 2007).

Taken together, endocytosis is a very complex process and further studies are needed to characterise endocytosis subclasses to reveal the class of proteins related to the particular type of endocytosis vesicle (Engqvist-Goldstein and Drubin 2003).

1.1.4.2 BDNF/TrkB endocytosis and endosomal trafficking are critical for neuronal function and require huntingtin protein

Zheng et al. (2008) showed that the endocytosis of BDNF/TrkB complex is clathrin-mediated and is required for neuronal survival and outgrowth of dendrites (Zheng, Shen et al. 2008). In the study, the authors demonstrated that: 1) TrkB endocytosis is induced by BDNF stimulation in cultured neurons including cortical, cerebellar granule and hippocampal neurons; 2) the components of clathrin-mediated endocytosis including clathrin, dynamin and adaptor protein 2 (AP2) are required for BDNF-induced TrkB endocytosis; 3) activation of PI3K/Akt induced by BDNF needs clathrin-mediated endocytosis in neurons; 4) BDNF endocytosis is required for neuronal survival and dendrite outgrowth in cerebellar granule cells and hippocampal neurons, respectively. Liu et al. (2015) recently demonstrated that TrkB endocytosis upon BDNF stimulation is impaired after exposure to amyloid beta (Aβ) and the impaired endocytosis and Akt activation are rescued by inhibiting glycogen synthase kinsase 3 beta (GSK-3β) by blocking its phosphorylation in cultured mouse cortical neurons. Furthermore, the authors showed that inhibiting GSK-3β facilitated neuronal survival and enhanced cognitive function in Alzheimer's disease (AD) mouse model (Liu, Geng et al. 2015). These studies suggest that BDNF/TrkB endocytosis and PI3/Akt signalling are necessary for neuronal survival and dendritic growth and the impaired BDNF/TrkB endocytosis could be related to pathology of neurological disorders

such as AD.

Mutated Huntingtin (htt) protein is known to cause Huntington's disease (Group 1993) and has been found to be involved in intracellular endosomal trafficking by interacting with other microtubule transport cargo molecules, dynein and kinesin and with adaptor molecules which are associated with actin (Caviston and Holzbaur 2009, Caviston, Zajac et al. 2011). Recently, Liot et al. (2013) have demonstrated that htt is critical in regulating BDNF/TrkB trafficking and signalling in striatal dendrites for neuronal survival. The authors used BDNF conjugated with quantum dots and added to dendritic terminals of cultured cortical neurons in microfluidic chamber. They then analysed retrograde trafficking of TrkB and ERK signalling. They found that htt is a key regulator of retrograde trafficking of TrkB and that Erk signalling activation was decreased in cultured cortical neurons of HD mouse model (Liot, Zala et al. 2013). This study suggests that BDNF/TrkB containing endosomal trafficking is critical for neuronal survival and is regulated by htt (Fig. 1-6).

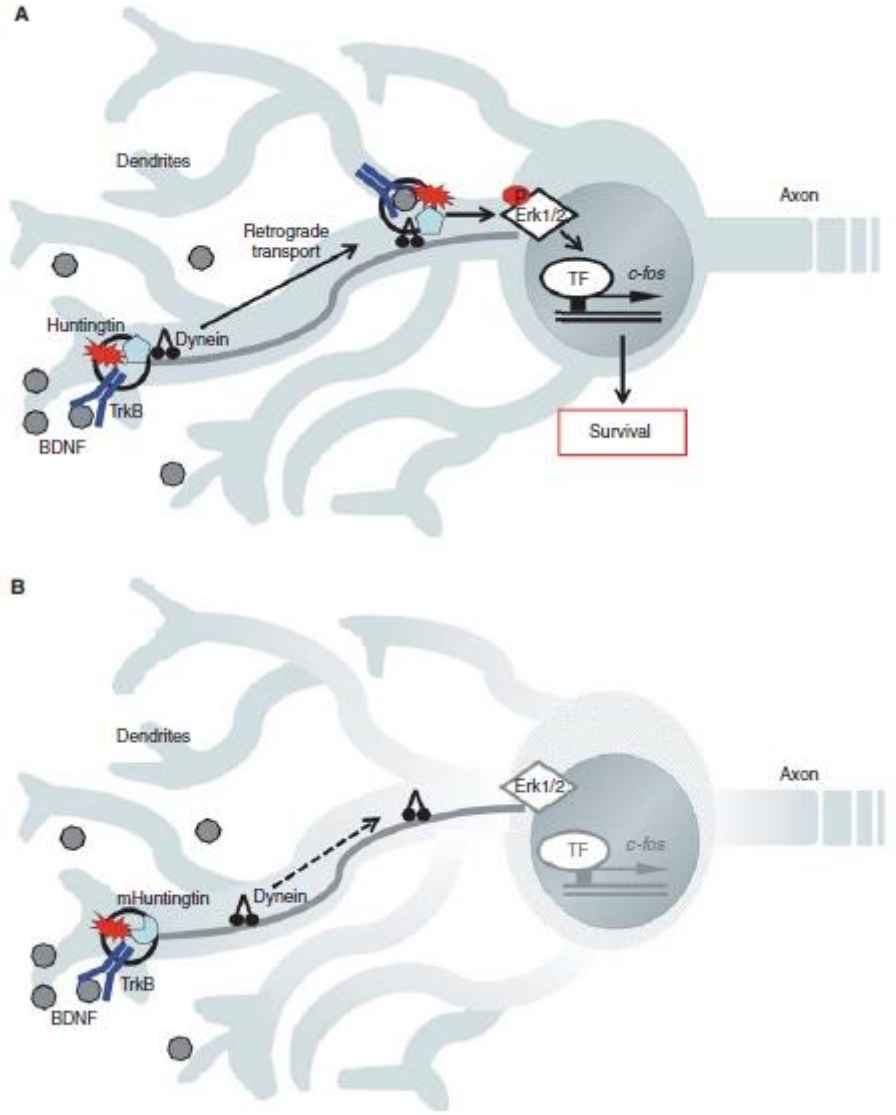


Figure 1-6 Retrograde transport of TrkB-BDNF signalling endosome in dendrites of striatal neurons is mediated by huntingtin protein (Cosker and Segal 2014).
A) Huntingtin protein is critical for BDNF/TrkB complex with dynein motors and for retrograde signals through Erk activation. B) BDNF/TrkB retrograde transport is inhibited by mutant huntingtin (mHuntingtin) and this leads to degeneration of striatal neurons in HD.

In neurons, some other studies suggest that huntingtin associated protein 1 (HAP1) is also involved in the trafficking and endocytosis of BDNF and related receptors and plays a critical role in the pathogenesis of Huntington's disease (Zimdahl, Gosele et al. 2001, Gauthier, Charrin et al. 2004, Sheng, Xu et al. 2008). One of the aims of this project is to

investigate the role of HAP1 in BDNF and its receptor endocytosis; therefore, the HAP1 protein and its function will be reviewed below.

1.1.5 The role of Huntingtin-associated protein 1 in BDNF and its receptor endocytosis and signalling

1.1.5.1 Huntingtin-associated protein 1 (HAP1)

In 1995, Li et al. identified Huntingtin-associated protein I (HAP1) for the first time through yeast two-hybrid screening with a rat brain cDNA library as one of the interacting proteins of htt (Li, Li et al. 1995). In rodents, there are two isoforms of HAP1 resulting from alternative splicing. HAP1A is a 75 kDa protein with 599 amino acids and HAP1B is an 85 kDa protein with 629 amino acids (Fig. 1-7). The difference between the two proteins is at the C-terminus of HAP1B; amino acids 600-629 in HAP1B (Li, Gutekunst et al. 1998, Li, Hosseini et al. 1998).

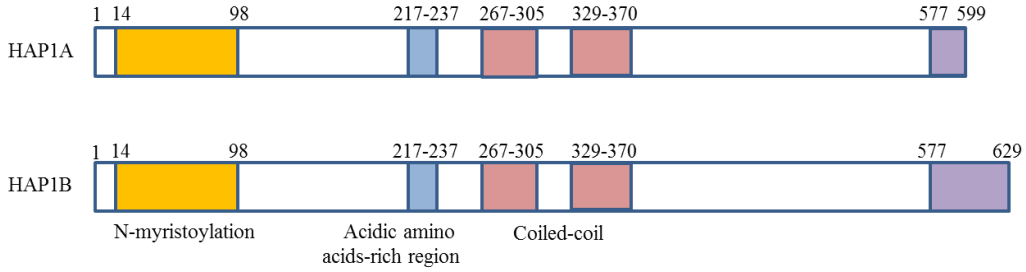


Figure 1-7 Structure of HAP1 (huntingtin-associated protein 1)

HAP1 does not contain conserved transmembrane domain or nuclear localization signal, suggesting that it is a cytoplasmic protein. Rat HAP1 possesses a few N-myristoylation sites at the N-terminal region (amino acids 14 – 98), acidic amino acids-rich region (amino acids 217 – 237) and several coiled-coil domains (amino acids 267 – 305 and 329 – 370, respectively) in the middle region, suggesting that the N-myristoylation and coiled-coil domains may mediate interactions with other proteins including htt (Li, Li et al. 1995,

Engelender, Sharp et al. 1997, Li, Hosseini et al. 1998, Li and Li 2005). To date, only one human HAP1 isoform has been identified which shows 96% homology to the rat HAP1A protein (Li, Hosseini et al. 1998).

HAP1 is mainly expressed in the central nervous system including cerebral cortex, basal forebrain, accessory olfactory bulb, cerebellum and the pedunculopontine nuclei, with predominant expression in hypothalamus, olfactory bulb and the supraoptic nucleus (Li, Li et al. 1995, Li, Sharp et al. 1996, Page, Potter et al. 1998, Dragatsis, Dietrich et al. 2000). Rat HAP1A and HAP1B show differential expression in diverse areas of the nervous system. In the spinal cord and olfactory bulb, HAP1B level is higher than the level of HAP1A (Li, Gutekunst et al. 1998) while in the other areas including striatum, cerebral cortex, hippocampus, brain stem and thalamus, the levels of HAP1A and HAP1B show little difference (Li, Gutekunst et al. 1998). Human HAP1 is mostly expressed in caudate and hippocampus whilst its levels in cerebellum and cerebral cortex are lower and there is no HAP1 detected in the white matter and thalamus (Li, Sharp et al. 1996, Li, Hosseini et al. 1998, Martin, Kim et al. 1999).

A few studies reported that HAP1 is present in axons, dendrites, cell bodies and nerve terminals in the CNS and HAP1 was found in soluble fraction and vesicle-rich fractions at nerve terminals (Li, Sharp et al. 1996, Gutekunst, Li et al. 1998, Martin, Kim et al. 1999). HAP1's presence in the vesicle-rich fractions suggests that HAP1 could play a role in vesicular trafficking in the cells.

1.1.5.2 HAP1-interacting proteins and vesicular transport

Given that htt itself plays a role in intracellular trafficking and HAP1 was initially identified as one of the htt interacting proteins, it was suggested that HAP1 also acts as one of elements

of cargo-motor molecules to facilitate intracellular trafficking (Li, Chin et al. 2002, Li and Li 2005, McGuire, Rong et al. 2006, Rong, McGuire et al. 2006). The two HAP1 isoforms, HAP1A and HAP1B, include a few conserved coiled-coil domains (Engelender, Sharp et al. 1997, Li, Chin et al. 2002) and a number of reports have demonstrated that coiled-coil domains are responsible for HAP1-interacting proteins (Table 1-1). Considering the functions of these HAP1-interacting proteins, it is suggested that HAP1 is included in the vesicle transport, membrane receptor trafficking, regulation of gene transcription, protein aggregation and calcium release. As vesicular trafficking-related and membrane receptor proteins are the major HAP1-interacting proteins, I will further discuss the interactions between HAP1 and these proteins.

It has been reported that HAP1 interacts with $p150^{Glued}$ (Engelender, Sharp et al. 1997, Bartlett, Brodie et al. 1998), kinesin light chain (KLC) (McGuire, Rong et al. 2006), 14-3-3 protein (Rong, Li et al. 2007), Duo (Colomer, Engelender et al. 1997), hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) (Li, Chin et al. 2002) and Abelson helper integration site 1 (AHI1) (Sheng, Xu et al. 2008) (see Table 1-1) and participates in the vesicular trafficking. $p150^{Glued}$ and KLC are the key molecules in retrograde (Engelender, Sharp et al. 1997) and anterograde transport (Bloom, Wagner et al. 1988) along microtubules, respectively, therefore I will focus on interactions between these two proteins and HAP1.

$P150^{Glued}$ is the biggest compartment of all the dynactin subunits. Dynactin consists of multiple subunits and binds to microtubule motor proteins such as dynein involved in retrograde transport in cells (Engelender, Sharp et al. 1997). The direct binding of dynactin to dynein results in the motor vehicle travelling a long distance (Schroer 2004) on microtubule. Li et al. (1998) showed that HAP1 interacts with $p150^{Glued}$ by protein-protein binding assay using GST-HAP1 and immunoprecipitation. Li's group demonstrated further

that htt and p150^{Glued} were found in the same fraction asHAP1 after sucrose gradient fractionation, suggesting that HAP1 may have a function in retrograde transport on microtubules (Bartlett, Brodie et al. 1998).

The kinesin complex (379–386 kDa) conventionally consists of two heavy chains (KHC, 110-120 kDa) and two light chains (KLC, 60-70 kDa) and it is responsible for anterograde transport along microtubules (Bloom, Wagner et al. 1988, Kuznetsov, Vaisberg et al. 1988). McGuire et al. (2006) demonstrated that 1) HAP1 co-localises and interacts with KLC to be involved in kinesin complex-associated transport during neuronal outgrowth; 2) the two molecules bind *in vitro* and *in vivo*; 3) vesicular transport is reduced in HAP1 lacking olfactory neuronal culture (McGuire, Rong et al. 2006). This study suggests that HAP1 plays a role in anterograde transport through the interaction with KLC.

Name	Function	Reference
Htt	Scaffold protein	(Li, Li et al. 1995)
p150^{Glued}	Microtubule-dependent transporter	(Engelender, Sharp et al. 1997, Li, Gutekunst et al. 1998)
Duo	GDP-GTP exchange factor	(Colomer, Engelender et al. 1997)
Hrs	vesicular trafficking	(Li, Chin et al. 2002)

$GABA_AR$	membrane receptor	(Kittler, Thomas et al. 2004)
$InsP_3R1$	membrane receptor	(Tang, Tu et al. 2003)
NeuroD	neuronal transcription factor	(Marcora, Gowan et al. 2003)
KLC	microtubule-dependent transport	(McGuire, Rong et al. 2006)
AR	membrane receptor	(Takeshita, Fujinaga et al. 2006)
14-3-3	protein trafficking complex assembly	(Rong, Li et al. 2007)
TBP	transcription factor	(Prigge and Schmidt 2007)
AHI1	Intracellular trafficking	(Sheng, Xu et al. 2008)
proBDNF	neurotrophin	(Wu, Fan et al. 2010, Yang, Lim et al. 2011)
APP	Membrane protein, produce Aβ	(Yang, Yang et al. 2012)

Table 1-1HAP1-interacting proteins and their proposed functions.
Htt, huntingtin; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; $GABA_AR$, γ-amino butyric acid type A receptors; $InsP_3R1$, inositol 1,4,5-trisphosphate receptor type 1; KLC, kinesin light chain; AR, Androgen receptor; TBP, TATA-binding protein; AHI1, Abelson helper integration site 1; proBDNF, precursor protein of BDNF; APP, amyloid precursor protein.

1.1.5.3 HAP1 regulates membrane receptor protein recycling and signalling

HAP1 plays a role in keeping stability of cell membrane receptors through interfering with the lysosomal degradation pathway and promoting the pathway of endocytic recycling. It has been shown that HAP1 interacts with several membrane receptors such as γ-amino butyric acid type A receptors ($GABA_AR$), tyrosine receptor kinase A (TrkA), type 1 inositol

(1,4,5)-triphosphate receptors ($InsP_3R1$) and androgen receptor (AR) (Rong, Li et al. 2007, Wu and Zhou 2009).

In 2002, the role of HAP1 in trafficking of the membrane receptors was first reported (Li, Chin et al. 2002). The authors showed that overexpression of HAP1 inhibited trafficking of the internalized epidermal growth factor receptor (EGFR) from early endosome to lysosome, resulting in reduced degradation of the internalized EGFR (Li, Chin et al. 2002). One study has shown that TrkA, a receptor of neuronal growth factor (NGF), is stabilized by HAP1 via prevention of TrkA from the lysosomal degradation (Rong, McGuire et al. 2006). Other studies have demonstrated that HAP1 binds to $GABA_AR$, specifically, β subunit and prevents the lysosomal receptor degradation and expedites recycling of $GABA_AR$ to the cell surface (Kittler, Thomas et al. 2004). Suppression of HAP1 expression by siRNA leads to the reduction of $GABA_AR$ level and activity in the hypothalamus and this phenomenon causes decreased food intake and body weight. HAP1 has also a critical function in modulating the major inhibitory synaptic transmission in the brain, in particular in the rodent hypothalamus (Sheng, Chang et al. 2006). Taken together, these data suggest that HAP1 can play a role in stabilising or facilitating recycling of the membrane receptor proteins by interacting with them.

1.1.5.4 HAP1 is required for proBDNF protein trafficking

Two our recent studies have offered a new insight into HAP1's role in trafficking of neurotrophic factors. Wu et al. (2010) showed that HAP1 binds to prodomain of proBDNF and further mapping identified amino acids 371-445 of HAP1 that bind to amino acids 65-90 of proBDNF (Yang, Lim et al. 2011). Wu et al. (2010) demonstrated that the axonal transport and proBDNF release are dramatically decreased in HAP1KO mice compared to WT mice, suggesting that HAP1 could be important in proBDNF transport and secretion

(Wu, Fan et al. 2010). Furthermore, the interaction of HAP1 and proBDNF is decreased by mutant htt and proBDNF(Val66Met) (Wu, Fan et al. 2010). As mutant htt causes neurodegenerative disorders such as Huntington's disease (Group 1993), it can be suggested that proBDNF transport and release may also be altered in Huntington disease (Wu, Fan et al. 2010). Yang et al. (2011) confirmed this finding by investigating the interaction between HAP1 and proBDNF (2011). This study demonstrated that 1) HAP1 is a component of the proBDNF-HAP1-Sortilin ternary complex; 2) proBDNF trafficking is attenuated in HAP1KO mice; 3) co-transfection of Sortilin and HAP1 immensely enhances proBDNF stability and its conversion to mBDNF when compared to HAP1 transfection only. Taken together, it is proposed that HAP1 may play a role with Sortilin in proBDNF trafficking and stabilising in neurons through direct binding and preventing it from degradation, which results in more mBDNF production. Thus, HAP1 loss or dysfunction may result in low mBDNF production and potentially can cause neurological disorders.

1.1.5.5 HAP1 is linked to BDNF receptor internalization

A few studies reported that HAP1 is related to internalization and stabilization of BDNF and TrkB. Sheng et al. (2008) showed that internalization of TrkB is suppressed in mouse brain stem cells infected with HAP1 siRNA. In addition, reduced BDNF endocytosis and activation of ERK and Akt were observed in cultured brain stem cells of HAP1 KO mice compared to WT mice (Sheng, Xu et al. 2008). In this study, TrkB expression level was also reduced in the brain stem and cerebellum of HAP1 KO mice compared to WT mice. Therefore, the reduced internalization of TrkB and BDNF could be owing to the reduced TrkB levels on the cell surface rather than owing to the loss of HAP1. Recently, Xiang et al. (2014) demonstrated that HAP1 and TrkB form an interaction complex and HAP1 suppresses TrkB lysosomal degradation by association with Sortilin in the hypothalamic

region of mice (Xiang, Yang et al. 2014). The authors also showed that TrkB expression level and neurogenesis are decreased in the hypothalamic region of HAP1 KO mice and the reduced neurogenesis is recovered by BDNF injection into brain (Xiang, Yang et al. 2014). The results of this study suggest that HAP1 may interact with TrkB to protect TrkB from lysosomal degradation and that reduced TrkB level in HAP1 KO mouse may be related to the impaired neurogenesis due to the decreased BDNF-TrkB signalling. As mentioned above, HAP1 participates in signalling endosome trafficking through interaction with other intracellular trafficking molecules and a few studies have shown association of HAP1 with BDNF endocytosis, TrkB and its expression level. It is still unclear, however, whether HAP1 is directly involved in BDNF and its receptor's endocytosis. Consequently, one aim of my PhD study is to establish HAP1's role in endocytosis of BDNF and its receptors, the TrkB (main receptor) and $p75^{NTR}$ (pan receptor), and downstream signalling.

1.2 BDNF and its measurement in neurological disorders

As neurotrophic factors have a number of neuronal roles in development and in adulthood (Chao, Rajagopal et al. 2006), BDNF as a neurotrophin has been implicated in pathophysiology of neurological disorders including neurodegenerative disorders such as Alzheimer's disease (AD), Huntington's disease (HD) and Parkinson's disease (PD) and psychiatric disorders such as major depression disorder (MDD), bipolar disease (BPD) and Schizophrenia. Below, we will review how BDNF levels are altered in these neurological disorders.

1.2.1 BDNF and neurodegenerative disorders

1.2.1.1 Alzheimer's disease (AD)

Alzheimer's disease (AD) is the most common form of dementia and a neurodegenerative disorder which is characterized by the progressive synapses loss resulting in neuron loss in the entorhinal cortex and hippocampus which play important roles in learning and memory , leading to gradual memory loss (Braak and Braak 1991, Masliah, Mallory et al. 1994, Kordower, Chu et al. 2001). BDNF which is produced in entorhinal cortex is transported to the hippocampus through anterograde trafficking and BDNF is released in an active-dependent manner in the hippocampus. It has been shown that BDNF application to dendrites stimulates the postsynaptic long term potentiation in the dentate granule cells in mouse hippocampus tissue by electrophysiological method (Kovalchuk, Hanse et al. 2002). This finding strongly suggests that BDNF could play a core role in supporting the electrophysiological role of memory circuitry (Altar, Cai et al. 1997, Kovalchuk, Hanse et al. 2002). Importantly, BDNF is widely expressed in the brain including the entorhinal cortex and hippocampus (Yan, Rosenfeld et al. 1997). Therefore, it is possible that disturbances in BDNF signaling/trafficking may be involved in memry loss in AD.

Amyloid beta (Aβ) plaques are a hall mark of AD (Sadigh-Eteghad, Sabermarouf et al. 2015) and Aβ is generated from amyloid precursor protein (APP)(Hartmann, Bieger et al. 1997). Nagahara et al. (2009) used an AD transgenic mice model which expresses a mutant APP (Nagahara, Merrill et al. 2009). The authors demonstrated that BDNF restored some gene expression which was disturbed by APP overexpression in entorhinal cortex and hippocampus; this effect was accompanied by the learning and memory improvements. Similarly, Blurton-Jones et al. (2009) reported that neural stem cell (NSC) transplantation to the hippocampus in an AD mice model (which overexpresses APP) increased synaptic density in hippocampus and improved mice cognitive function. It was also found that BDNF

expression is elevated in the hippocampus after NSC transplantation. Therefore, it is suggested that the improved cognition function may be mediated by BDNF (Blurton-Jones, Kitazawa et al. 2009) . Taken together, these data suggest that BDNF plays crucial protective roles in the maintenance of neuronal circuitry responsible for cognitive functions and that cognitive impairment in AD may be associated with low BDNF levels.

It has been suggested that low BDNF serum levels are related to cognitive dysfunctions or AD, (Carlino, De Vanna et al. 2013) while higher serum BDNF levels are protective of cognitive decline according to the epidemiological data (Weinstein, Beiser et al. 2014). It has been also shown that BDNF Met carriers are more likely to cognitively decline faster, although the BDNF SNP is not associated with AD diagnosis (Honea, Cruchaga et al. 2013). There is a relationship between the female BDNF Met carriers and AD risk in a meta-analysis (Chen, Liang et al. 2014). These data suggest the relationship between AD and BDNF serum levels or the BDNF SNP, however the inconsistent results among different studies could be owing to the inappropriate methods such as sample cohort size, AD diagnosis standard and non-specific BDNF ELISA. This will be discussed further later in this chapter.

1.2.1.2 Huntington's disease (HD)

Huntington's disease (HD) is a neurodegenerative disorder characterized by cognitive decline and dementia eventually leading to death after 10-20 years of onset of symptoms (Walker 2007). It has been known that HD is caused by mutated huntingtin (htt) protein (Group 1993) which is expressed abundantly in neurons in the CNS of humans and rodents (Kuruvilla, Ye et al. 2000). As mentioned previously htt is associated with vesicles in cytoplasm (Russo-Neustadt, Beard et al. 1999) and microtubules (Nibuya, Nestler et al. 1996) and is involved in vesicular trafficking (Liot, Zala et al. 2013). Although polyglutamine expansion in htt (PolyQ-htt) is found to be responsible for neuronal toxicity displayed in this

disease (especially in striatum) (Orr and Zoghbi 2007), the pathogenic mechanisms caused by polyQ-htt are not clearly understood.

In the HD patients, striatal degeneration is a pathological hallmark and BDNF levels are decreased in the striatum and the cerebral cortex (Zuccato, Ciammola et al. 2001) in these patients. Baquet et al. (2004) have demonstrated that BDNF KO mice develop striatal degeneration at 35 day of age (Baquet, Gorski et al. 2004) . Furthermore, it has been shown that the gene expression profile in striatum of the BNDF KO mice is similar to human HD gene expression profile as assessed by differential gene expression array method (Strand, Baquet et al. 2007). These findings suggest that neuronal degeneration in HD could be caused by deficiency of BDNF transport to striatum from cortical neurons as most of the striatal BDNF is synthesized in cortical neurons (Altar, Cai et al. 1997).

Defective endocytosis and vesicular trafficking have been linked to many neurological diseases (Perlson, Maday et al. 2010, Wang, Chan et al. 2013). Gauthier et al. showed huntingtin protein facilitates BDNF transport along microtubules (Gauthier, Charrin et al. 2004). The authors have also demonstrated that 1) huntingtin-associated protein 1 (HAP1) and $p150^{Glued}$, a subunit of dynactin, are involved in the huntingtin-mediated transport; 2) attenuated BDNF transport is associated with reduced levels of huntingtin; 3) mutant huntingtin leads to altered transport, which results in declined neurotrophic support and increased cell death. These findings suggest that the deficit of BDNF transport in neurons can contribute to the HD pathogenesis. Another study has shown that cell death is reduced with BDNF treatment of cultured neurons transfected with mutant huntingtin (Zala, Benchoua et al. 2005). The survival of enkephalin-immunoreactive striatal neurons was increased and motor functions were improved in huntingtin -mutant mice by BDNF protein infusion (Canals, Pineda et al. 2004). Although there have been no BDNF therapy trials in humans who have HD to date, BDNF can be a hopeful candidate to prevent or delay the progress of Huntington's disease.

1.2.1.3 Parkinson's disease (PD)

Parkinson's disease (PD) is neurodegenerative disorder which is characterized by impairment of motor and cognitive function. The loss of dopaminergic neurons from the substantia nigra and striatum is the pathological distinctive feature (Baquet, Bickford et al. 2005). Hyman et al. (1991) showed that survival of nigral dopaminergic neurons was increased by BDNF in cultured E14 rat ventral mesencephalic cells (Hyman, Hofer et al. 1991). In human study, the levels of BDNA mRNAs and protein expression were reduced in nigral neurons in PD (Mogi, Togari et al. 1999, Parain, Murer et al. 1999). It was also found that reduced BDNF serum levels correlated with impaired motor function in early stages of PD patients (Scalzo, Kummer et al. 2010). Tsukahara et al. (1995) used 1-methyl-4-phenylpyridinium (MPP) to induce parkinsonism in Japanese monkeys and demonstrated that BDNF treatment group showed slower and milder parkinsonism with decreased neuronal cell loss in the substantia nigra compared to non-treatment group (Tsukahara, Takeda et al. 1995). Taken together, it is suggested that BDNF is highly linked to PD and BDNF treatment could help PD patients through protecting dopaminergic neurons in the nigrostriatal pathway.

1.2.2 BDNF and psychological disorders

A number of clinical studies have been performed to find a link between the BDNF levels and psychiatric diseases including the major depression disorder, bipolar disease and schizophrenia (Schumacher, Jamra et al. 2005; Post 2007; Kato and Serretti 2010**).**

1.2.2.1 Major depression disorder (MDD) and bipolar disorder (BPD)

Mood disorders including major depressive disorder (MDD) and bipolar disease (BPD) are the most predominant psychiatric conditions. It affects 1 in 6 people sometime in their life, severely disrupts their quality of life and working efficiency, and is one of the leading causes of mortality (from suicide) and morbidity in the young population (Kessler, McGonagle et al. 1994). Growing number of reports have demonstrated that BDNF level and polymorphism in the BDNF gene are related to mood disorders including depressive disorder and bipolar disease (Schumacher, Jamra et al. 2005, Post 2007, Kato and Serretti 2010).

It has been shown that blood BDNF levels decline in manic (Cunha, Frey et al. 2006, Palomino, Vallejo-Illarramendi et al. 2006, de Oliveira, Cereser et al. 2009) and depressed states (Cunha, Frey et al. 2006, Yoshimura, Nakano et al. 2006, de Oliveira, Cereser et al. 2009) . Furthermore, a meta-analysis study showed that pharmacological treatment improves mood states in manic patients and also increases blood BDNF levels in BPD patients in manic state (Lin 2009). Given that BDNF levels decrease in manic and depressed states of BPD and increase after pharmacological treatment, it is suggested that blood BDNF levels could be a potential state-dependent biomarker of BPD for diagnosing or monitoring process of treatment.

Similarly, decreased serum BDNF in MDD patients and increased BDNF levels in antidepressant-treated patients have been reported (Gervasoni, Aubry et al. 2005, Karege, Bondolfi et al. 2005, Aydemir, Yalcin et al. 2006, Aydemir, Deveci et al. 2007, Yoshimura, Mitoma et al. 2007, Monteleone, Serritella et al. 2008, Piccinni, Marazziti et al. 2008). Furthermore, it has been shown that antidepressant treatment significantly increases blood BDNF levels and the BDNF level changes significantly correlate with depression score changes ($P = 0.002$) by meta-analysis (Brunoni, Lopes et al. 2008).

Several studies have suggested that BDNF expression could be a subsequent target of anti-depressant drug treatments. Anti-depressant administrations were found to up-regulate

BDNF mRNA expression in rat limbic system by chronic treatment (Nibuya, Morinobu et al. 1995, Nibuya, Nestler et al. 1996, Russo-Neustadt, Beard et al. 1999). Additionally, it has been demonstrated that BDNF protein levels increase after chronic anti-depressant treatment in mice hippocampus (Deltheil, Guiard et al. 2008).

Taken together, it is suggested that BDNF is a critical factor in pathophysiology of mood disorders such as MDD and BPD and can be a biomarker for diagnosis and/or monitoring the efficacy of therapeutic treatment.

1.2.2.2 Schizophrenia

Schizophrenia is a mental disease characterised by impairment of neurocognitive function (Sharma 2003; Condray and Yao 2011). Numerous studies have reported that BDNF levels in blood are decreased in patients with schizophrenia, however some reports were unable to reproduce those findings. For example, many studies have shown that BDNF levels in blood were decreased in first-episode and treated patients with schizophrenia (Shimizu, Hashimoto et al. 2003; Pirildar, Gonul et al. 2004; Palomino, Vallejo-Illarramendi et al. 2006; Grillo, Ottoni et al. 2007; Rizos, Rontos et al. 2008; Chen da, Wang et al. 2009; Zhang, Liang et al. 2012). In contrast, some authors have reported no differences between untreated and schizophrenic patients and healthy control subjects (Shimizu, Hashimoto et al. 2003; Huang and Lee 2006). In other studies, increased BDNF levels in serum in treated schizophrenic patients were demonstrated (Gama, Andreazza et al. 2007; Reis, Nicolato et al. 2008). Therefore, BDNF level alteration in schizophrenia has been controversial and further studies are required.

In summary, a growing number of studies support that BDNF is linked to neurological disorders and BDNF levels also alter in disease status. For example, BDNF levels correlate with mood disorder status, it is therefore suggested that BDNF can be a biomarker for

diagnosis and/or monitoring of treatment progress in mood disorders. It is important to note that although most clinical studies measure BDNF levels in blood, the main BDNF neurotrophic functions occur in the brain. Therefore, it is important to investigate the correlations between BDNF levels in blood including whole blood, serum and plasma and brain in order to establish if blood BDNF can reflect brain BDNF levels. This type of studies can only be performed in animals and will be covered below.

1.2.3 Correlation between blood BDNF and brain BDNF levels in animal studies

Although BDNF is abundant in the brain, it is also expressed in peripheral tissues including muscle (Matthews, Astrom et al. 2009) and liver (Cassiman, Denef et al. 2001). BDNF protein levels in serum, plasma, whole blood and brain tissue and cerebrospinal fluid (CSF) were demonstrated by commercial ELISA kits in human (Laske, Stransky et al. 2007, Yoshimura, Mitoma et al. 2007, Aznar, Klein et al. 2010, Kang, Chang et al. 2010). BDNF levels even though Pan et al. (1998) showed that BDNF passes over the blood-brain barrier (BBB) (Pan, Banks et al. 1998). A few animal studies compared serum and brain levels of BDNF in different species. First study compared BDNF levels in serum, platelets, hippocampus and frontal cortex in rats, by ELISA and RT-PCR to measure proteins and mRNA levels, respectively. Postnatal, young adult rats and aged rats were used in that study. Interestingly, it was found that serum BDNF levels correlated with the brain BDNF levels in hippocampus and frontal cortex from postnatal (1 week of age) state to aged (2 years of age) state (Karege, Schwald et al. 2002). Another study demonstrated that changes in BDNF levels in serum correlated with the changes in BDNF levels in brain tissue in adult rats after electroconvulsive treatment (Sartorius, Hellweg et al. 2009). Recently, Klein et al. (2011) investigated BDNF levels in blood, serum, plasma and brain tissue in rats, pigs and mice by

ELISA. The results showed that there was a positive correlation between blood BDNF and hippocampal BDNF levels in rat and pig (Klein, Williamson et al. 2011). These results suggest that there could be a correlation between the brain and blood BDNF levels and that blood BDNF levels may potentially be an indicator of brain BDNF levels. Interstingly, several stidies have reported other potential peripheral sources of BDNF such as smooth muscle cells (Donovan, Miranda et al. 1995), activated macrophages, lymphocytes (Braun, Lommatzsch et al. 1999, Kerschensteiner, Gallmeier et al. 1999) and vascular endothelial cells (Nakahashi, Fujimura et al. 2000). However, it is unclear whether blood BDNF levels correlate with those in peripheral tissues. As these peripheral BDNF sources can affect the blood BDNF level, further studies are required to clarify whether there is a correlation between blood BDNF and peripheral sources of BDNF. It should be also emphasised here that most of the studies that investigated BDNF levels in the brain and blood in different species used commercially BDNF ELISA kits that may not be able to distinguish mature BDNF and proBDNF. As described in section1.4, there are two forms of BDNF, proBDNF and mature BDNF, which have different functions, therefore further studies are required to confirm the above findings using methods that allow measuring specific forms of BDNF. Consequently, one of the aims of this project is to establish the ELISA technique that will be able to specifically measure mature BDNF in human and animal tissue/fluid samples. The principles of the ELISA method and the limitations of current BDNF ELISA kits are discussed below.

1.2.4 ELISA is a high-throughput method to measure BDNF levels in a number of tissue samples

Enzyme-linked immunosorbent assay (ELISA) is an immunoassay which uses one or two antibodies against a specific protein and an enzyme conjugated to an antibody. A standard

curve employing standard molecule with known concentration allows a specific protein in certain sample matrix to be quantified. Most commercial ELISA kits use sandwich ELISA format using two antibodies, one to capture and the other to detect the protein of interest (Fig.1-8).

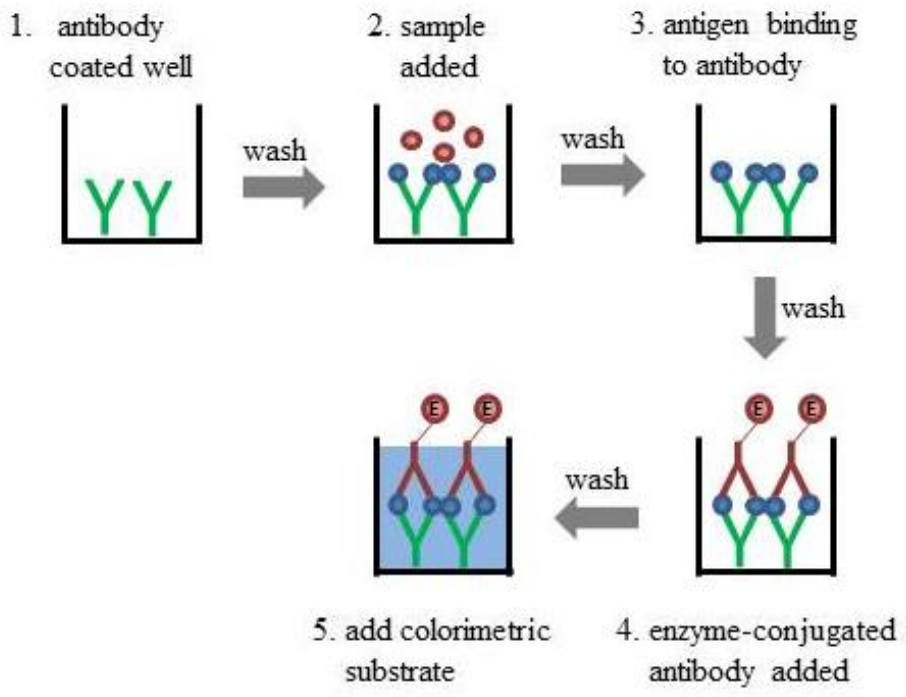


Figure 1-8 Schematic representation of the sandwich ELISA.
A capture antibody is coated on the plate and the plate is blocked except capture antibody bound sites. Sample is then added and any antigen binds to the capture antibody. An enzyme conjugated detection antibody is added and binds to the antigen which is captured by the capture antibody. Colorimetric substrate is added and changed to a detectable form by the enzyme which is conjugated to detection antibody.

Although it is notable that Western blot technique can distinguish the pro- and mature BDNF with higher specificity than ELISA (Gharami, Xie et al. 2008), there are a few advantages in using ELISA to determine BDNF levels. First, it allows measuring BDNF levels in both blood and the brain (Klein, Williamson et al. 2011) while western blotting is

not suitable for measuring blood BDNF levels; second, ELISA offers higher sensitivity than Western blot which allows measuring endogenous BDNF levels (which are usually very low) in samples including blood and tissue easily; last, ELISA is a high-throughput quantitative method so it is more time-efficient and accurate than Western blot.

1.2.4.1 Commercial BDNF ELISA kit may not be able to distinguish mature BDNF and proBDNF

As mentioned above, most of studies which investigated BDNF levels by ELISA used commercially available BDNF ELISA kits supplied from either Promega (Umene-Nakano, Yoshimura et al. 2009, Hu, Yu et al. 2010, Serra-Millas, Lopez-Vilchez et al. 2011), R&D Systems (Minneapolis, MN, USA) (Basterzi, Yazici et al. 2009, Mackay, Forrest et al. 2009, Kang, Chang et al. 2010, Bot, Pouwer et al. 2011) or EMD Millipore Corporation (Billerica, MA, USA) (Rojas, Fritsch et al. 2011).

Mature BDNF is produced by enzymatic cleavage of proBDNF (Mowla, Farhadi et al. 2001, Matsumoto, Rauskolb et al. 2008) and biological function of proBDNF itself is to promote neuronal cell apoptosis (Pang, Teng et al. 2004, Teng, Teng et al. 2005, Woo, Teng et al. 2005). Therefore, it is essential to measure two different forms with high specificity. Moreover, a few researchers suggested that mature and proBDNF levels should be determined separately because of the opposite functions of mature and proBDNF (Hashimoto 2010, Teche, Nuernberg et al. 2013) .

Recently, Yoshida et al. (2012) reported no change in proBDNF but reduced levels of mature BDNF in major depression disorder patients serum (Yoshida, Ishikawa et al. 2012). In that study, mature and proBDNF specific commercial ELISA kits (AdipoBiosciences, Santa Clara, CA, USA) were used. However, there are some apparent problems with the ELISA

kits used in this study. According to the manufacturer's instructions, the specificity of mature BDNF ELISA kit was tested for NT-3, NT-4, NGF and prodomain part (amino acid 19 – 128) of full-length proBDNF (amino acid 1 -274) but not for the full-length proBDNF, therefore it is possible that this kit may also detect the full length proBDNF . Therefore, there is no evidence to suggest that the mature BDNF ELISA kit detects only mature BDNF. Therefore, it seems that the mature BDNF ELISA kit from AdipoBiosciences is not suitable to measure specific forms of BDNF.

In conclusion, although previous studies have provided some evidence that blood BDNF levels can reflect brain BDNF levels, current methods clearly have some limitations with respect to the measurements of mature BDNF in the brain and blood. Therefore, it is important to develop new methods that will be able to specifically measure only mature BDNF levels, because this will allow to further elucidate the possible roles of mature BDNF in neurological disorders and to establish if blood BDNF levels can be used as a biomarker with other parameters and biomarkers in neurological disorders.

Thus one aim of my PhD study is to establish a mature BDNF-specific ELISA that will allow investigation of mature BDNF levels in tissues and blood.

1.3 BDNF levels in major depression disorder (MDD) and Alzheimer's disease (AD)

1.3.1 Mature BDNF and proBDNF and their receptors in MDD

As mentioned above, the role of mature BDNF in MDD has been intensively studied.

As TrkB is a receptor for mature BDNF with high affinity (Huang and Reichardt 2003, Chao, Rajagopal et al. 2006), it has been shown that *trkB* mRNA expression is increased in rat brain including frontal cortex and hippocampus by anti-depressant drug treatment (Nibuya,

Morinobu et al. 1995). Saarelainen et al. (2003) used transgenic mice which overexpress TrkB-T1, a truncated isoform of TrkB, and compared the effects of anti-depressant drugs to WT mice (Saarelainen, Hendolin et al. 2003) in a forced swim test. The results demonstrated that TrkB-T1 overexpression leads to reduced TrkB activation by forming heterodimer with TrkB, suggesting that normal TrkB is required for anti-depressant drug effect. As described in section 1.4, proBDNF has opposite functions to mature BDNF and its signalling is mediated through the main receptor $p75^{NTR}$ and co-receptor Sortilin (Teng, Teng et al. 2005). Considering different cellular functions of mature BDNF, proBDNF and their receptors, it would be important to analyse these molecules and investigate their correlations in MDD. Therefore, one of the aims of my PhD study is to determine whether mature BDNF/proBDNF and their receptors are altered in MDD using mature BDNF-specific ELISA which is one of aims of this PhD study.

1.3.2 BDNF levels in Alzheimer's disease (AD) patient samples show inconsistent results

BDNF expression levels in AD are not conclusive. While majority of studies have demonstrated that BDNF level is reduced in the entorhinal cortex and the hippocampus in AD (Narisawa-Saito, Wakabayashi et al. 1996, Connor, Young et al. 1997, Hock, Heese et al. 2000, Peng, Wuu et al. 2005) and in the brain of AD mice (Kimura, Devi et al. 2010, Devi and Ohno 2012), other studies have reported that BDNF expression levels increase in the hippocampal region of AD patient brain (Durany, Michel et al. 2000, Savaskan, Muller-Spahn et al. 2000).

A few studies have reported that BDNF levels in serum is decreased in AD patients compared to healthy subjects who are normal cognitively (Laske, Stransky et al. 2007), whereas other studies have reported that there was no differences in BDNF levels between

AD and healthy control subjects (O'Bryant, Hobson et al. 2009, Woolley, Strobl et al. 2012). Recently, increased serum BDNF levels were found in mild cognitive impairment and AD patients compared to healthy control subjects regardless of acetylcholinesterase inhibitor treatment (which is one of the AD treatment drugs) or antidepressant medication (Angelucci, Spalletta et al. 2010). These conflicting results regarding BDNF levels in AD could be affected by a few factors. One factor could be the variables in different cohorts. Firstly, many studies used insufficient sample numbers. Angelucci et al. (2010) used cohort which contained 27 healthy control, 54 mild cognitive impairment, 54 mild AD and 35 moderate-severe AD (Angelucci, Spalletta et al. 2010) patient samples. Laske et al. (2007) investigated 27 AD and 28 age-matched healthy controls (Laske, Stransky et al. 2007). In some studies, blood collection was done in a fasting state (Angelucci, Spalletta et al. 2010, Laske, Stellos et al. 2011, Sonali, Tripathi et al. 2013), while others did not state the procedure (Laske, Stransky et al. 2007). BDNF levels may also change in accordance with participant's physiological conditions, for example weight, menstrual cycle, cholesterol levels and severity of depression (Trajkovska, Marcussen et al. 2007). Another possible factor is the methodology to measure BDNF levels in blood samples. As mentioned in section 2.4.1, current commercial BDNF ELISA kits which are used widely do not supply clear evidences with regard to the cross-reactivity with proBDNF. Moreover, the standard BDNF molecules which are supplied with other ELISA reagents may not be standardized among different kit suppliers, so that it can result in different BDNF levels in different supplier ELISA kits.

To solve the sample variation problem, a large well-characterised cohort could be essential. Recently, the Australian Imaging, Biomarker and Lifestyle (AIBL) study recruited an AD cohort consisting of over 1000 participants (Ellis, Bush et al. 2009). I will be using samples for my PhD study from this cohort. The more details about the AIBL cohort and studies with the cohort are discussed below.

1.3.2.1 Australian Imaging, Biomarker and Lifestyle (AIBL) cohort

In 2005, the Australian Commonwealth Scientific Industrial and Research Organisation (CSIRO) made a partnership with many researchers and research institutes in Melbourne and Perth (Australia). The goal was 1) to pool a cohort of individuals to be evaluated and compared over a long period of time; 2) to assess the participant tissues, amyloid imaging in brain and lifestyle factors and to compare these in relation to the participant's cognitive function with aiming to stimulate future AD research. As a result, over 1000 participants were recruited; 211 patients with AD, 133 patients with mild cognitive impairment and 768 healthy controls; as such the AIBL cohort has become the largest AD cohort in Australia and one of the largest in the world. Cognitive and mood tests were conducted to categorise the participants based on internationally acknowledged method such as Minimental State Examination (MMSE) (Folstein et al. 1975). Volunteers visited clinics at base-line and 18 months follow-ups. Therefore, the AIBL cohort is a large and highly characterised cohort (Ellis et al. 2009) and includes sufficient numbers in each category based on their clinical stages.

1.3.2.2 Biomarkers studied in the AIBL cohort

Several studies have reported the correlations of potential biomarkers in AD with AIBL cohort (Lui, Laws et al. 2010, Gupta, Laws et al. 2011, Villain, Chetelat et al. 2012, Brown, Peiffer et al. 2013, Lim, Ellis et al. 2013, Lim, Villemagne et al. 2013, Lim, Villemagne et al. 2014). Lui et al. (2010) measured plasma $A\beta_{1-40}$, $A\beta_{1-42}$ and their N-terminal fragments ($A\beta_{n-40}$ and $A\beta_{n-42}$) by ELISA in 1032 human subjects from the AIBL cohort. Further analysis showed the lower level of $A\beta_{1-42}$ and ratio of $A\beta_{1-40}/A\beta_{1-42}$ in AD patients and negative correlation with Pittsburgh compound B (PiB) positron-emission tomography (PET)

(Lui, Laws et al. 2010). This result suggests that the higher Aβ $_{1-42}$ exists in plasma the less amyloid plaque forms in brain.

Although several studies have been reported using the AIBL cohort, the levels of mature BDNF levels have not been assessed in this cohort yet. Furthermore, studies investigating serum BDNF levels in AD have produced inconsistent results as described above. In addition, cognitive decline and amyloid burden are hallmarks of AD or aged poeple. Therefore, we aim to analyse mature BDNF serum levels in AD using the AIBL cohort and to examine whether serum mBDNF is correlated with cognitive decline or amyloid burden which has been previously assessed in the AIBL cohort.

1.4 Hypotheses and aims

1.4.1 Hypotheses

1. HAP1 is required for BDNF and its receptors endocytosis to regulate TrkB downstream signalling pathway for neuronal proliferation.

2. The balance of mature BDNF/proBDNF and their receptors will alter in MDD.

3. Serum mature BDNF levels will correlate with cognitive decline and amyloid burden in AD patients

1.4.2 Aims

1. To establish the role of HAP1 in BDNF and its receptor endocytosis in neurons

2. To develop mature BDNF-specific ELISA

3. To investigate the levels of mature BDNF/proBDNF and their receptors in MDD

4. To analyse serum mature BDNF levels in AD and examine its correaltion with cognitive decline or amyloid burden

The aims of this project are presented in Fig. 1-9.

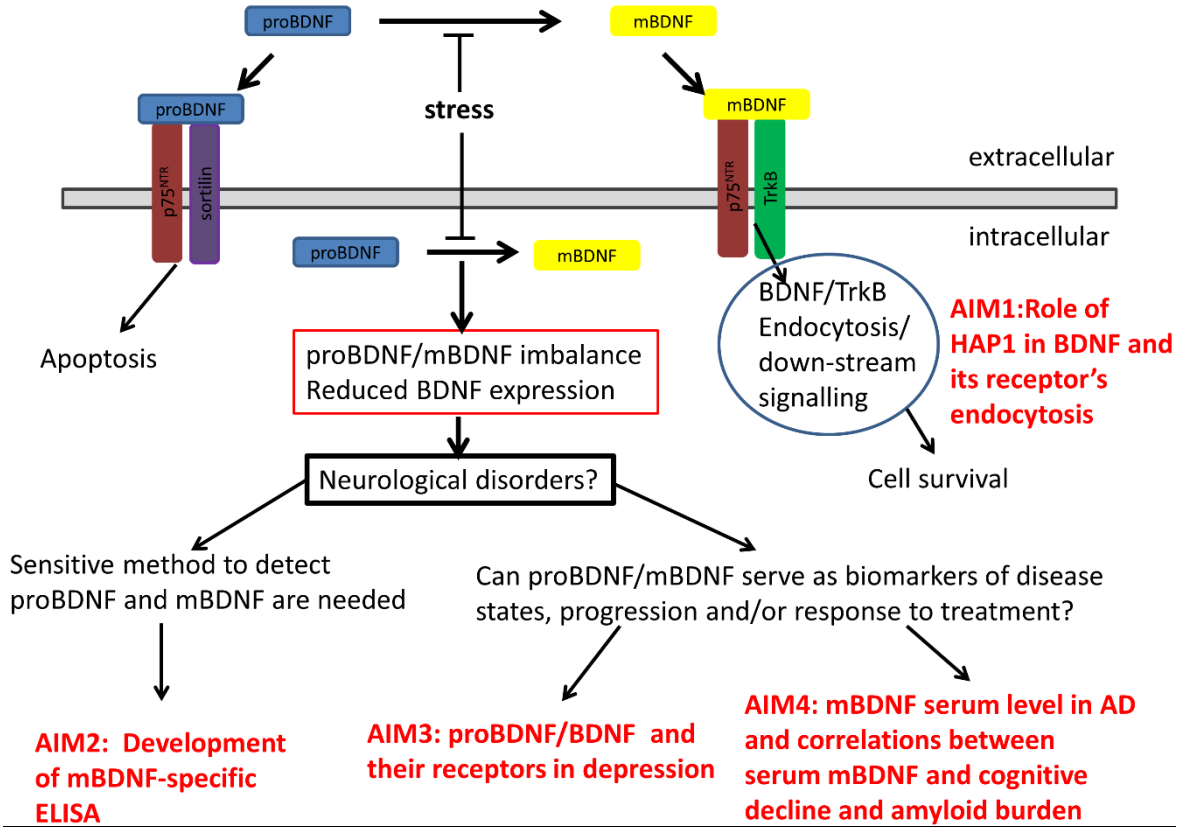


Figure 1-9 Graphical summary of aims of this project.

Chapter 2: HAP1 is required for endocytosis and signalling of BDNF and its receptors in neurons

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Yoon Lim: Designed and performed experiments, analysed data, interpreted the results of the experiments, prepared figures, drafted the manuscript. (Fig. 2-4, Fig. 2-5, Fig. 2-6, Fig. 2-7A, B)

Linda Lin-yan Wu: designed and performed experiment, analysed data and prepared figure (Fig. 2-1A, C)

Si Chen: performed experiment, analysed data (Fig. 2-1B, D and Fig.2-3)

Ying Sun: performed experiment, analysed data (Fig. 2-7C,D)

Swarna Lekha: designed and performed experiment (Fig. 2-2A,B)

Miao Yang: performed experiment (Fig. 2-2C, D)

Larisa Bobrovskaya: contributed to the critical manuscript revision

Xin-Fu Zhou: Conceived and designed experiments, contributed to the critical manuscript revision and acted as the corresponding author.

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2.1 Abstract

When brain derived nerve growth factor (BDNF) binds to its receptors, tyrosine receptor kinase B (TrkB) and p75 neurotrophin receptor ($p75^{NTR}$) the BDNF-receptor complex is endocytosed and trafficked to the cell body for down-stream signal transduction, which plays a critical role in neuronal survival, development, learning and memory. Huntingtin-associated protein 1 (HAP1) is involved in retrograde and anterograde trafficking of vesicles intracellularly and also interacts with several membrane proteins including amyloid precursor protein (APP), epidermal growth factor receptor, γ-amino butyric acid type A receptor androgen receptor and tyrosine receptor kinase A (TrkA) and TrkB. Although it has been known that HAP1 has functions in vesicular trafficking and receptor stabilization, it is not yet established whether HAP1 has a role in BDNF and its receptor endocytosis. Therefore, the aim of the present study is to investigate if HAP1 regulates endocytosis of BDNF and its receptors- the TrkB (cognate receptor) and $p75^{NTR}$ (pan receptor), and downstream signalling in neurons. We found that HAP1 is in an interacting complex with $p75^{NTR}$, TrkB and BDNF, especially newly endocytosed BDNF. BDNF and TrkB internalisation is abolished in HAP1 knock-out (KO) cortical neurons. TrkB down-stream signalling pathways such as extracellular signal-regulated kinase (ERK), Akt and phospholipase Cγ-1 (PLCγ-1) are also impaired in HAP1 KO cortical neurons upon BDNF stimulation. Proliferation of cerebellar granule cells is also impaired in cell culture and cerebellum of HAP1 KO mice. Our findings suggest that HAP1 may play a key role in BDNF and its receptor endocytosis and may promote neuronal survival and proliferation.

Key words: HAP1, BDNF, endocytosis, signalling endosome model, TrkB and $p75^{NTR}$

2.2 Introduction

Brain-derived neurotrophic factor (BDNF) is a member of neurotrophin growth factors which have pivotal roles in neuronal development and maintaining the nervous system functions in adult (Levi-Montalcini 1987, Lewin and Barde 1996, Huang and Reichardt 2001). When BDNF is initially synthesized, it is a precursor protein which is called pre-proBDNF. Cleaving of the signal peptide of pre-proBDNF generates proBDNF (34 kDa), followed by further processing of the prodomain of proBDNF to produce mature BDNF (will be called BDNF, 13 kDa) and this processing occurs intracellularly by furin or extracellularly by tissue plasminogen activator or matrix metalloproteases (Seidah, Benjannet et al. 1996, Mowla, Farhadi et al. 2001, Pang and Lu 2004, Nagappan, Zaitsev et al. 2009). A growing body of evidence has shown that proBDNF causes neuronal apoptosis with $p75^{NTR}$ sortilin and long-term depression while BDNF has a critical function in neuronal survival and long-term potentiation (Lee, Kermani et al. 2001, Pang and Lu 2004, Woo, Teng et al. 2005, Sun, Lim et al. 2012), suggesting that proBDNF and BDNF have opposite functions.

BDNF binds to tyrosine kinase B (TrkB) with high affinity and $p75^{NTR}$, a pan receptor, with low affinity (Huang and Reichardt 2003). When neurotrophins including BDNF bind to receptors at axonal terminals, the ligand-receptor complex is endocytosed and transported retrogradely along the axon to the cell body, which results in the initiation of signalling cascades locally (Seidah and Chretien 1999). The process involving receptor-mediated endocytosis and retrograde transport of neurotrophins and growth factors is called the signalling endosome model (Grimes, Zhou et al. 1996, Bartlett, Reynolds et al. 1998, Howe and Mobley 2004). It has been shown that the endocytosis of BDNF/TrkB complex is clathrin dependent and is essential for outgrowth of dendrites and neuronal survival (Zheng, Shen et al. 2008).

Huntingtin-associated protein 1 (HAP1) is the first identified protein that interacts with huntingtin (Htt) protein which causes Huntington disease (HD) when mutated (Li, Li et al. 1995). HAP1 KO mice often do not survive long after birth and die before the third day after birth owing to hypothalamic neuronal degeneration, resulting in feeding behaviour inhibition (Li, Yu et al. 2003). There have been two HAP1 isoforms reported, HAP1A and HAP1B, in rodents (Li, Gutekunst et al. 1998, Li, Hosseini et al. 1998) and there is only one human HAP1 isoform which shows 96% identity to the rat HAP1A (Li, Hosseini et al. 1998).

Growing evidence has shown that HAP1 is involved in retrograde and anterograde vesicular trafficking intracellularly by interacting with $p150^{Glued}$ (Engelender, Sharp et al. 1997, Bartlett, Brodie et al. 1998) and kinesin light chain (KLC) (McGuire, Rong et al. 2006), respectively. It has been also demonstrated that HAP1 interacts with other proteins which are involved in vesicular transport including Duo (Colomer, Engelender et al. 1997), hepatocyte growth factor-regulated tyrosine kinase substrate (Li, Chin et al. 2002), 14-3-3 protein (Rong, Li et al. 2007) and Abelson helper integration site 1 (Sheng, Xu et al. 2008). Our previous studies have shown that HAP1 directly interacts with the prodomain of proBDNF and sortilin to prevent its lysosomal degradation and regulates the anterograde transport and release of proBDNF (Wu, Fan et al. 2010, Yang, Lim et al. 2011).

HAP1 also interacts with several membrane receptor proteins including epidermal growth factor receptor (EGFR), γ-amino butyric acid type A receptor ($GABA_AR$), androgen receptor (AR) and tyrosine receptor kinase A (TrkA) and TrkB and plays a role in facilitating recycling of these receptors to the plasma membrane or stabilizing them to prevent lysosomal degradation (Li, Chin et al. 2002, Kittler, Thomas et al. 2004, Rong, McGuire et al. 2006, Rong, Li et al. 2007, Wu and Zhou 2009, Twelvetrees, Yuen et al. 2010, Xiang, Yang et al. 2014). Sheng et al. (2008) demonstrated that TrkB internalisation is reduced in cultured brainstem neurons when HAP1 expression is suppressed by HAP1 siRNA in the presence of

the BDNF and BDNF internalisation is reduced in the cultured brain stem cells of HAP1 KO mice (Sheng, Xu et al. 2008). However, in the study, TrkB expression level in the brainstem of HAP1 KO mice was also significantly lower than in WT mice, therefore the reduced BDNF internalization could be owing to reduced TrkB level. Recently, it has been also shown that HAP1 is associated with TrkB to protect TrkB from lysosomal degradation (Sheng, Xu et al. 2008). Although HAP1 is involved in signalling endosome trafficking and a few reports have demonstrated HAP1's association with internalisation of BDNF and TrkB, it is still uncertain whether HAP1 regulates BDNF and its receptors' endocytosis directly.

In the present study we have investigated whether HAP1 regulates endocytosis and signalling of BDNF and its receptors, TrkB and $p75^{NTR}$, using cell lines (PC12 and HEK293T) and cortical neurons from HAP1 KO mice (Li, Yu et al. 2003). We have found that endocytosed BDNF and HAP1 highly co-localize and BDNF endocytosis is blocked by antibodies to BDNF and $p75^{NTR}$. Also, TrkB and $p75^{NTR}$ interact with HAP1 and these interactions are increased by BDNF stimulation. In cultured HAP1 KO cortical neurons, TrkB internalisation and down-stream signalling are abolished. Moreover, the loss of HAP1 showed proliferation defect of cerebellar granule cells in response to BDNF both *in vitro* and *in vivo*.

2.3 Materials and Methods

2.3.1 Animals

All procedures involving animals were approved by the Animal Ethic Committee of SA Pathology (Adelaide, Australia) and the Animal Welfare Committee of Flinders University (Adelaide, Australia). All the procedures were undertaken according to the guidelines of the National Health and Medical Research Council of Australia. All animals were maintained

under 12-hour light/dark cycle and free access to food and water. HAP1 KO mice were generated as described previously (Li, Yu et al. 2003). HAP1 KO, HET (heterozygous for the HAP1 knockout allele) or WT neonatal mice were bred from transgenic breeding pairs heterozygous for the HAP1 knockout allele (C57/Black6 genetic background). PCR genotyping of HAP1 KO mice was carried out using primers 5'- TTTTGGAGG TCTGGTCTCGCTCTG- 3'/5'-CGTCTTCCATCTTAGTGCGTTCAC-3' for wild type (WT) and 5'-TTTTGGAGGTCTGGTCTCGCTCTG-3'/'5-CTTCATGTGGATGCTAGGGATCC-3' for KO animals.

2.3.2 Plasmids

The following plasmids were used in this study; $p75^{NTR}$- cyan fluorescent protein (CFP), $p75^{NTR}$- yellow fluorescent protein (YFP), $p75^{NTR}$- Hemagglutinin (HA) WT (all $p75^{NTR}$ plasmids were kindly donated by Prof. Goldstein University of California, San Diego, CA, USA), TrkB-YFP (kindly donated by Dr. Wayman, Washington State University, Pullman, WA, USA), $p75^{NTR}$-CFP-YFP (kindly donated by Dr. Coulson, University of Queensland, QLD, Australia), TrkB-Flag (kindly donated by Dr. Chen, Shandong University, Shandong, China) and pEYFP-N1 was obtained from Clontech (Mountain View, CA, USA). HAP1A-green fluorescent protein (GFP), HAP1A-CFP and HAP1-YFP were derived by subcloning a PCR product generated from PRK-HAP1A (kindly donated by Prof. Xiao-Jiang Li, Emory University, Atlanta, GA, USA) using primers (Forward: 5'-TAGCTAGCATGCGCCCGAAGGAC-3' and Reverse: 5'-GAGGTACCAGGGTTGATGATCGGTAGC-3') into the *NheI* and *KpnI* sites of pEGFP-N1, pECFP-N1 and pEYFP-N1 (Clontech, Mountain View, CA, USA). All cloned constructs were confirmed by DNA restriction enzyme and DNA sequence analysis in both directions. To produce BDNF with red fluorescence tag, the rat cDNA sequences of mature

BDNF (384bp) was amplified from a proBDNF-EGFP construct (kindly donated by Dr. Kojima**,** National Institute of Advanced Industrial Science and Technology, Osaka, Japan) using the following PCR primers (Forward: 5'-GCGAATT CATGCACTCCGACCCT-3' and Reverse: 5'- ATGGCGACCGGTGGATCCCT-3') with added *BamHI* and *EcoRI* site. The amplified fragment was cut with *BamHI* and *EcoRI* and subcloned in-frame into pDsRed-Express-N1 (Clontech, Mountain View, CA, USA) and the final constructs were verified by DNA restriction enzyme digestion and DNA sequencing.

2.3.3 Cell culture

HEK293T cells (ATCC, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technology, VIC, Australia) containing 10% fetal bovine serum (FBS), penicillin (50 IU/ml), streptomycin (50 µg/ml) and L-glutamine (2 mM) (Life Technology, VIC, Australia). PC12 cells (ATCC, Rockville, MD, USA) were cultured in DMEM medium supplemented with horse serum (5%) (Life Technology, VIC, Australia), FBS (10%), penicillin (50 IU/ml), streptomycin (50 µg/ml). All cells were maintained at 37°C in a humidified incubator supplemented with 95% O_2 and 5% CO_2 as described previously (Gueorguiev, Zeman et al. 1999).

2.3.4 Biotinylation of BDNF

To analyse endocytosed BDNF in cell imaging and biochemical assay, BDNF was biotinylated as described previously (Du, Feng et al. 2003) with minor modification.

It has been shown that biotinylated BDNF has biological activity (Lu, Ji et al. 2011, Liu, Obianyo et al. 2014). BDNF (100 µg, kindly donated by Amgen, Thousand Oaks, CA, USA) was dialysed against 1 x PBS (pH7.4) for 3 hours and PBS was changed two more times every 3 hours. The dialysed BDNF was incubated with 2 mg Sulfo-NHS-SS-biotin (Thermo

Fisher Scientific, Rockford, IL, USA) for 2 hours on ice. Free biotin was removed by dialysis against PBS.

2.3.5 BDNF- red fluorescent protein (RFP) preparation

As BDNF-RFP is secreted into the cell culture medium, we used the culture medium containing BDNF-RFP for the co-localisation assay. To prepare the culture medium containing BDNF-RFP, we transfected PC12 cells with pDs-Red-BDNF plasmid for 48 hours. The culture medium containing BDNF-RFP was then used immidately in the co-localisation assay.

2.3.6 The co-localisation assay

To investigate whether HAP1, BDNF and $p75^{NTR}$ co-localise with each other, co-localisation assay was performed. PC12 cells were seeded onto Poly-L-ornithine (mol. weight 30,000 – 70,000, 15 μg/ml; Sigma-Aldrich, St Louis, MO, USA) - coated 13 mm coverslips (Thermo Fisher Scientific, Rockford, IL, USA) and cultured for 3 days in differentiation culture medium containing 100 ng/ml nerve growth factor (NGF) before transfection to make an *in vitro* neuron like model (Das, Freudenrich et al. 2004). pEGFP-HAP1A only or pEGFP-HAP1A/pDs-Red-BDNF pair were mixed with NeuroPorter (Sigma-Aldrich, St Louis, MO, USA) in a ratio of 5 μl to 5 μl according to the instructions of NeuroPorter Transfection kit (Sigma-Aldrich, St Louis, MO, USA), followed by adding the mixture to the PC12 cells. The cells were then incubated for four hours at 37°C and the transfection mixtures were removed, followed by washing with PBS twice and adding 5 ml fresh NGF containing culture medium with incubation at 37°C for 48 hours in humidified air containing 5% CO2. The cells were cultured with sheep anti-BDNF antibody (10 μg/ml, in-house) alone, cell culture medium containing BDNF-RFP alone and either cell culture medium containing

BDNF-RFP with sheep anti-BDNF (10 µg/ml) or cell culture medium containing BDNF-RFP with rabbit anti-$p75^{NTR}$ antibody (10 µg/ml, a kind gift from Prof. Chao, Skirball Institute, New York, NY, USA). The cells were then fixed and images of fluorochromes (green and red) were obtained by Leica TCS SP5 (Leica Microsystems, Mannheim, Germany) for quantitative assessment of co-localisation between HAP1A and BDNF-RFP.

For immunocytochemistry co-localisation assay, differentiated PC12 cells were treated as above and BDNF-biotin (BDNF-B) was added at 100 ng/ml for 30min in DMEM containing horse serum (5%) (Life Technology, VIC, Australia), FBS (10%), penicillin (50 IU/ml) and streptomycin (50 µg/ml) (Life Technology, VIC, Australia), followed by acid wash (0.25 M acetic acid, 0.25 M NaCl) for 20 min to remove surface-bound BDNF-B. Immunostaining was performed with mouse anti-HAP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-$p75^{NTR}$ (a gift by Prof. Chao, Skirball Institute, New York, NY, USA), followed by anti-mouse-Alexa488 and anti-rabbit-Cy3 (Life Technology, VIC, Australia) and streptavidin-Cy5 (Life Technology, VIC, Australia) for BDNF-B after fixing cells. The images of fluorochromes were obtained by Leica TCS SP5 (Leica Microsystems, Mannheim, Germany) for quantitative assessment of co-localisation of HAP1, $p75^{NTR}$ and BDNF-B.

2.3.7 Co-immunoprecipitation (Co-IP) assay

Co-IP assay was performed to analyse the interaction between HAP1A/TrkB or HAP1A/$p75^{NTR}$ as described previously (Yang, Lim et al. 2011). HEK293T cells were co-transfected with HAP1A-YFP/TrkB-FLAG or HAP1A-YFP/$p75^{NTR}$-HA pair. The cells were treated with or without BDNF at 100 ng/ml for 1 hour in DMEM supplemented with 10% FBS, penicillin (50 IU/ml), streptomycin (50 µg/ml) and L-glutamine (2 mM) (Life Technology, VIC, Australia) and then lysed with RIPA buffer (50 mM Tris-HCl, 0.5% Sodium deoxycholate,1% NP-40, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, pH7.4)

supplemented with protease inhibitor cocktail (Roche, Castle Hill, NSW, Australia), followed by sonication and centrifugation at 12,000 g for 10 min at 4°C. The resultant supernatants were collected and BCA protein assay (Thermo Fisher Scientific, Rockford, IL, USA) was performed to determine protein concentrations. The supernatants (400 µg) were then incubated with 2 µg antibody (mouse anti-FLAG (Sigma-Aldrich, St Louis, MO, USA) for TrkB-FLAG/HAP1A-YFP, goat anti-GFP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for HAP1A-YFP/ $p75^{NTR}$-HA and mouse or goat IgG as a control for antibody specificity at 4°C overnight with rotation at 60 rpm. Protein G beads (30 µl; Thermo Fisher Scientific, Rockford, IL, USA) were added to the mixture and rotated for further two hours at 4°C in order to immobilize antibody and the control IgG. The beads were washed four times with PBST (PBS, 0.1% Tween-20) and boiled in 30 µl of 2 x SDS PAGE loading buffer (0.1 M Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue). The samples were then analysed by Western blotting to detect HAP1A-YFP with goat anti-GFP antibody and $p75^{NTR}$-HA using rabbit anti-HA antibody (Sigma-Aldrich, St Louis, MO, USA) respectively. At the same time, 2 µg of the original sample supernatant was loaded as an input control.

2.3.8 Fluorescence resonance energy transfer (FRET)

FRET (Fluorescence resonance energy transfer or Förster resonance energy transfer) is a non-radiative, distance-dependent (1-10nm), energy transfer from an excited fluorophore (donor) to another fluorophore (acceptor) and was performed as described previously (Pollok and Heim 1999, Yang, Lim et al. 2011). HEK293T cells were seeded (at $2.5x10^4$) on coverslips for overnight and then transfected with HAP1A-CFP/$p75^{NTR}$-YFP pair HAP1A-CFP/TrkB-YFP pair, positive control ($p75^{NTR}$-CFP-YFP) or negative control ($p75^{NTR}$ -CFP/YFP pair). BDNF was added to the pairs of HAP1A-CFP/$p75^{NTR}$-YFP and HAP1A-

CFP/TrkB-YFP at various concentrations (0, 5, 50 and 100 ng/ml). A Leica SP5 spectral confocal microscope was used to perform FRET according to the manufacturer's recommendations (Leica Microsystems, Mannheim, Germany). Briefly, acceptor bleaching was used to compare the donor fluorescence intensity in the same sample before and after photo bleaching the acceptor. If there is FRET, the intensity of the donor fluorescence increases after photo-bleaching the acceptor. The efficiency of FRET can be determined as: $FRET_{\text{eff}}$ $(D_{\text{post}}_D_{\text{pre}})/D_{\text{post}}_100\%$, where D_{post} is the fluorescence intensity of the Donor after acceptor photo bleaching, and D_{pre} is the fluorescence intensity of the donor before acceptor photo bleaching. When $FRET_{\text{eff}}$ is higher than background and negative controls, the $FRET_{\text{eff}}$ is considered positive. The higher the $FRET_{\text{eff}}$ the closer the two molecules are to each other based on the FRET principle. Each $FRET_{eff}$ value from the observed samples including positive and negative controls was determined according to a mean of six individual tests from different regions of interest. Three different experiments were conducted and the results were analysed by IBM SPSS Statistics 21.0 software (IBM, supplied by University of South Australia).

2.3.9 Primary Neuronal culture

Transgenic pups at postnatal day 0 (P0) to 1 (P1) were anaesthetised on ice-water slurry and killed by decapitation. After the brain meninges removal, the cortex from both hemispheres of each pup was separated from other parts of brain and transferred into 15 ml falcon tubes containing 2 ml DMEM and centrifuged at 2,000 rpm for 2 min at 4°C. DMEM was then removed and tissues were digested with 2 ml of 0.5% trypsin including 0.1% DNaseI (NEB, Ipswich, MA, USA) for 20 min at 37ºC with shaking every 5 min. FBS (final 15%) was added to stop the tissue digestion and the digested tissue was triturated by passing through a 10 ml serological pipette (Thermo Fisher Scientific, Rockford, IL, USA) for 10 times. When

tissue pieces settled, the supernatant was transferred into a new tube and spun at 2,000 rpm for 2 min at 4°C. The cell pellet was resuspended in 1 ml of cortical neuron culture media (Neurobasal medium containing B27 supplement (2%), L-glutamine (2 mM) and penicillin/streptomycin (100 IU/ml) (Life Technology, VIC, Australia) and β-mercaptoethanol (0.1 mM). In order to harvest neurons after treatment for western blotting or for immunocytochemistry assay, cortical cells were placed into poly-D-lysine and laminin-coated 6 well plate (at 2 x 10^6) and on poly-D-lysine-and laminin- coated coverslips (Thermo Fisher Scientific, Rockford, IL, USA) in 4- or 24-well plates (at 2.5 x 10^4) (Thermo Fisher Scientific, Rockford, IL, USA), respectively, and after 72 hours incubation at 37ºC supplemented with 95% O2, 5% CO2 incubator, neurons were ready for treatment.

2.3.10 Cell surface biotinylation assay

To analyse internalised TrkB and $p75^{NTR}$ in cultured cortical neurons, cell surface biotinylation assay was conducted as previously described (Zheng, Shen et al. 2008). Briefly, cortical neurons from P0 – P1 HAP1 WT and KO mice were isolated and cultured in 6-well plates for 72 hours in cortical neuron culture media. Thereafter, the cultured neurons were washed with Neurobasal medum twice and then kept in Neurobasal medium only without B27 supplement for 2 hours, followed by changing to cortical neuron culture media containing 0 (control) or 100 ng/ml of BDNF and incubation for 30 min in a 37°C incubator supplemented with 5% CO_2 and 95% O_2. Culture plates were then transferred onto ice and left for 10 min. The plates were then gently rinsed three times with chilled PBS-CM (PBS containing 1mM $CaCl_2$, 0.5mM $MgCl_2$, pH7.4), followed by incubation with 0.5 mg/ml Biotin-S-S-NHS (Thermo Fisher Scientific, Rockford, IL, USA) on ice for 60 min. The cell surface biotinylation reaction was quenched by washing twice (10 min each wash) with 100 mM glycine in PBS-CM with gentle rocking, followed by washing three times (5 min each

wash) with chilled PBS-CM with gentle rocking. Neurons were then lysed with RIPA buffer containing proteinase inhibitor cocktails (Roche, Castle Hill, NSW, Australia) and the lysates were collected by cell scraper. Cell extracts were then sonicated and centrifuged at 12,000 rpm for 10 min at 4°C. The resultant supernatants were transferred to fresh tubes and protein concentrations were determined by BCA protein assay (Thermo Fisher Scientific, Rockford, IL, USA). Total protein (40 - 60 µg) was mixed with streptavidin-agarose beads (Sigma-Aldrich, St Louis, MO, USA) and incubated overnight at 4°C and 20-30 µg of total protein was kept for total receptor detection. Next day, beads were washed 4 times with RIPA buffer and 30 µl of 2 x SDS-PAGE loading buffer (100 mM Tris-Cl (pH 6.8), 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM DTT (dithiothreitol)) was added, followed by boiling for 5 min. The samples were then subjected to Western blot analysis with goat anti-TrkB (R & D systems, Minneapolis, MN, USA), rabbit anti-$p75^{NTR}$ (from Prof. Reichardt, University of California, San Francisco, CA, USA) and mouse anti-β-actin (Sigma-Aldrich, St Louis, MO, USA) to detect surface and total TrkB and $p75^{NTR}$ and β-actin as internal control, respectively. The band densitometry of the cell surface protein versus total protein and β-actin were determined using ImageJ software (Research Service Branch; National Institute of Health, http://rsbweb.nih.gov/ij/index.html).

2.3.11 Reverse Transcription (RT)-PCR

Determination of TrkB mRNA expression levels was conducted according to the standard procedures. Total RNA was extracted from the cortex and brainstem of neonatal HAP1 WT (n=3) and KO (n=3) mice using an RNAeasy mini kit (Qiagen, Doncaster, Vic, Australia). The concentration and purity of RNA were determined with a NanoDrop 2000 (Thermo Fisher Scientific, Rockford, IL, USA). Five hundred ng of total RNA was used to generate first strand cDNA using Superscript III First-Strand Synthesis System (Life Technology,

VIC, Australia). PCR was conducted with GoTaq Green Master Mix (Promega, Madison, WI, USA) with primers for mouse TrkB (forward primer, 5'-CCTCCACGGATGTTGCTGAC-3'; and reverse primer, 5'-GCAACATCACCAGCAGGCA-3') and normalised against the housekeeping gene GAPDH (forward primer, 5'-AACATCATCCCTGCATCCAC-3'; and reverse primer, 5'-TTGAAGTCT CAGGAGACAAC-3'). RT-PCR was performed for 25 cycles in Veriti 96-well Thermal Cycler (Life Technology, VIC, Australia) at 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s. The densitometry of the PCR bands (TrkB and GAPDH) was measured using ImageJ software (Research Service Branch; National Institute of Health, http://rsbweb.nih.gov/ij/index.html). GAPDH was used for normalising TrkB mRNA expression.

2.3.12 TrkB signalling Western blot analysis

Cortical neurons were washed with Neurobasal medium and the neurons were incubated in Neurobasal medium only for 2 hours before treatment. Neurons were treated with 0 (control) or 100 ng/ml BDNF for 20 min at 37°C in an incubator supplemented with 5% CO_2 and 95% O_2. The tissue culture plate was transferred onto ice-water slurry to stop the treatment, followed by three washes with ice-cold PBS. Ice-cold phosphorylation lysis buffer containing 0.1% SDS, 1% Nondiet P-40, 0.5% sodium deoxycholate, 2 mM EDTA, 2 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride (PMSF),10 mM sodium fluoride, and protease inhibitor cocktail (Roche, Castle Hill, NSW, Australia) in PBS was then added to the cells. The cell lysates were sonicated for 15 s on ice-water slurry and centrifuged for 10 min at 12,000 rpm at 4 °C. The supernatants were then transferred to fresh microtubes followed by determining protein concentrations. The neuron lysates were separated by 10 % SDS-PAGE and transferred to Hybond-C membrane (GE Healthcare

Australia, NSW, Australia) and blocked with blocking buffer (5% skim milk in Tris-buffered saline (TBS) (150 mM NaCl, 50 mM Tris-Cl, pH 7.5) for 1.5 hours at 25°C with gentle shaking. After blocking, membranes were incubated on a rocker at 4°C with one of the following primary antibodies: rabbit anti-phospho- extracellular-regulated kinases 1 and 2 (ERK1/2), rabbit anti- ERK1/2, rabbit anti-phospholipase Cγ 1 (PLCγ-1), rabbit anti-phospho-Akt1/2/3, rabbit anti-Akt1/2/3 (Cell Signalling, Danvers, MA, USA), anti-phospho-PLCγ1 (Abcam, Cambridge, UK), and mouse anti-β-actin (Sigma-Aldrich, St Louis, MO, USA). Thereafter, corresponding secondary antibodies linked to horse radish peroxidase were applied onto the membranes for 1 hour at 25°C. Band densities were determined using Image Quant LAS 4000 (GE Healthcare Australia, NSW, Australia). All phosphorylated protein bands were normalised to total protein and GAPDH band to yield the densitometry ratio value. For TrkB Western blot, the cortex and brainstem tissue of neonatal HAP1 WT (n=3) and KO (n=3) mice were homogenised in RIPA Buffer. The proteins (30 µg) were subjected to Western blot analysis as described above. Goat anti-TrkB (R & D systems, Minneapolis, MN, USA) was used and mouse anti-β-actin (Sigma-Aldrich, St Louis, MO, USA) was used for loading control.

2.3.13 Proliferation assay of cerebellar granule cells *in vitro* and *in vivo*

For an *in vitro* experiment, cerebellar neurons were prepared similar to cortical neuron culture method. Briefly, cerebella from P0 HAP1 WT (n=2) and KO (n=2) mice were dissected after meninges removal from the whole brain and cut into 3-4 pieces followed by digestion in 2 ml of 0.5% trypsin including 0.1% DNaseI (NEB, Ipswich, MA, USA) for 20 min at 37ºC with shaking every 5 min. To stop the digestion, 300 µl of FBS was added. The tissue pieces were triturated 10 times with a fire-polished pasteur glass pipette and the tube was left undisturbed for 5 min for the tissue debris to settle. The cell suspension was then

centrifuged at 2,000 rpm for 2 min at 4°C and the cell pellet was resuspended in Neurobasal medium containing FBS (2%), B27 supplement (2%), N-2 (1%), L-glutamine (2 mM) (Life Technology, VIC, Australia), β-mercaptoethanol (0.1 mM). The viable cells were counted using trypan blue (Sigma-Aldrich, St Louis, MO, USA) to exclude dead cells and 5 x 10^4 cerebellar granule cells were seeded onto poly-D-lysine-coated 13 mm cover slip (Thermo Fisher Scientific, Rockford, IL, USA) and cultured. Next day, cultured cerebellar neurons were treated with cerebellar neuron culture medium containing BDNF (0, 10, 100 ng/ml) and 10 µg/ml of bromodeoxyuridine (BrdU) (Sigma-Aldrich, St Louis, MO, USA) to label cells in S phase for 24 hours. Then the cells were subjected to immunocytochemistry. Cultured cells were fixed in 4% Paraformaldehyde, PBS pH 7.4 for 10 min at room temperature, followed by TBS wash. The cells were then kept in 2N HCL at 37ºC for 1 hr to expose BrdU, followed by TBS wash. Thereafter, the cells were then incubated with blocking buffer (3% Bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO, USA), TBS, 0.1% Triton X-100, 0.01% NaN3, pH 7.4) for 1 hour at 25° C, then incubated with an anti-BrdU monoclonal antibody (G3G4) (DSHB, Iowa city, Iowa, USA), followed by a Cy3-conjugaetd goat anti-mouse IgG (Sigma-Aldrich, St Louis, MO, USA).The images were taken by CX40 fluorescence microscope (Olympus, VIC, Australia) and BrdU-positive cells were counted using ImagaJ software (Research Service Branch; National Institute of Health, http://rsbweb.nih.gov/ij/index.html) . Six fields were checked in each cover slip and the same locations of the six fields were applied to the all observed cover slips. Three and four cover slips were observed for 0 and 10 and 100 ng/ml of BDNF treatment samples of each genotype, respectively.

In vivo experiment was conducted as described previously (Xu, Sun et al. 2011). Briefly, neonatal mice were immobilized under a stereomicroscope with anaesthesia on ice. PBS (1 µl, control) or BDNF (1 µl, 5 µg) were injected into the lateral ventricle slowly (n = 6/group) at the injection target (0.5 mm lateral to the midline, 1 mm caudal to bregma, and 1.5 mm in

depth). To label proliferating cells, BrdU (50 mg/kg; Sigma-Aldrich, St Louis, MO, USA) was injected subcutaneously after the lateral ventricle injection and the animals were kept for 24 hours in their home cage. Mice were perfused with cold 4% paraformaldehyde after anaesthesia. Dissected brain tissues were submerged into 30% sucrose in 0.1M phosphate buffer for cryoprotection. Sagittal section cutting (25 μm) was performed on a microtome (Leica, Mannheim, Germany) and the cut sections were mounted on gelatin-treated slides. The sections were incubated in 0.5% Triton X-100, followed by incubation in 2N HCl at 37ºC for 1 hour to expose BrdU incorporated into DNA. Thereafter, the sections were washed three times with PBS and blocked with blocking solution containing 5% donkey serum (Sigma-Aldrich, St Louis, MO, USA) for 1 hour at 25°C. Anti-BrdU monoclonal antibody (G3G4) (DSHB, Iowa city, Iowa, USA) was added and incubated overnight at 4ºC. After washing, samples were then incubated Cy3-conjugated goat anti-mouse IgG (Sigma-Aldrich, St Louis, MO, USA). The number of BrdU-positive granule cells was counted with Olympus BX50 (Olympus, VIC, Australia) with 20 × objective.

2.3.14 Statistical analysis

The data are presented as mean ± SEM (standard error of the mean) and it was considered significant when $p < 0.05$. Student's *t* test or one-way ANOVA with post hoc test were used to analyse the data with intra or inter group. All statistical analyses were conducted using IBM Statistics 21 software (IBM, supplied by University of South Australia).

2.4 Results

2.4.1 Endocytosed BDNF highly co-localises with HAP1

As HAP1 co-localises with proBDNF and regulates its anterograde transport and release (Wu, Fan et al. 2010), we examined whether HAP1 co-localises with BDNF and $p75^{NTR}$ in neuron-like PC12 cells; we could not investigate HAP1 co-localisation with TrkB in these cells, because PC12 cells do not express TrkB (Lou, Yano et al. 2001, Howe and Mobley 2004, Saxena, Howe et al. 2004). We co-transfected these cells with the two plasmids, HAP1A-GFP and BDNF-RFP and found that HAP1A-GFP and BDNF-RFP co-localise (34%) in PC12 cells after co-transfection (Fig. 2-1A-a, B). This result indicates that HAP1 may have a relationship with BDNF. However, as transfected BDNF-RFP can be released and then endocytosed, it is not clear whether HAP1 co-localises with newly endocytosed BDNF or intracellular BDNF. To examine this issue, we incubated transfected cells with antibody to BDNF to neutralise secreted BDNF-RFP and found that the co-localisation significantly decreased to 13%, $p < 0.01$) (Fig. 2-1A-a, B). This result indicates that HAP1 co-localises with a large proportion of endocytosed BDNF rather than intracellular BDNF within the transfected cells. To confirm whether endocytosed BDNF highly co-localises with HAP1, PC12 cells were transfected with HAP1A-GFP only and the cell culture medium containing RFP-BDNF was added exogenously (BDNF-exo) in the absence or presence of antibodies to BDNF or $p75^{NTR}$ extracellular domain which is a pan receptor of BDNF (Huang and Reichardt 2003). While the newly endocytosed BDNF-RFP showed high co-localisation (90%, $p < 0.001$) with HAP1A-GFP (Fig. 2-1A-c, B), the co-localisation of BDNF-RFP with HAP1A-GFP was dramatically decreased (to 10%, $p < 0.001$) in the presence of antibody to BDNF (Fig. 2-1A-d, B) or $p75^{NTR}$ (Fig. 2-1e, B). These results demonstrate that HAP1 is clearly associated with endocytosed BDNF but not with the

intracellular BDNF which is synthesised within the cells, suggesting HAP1 may be associated with BDNF endocytosis.

As HAP1 is associated with TrkA (Rong, McGuire et al. 2006) and TrkB (Xiang, Yang et al. 2014), we next investigated whether $p75^{NTR}$ co-localises with HAP1 and endocytosed BDNF. In this experiment, BDNF-biotin (BDNF-B) was used as it is a pure single molecule. PC12 cells were treated with or without BDNF-B and HAP1; $p75^{NTR}$ and BDNF-B were immunostained for co-localisation analysis. Whereas only a small fraction (less than 20%) of HAP1 (green) co-localises with $p75^{NTR}$ (red) in PC12 cells (Fig. 2-1C-d, e, f, j, D) in the absence of BDNF-B, BDNF-B induced over 90% of co-localisation (Fig. 2-1C-a, b, c, g, D; $p < 0.001$ vs without BDNF-B). Furthermore, internalised BNDF-B (blue) (Fig. 2-1C-h) also co-localises with the HAP1/$p75^{NTR}$ vesicles (Fig. 2-1C-i). The co-localisation of $p75^{NTR}$/BDNF-B and HAP1/BDNF-B was 81% ($p < 0.001$) and 91% ($p < 0.001$), respectively. This result shows that BDNF stimulation induces the recruitment of HAP1 molecule to the complexes of $p75^{NTR}$/BDNF.

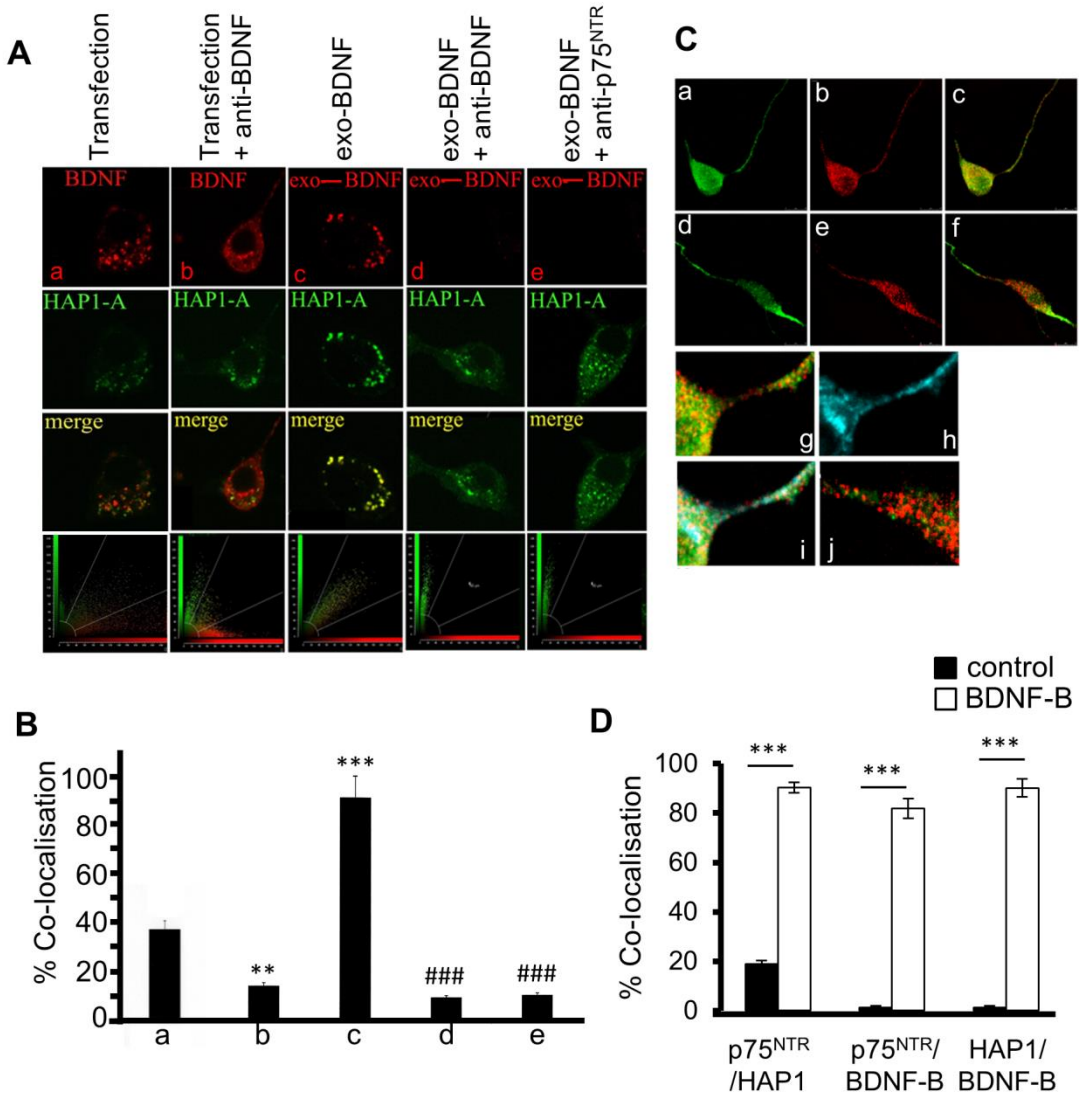


Figure 2-1 HAP1 and p75NTR are highly co-localise with endocytosed BDNF.
A) PC12 cells were transfected with HAP1A-GFP (green)/BDNF-RFP (red) or HAP1A-GFP only. Cells were co-transfected with HAP1A-GFP/BDNF-RFP (a) or cultured in the presence of sheep anti-BDNF to neutralise the secreted BDNF after the co-transfection (b). (c), (d), and (e) show that PC12 cells were transfected only with HAP1A-GFP plasmid. Two days later, cells were incubated with either cell culture medium containing RFP-BDNF (exo-BDNF) only (c), or exo-BDNF with sheep anti-BDNF antibody (d), or exo-BDNF with $p75^{NTR}$ antibody which is against the extracellular domain of $p75^{NTR}$ (e). Co-localisation is indicated in merged panels (yellow). **B)** Plots presenting co-localisation results in (A). Co-localisation of HAP1A-GFP/BDNF-RFP after co-transfection with anti-BDNF treatment was reduced (**$p < 0.01$ vs (a)). Addition of exo-BDNF increased the co-localisation with HAP1A-GFP (***$p < 0.001$ vs (a)). Addition of antibody to BDNF or $p75^{NTR}$ with exo-BDNF decreased co-localisation of HAP1A-GFP/exo-BDNF (###$p < 0.001$ vs (c)). The data are presented as mean ± SEM (n=20 per group, One-way ANOVA). **C)** PC12 cells were cultured with BDNF-biotin (BDNF-B) (a, b, c, g) or without BDNF-biotin (BDNF-B) (d, e, f, j) for 30 min and immunostained for HAP1 (a, d; green) and $p75^{NTR}$ (b, e; red); c) and f), merged images (yellow); g) and j), enlarged images of parts of c) and f), respectively; h), stained for -BDNF-B (blue); i) merged image of h) and g). **D)** Plots presenting the co-localisation results in (C). The data are presented as mean ± SEM (n=9 per group, ***$p < 0.001$ vs corresponding control; student's t-test).

2.4.2 BDNF stimulates the interactions of HAP1/TrkB and HAP1/p75^{NTR}

As TrkB is the receptor for BDNF (Huang and Reichardt 2003), we performed two different experiments, FRET and Co-IP, to confirm that HAP1 interacts with TrkB and p75^{NTR} using HEK293T cells. The result from the FRET experiment revealed that HAP1A highly interacts with TrkB ($p < 0.001$) compared to negative control (pEYFP/p75^{NTR}-CFP) and that BDNF significantly increased the HAP1A/TrkB interaction ($p < 0.001$) at 100 ng/ml (Fig. 2-2A). Compared to negative control, there was a FRET signal between HAP1A and p75^{NTR} ($p < 0.001$), and BDNF also triggered significant increase in the FRET signals between HAP1A and p75^{NTR} in a dose dependent manner up to 100 ng/ml (50 ng/ml, $p < 0.01$; 100 ng/ml, $p < 0.001$) compared to control (0 ng/ml) (Fig. 2-2B These data indicate that HAP1 interacts with both BDNF receptors TrkB and p75^{NTR} and TrkB, and BDNF can increase such interactions. Co-IP was employed to confirm the interaction of HAP1A/TrkB and HAP1A/p75^{NTR} in HEK293T cells. The Co-IP result demonstrated that TrkB was present in the cell lysate immunoprecipitated by anti-FLAG antibody and addition of exogenous BDNF increased the interaction of TrkB-HAP1A complex significantly by 2 fold ($p < 0.001$) (Fig. 2-2C, E). There was no immunoprecipitated TrkB using mouse IgG used as a negative control (Fig. 2-2C). BDNF also increased the level of p75^{NTR} pulled down by anti-GFP antibody significantly by 3.5 fold ($p < 0.001$) (Fig. 2-2D, F). Given that HAP1 appears to co-localise with p75^{NTR} when TrkB is not expressed in PC12 cells, TrkB and p75^{NTR} may be in separate complexes with HAP1 recruited in response to BDNF stimulation.

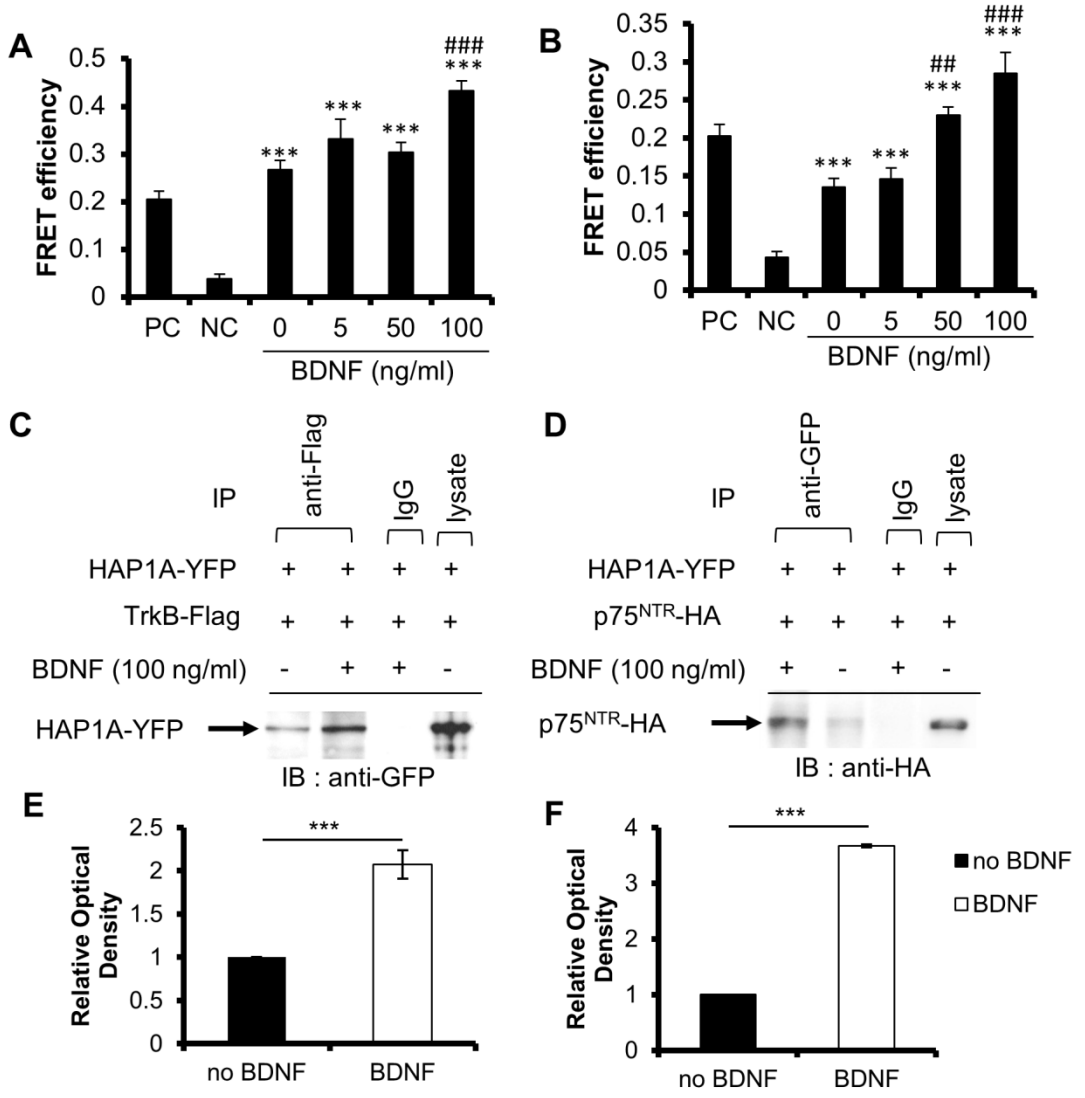


Figure 2-2 Interaction of HAP1 /TrkB and HAP1 /p75NTR is increased by BDNF treatment in HEK293T cells.

A) Graph presenting FRET acceptor bleaching analysis for HAP1A-YFP/ TrkB-CFP interaction compared to NC or control (0 ng/ml BDNF). BDNF increases the HAP1A/TrkB interaction (***$p < 0.001$ vs NC; ###$p < 0.001$ vs 0 ng/ml BDNF). B) Graph presenting FRET acceptor bleaching analysis for HAP1A-YFP/ p75NTR-CFP interaction compared to NC or control (0 ng/ml BDNF). BDNF increases the HAP1A/ p75NTR interaction (***$p < 0.001$ vs NC; ##$p < 0.01$, ###$p < 0.001$ vs 0 ng/ml BDNF). For A) and B), data represent mean ± SEM (n=5-11 per group, One-way ANOVA, Tukey's post-hoc test; PC, Positive Control (p75NTR-EYFP-CFP); NC, Negative Control (pEYFP/p75NTR-CFP). C) BDNF increases the level of HAP1A-YFP pulled down by mouse anti-flag antibody (TrkB fused with Flag) as indicated. Lane 1: no BDNF; lane 2: BDNF (100 ng/ml); lane 3: mouse IgG as negative control; lane 4: cell lysate input; IP, immunoprecipitation; IB, immunoblot. D) BDNF increases the level of p75NTR-HA pulled down by anti-GFP antibody (HAP1 fused with YFP) from lysate of HEK293T cells co-transfected with p75NTR-HA and HAP1A-YFP as indicated.Lane 1: BDNF (100 ng/ml); lane 2: no BDNF; lane 3: goat IgG as negative control; lane 4: cell lysate input; IP, immunoprecipitation; IB, immunoblot. E) and F) Bar graphs presenting relative optical density to 'no BDNF' in C) and D) (n=3 per plasmid pair, mean ± SEM from 3 experiments, ***$p < 0.001$, Student's t-test).

2.4.3 HAP1 is required for the endocytosis of BDNF

It has been shown that BDNF endocytosis is reduced significantly in cultured brainstem cells of HAP1 KO (Sheng, Xu et al. 2008) and BDNF is endocytosed through TrkB preferably (McKay, Garner et al. 1996). We therefore tested whether BDNF endocytosis could be impaired in cortical neurons of HAP1 KO mice. Cultured cortical neurons from HAP1 WT, HET or KO were treated with BDNF-B for 30 min and the cells were then immunostained to determine endocytosed BDNF-B. We found that BDNF-B was internalized in cortical neurons from HAP1 WT and HET mice but not KO mice ($p < 0.001$) (Fig. 2-3A, B). We then transfected HAP1 KO neurons with HAP1A-CFP plasmid toexamine whether the HAP1A cDNA could rescue the impaired endocytosis. Interestingly, the result showed that the transfection of HAP1 KO neurons with HAP1A plasmid rescued the internalisation of BDNF-B ($p < 0.001$) (Fig. 2-3A bottom panel, B). To support this finding, endocytosed BDNF-B was pulled-down by streptavidin-agarose beads in cortical neuron lysate of HAP1 WT or KO mice after BDNF-B treatment. We found that BDNF-B was detected in only WT neuronal lysate but not in KO (Fig. 2-3C) neurons. Taken together, these findings propose that HAP1 has a critical role in BDNF endocytosis in cortical neurons.

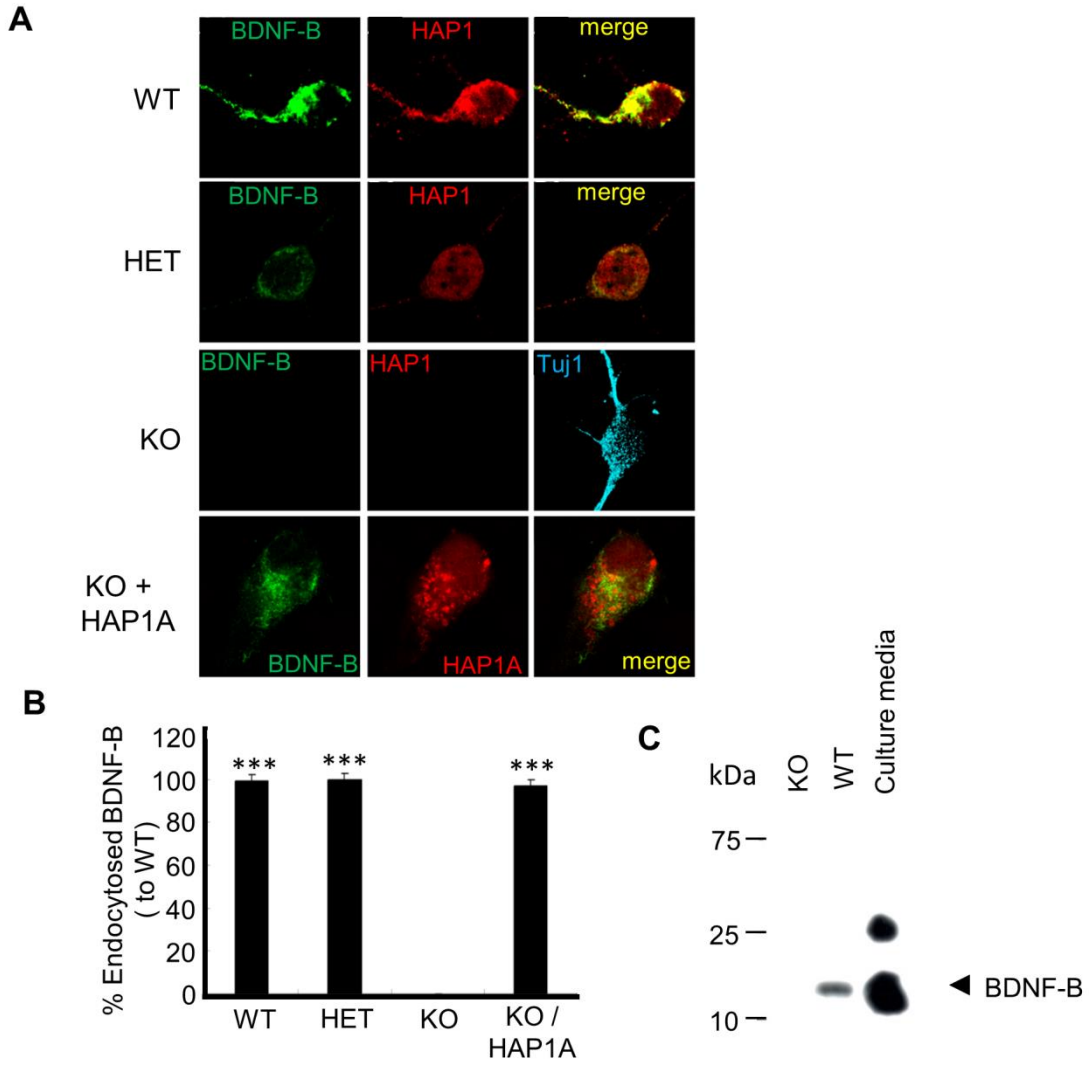


Figure 2-3 Endocytosis of BDNF-B is abolished in cultured cortical neurons from HAP1 KO mice.

A) Cortical neurons from P1 HAP1 WT, HET or KO mice were cultured for 3 days, incubated with BDNF-B for 30 min and immunostained for BDNF-B (green), HAP1 and Tuj1 (blue). Genotypes and transfection of KO are labelled at left side of image panels. For rescue experiment, HAP1KO cortical neurons were transfected with HAP1A-CFP. Merged image is indicated (yellow). **B)** Quantification of endocytosed BDNF-B; data are represented as the mean ± SEM (n=50 per group, ***p < 0.001 vs KO, One-way ANOVA, Tukey's post-hoc test). **C)** Cultured cortical neurons from HAP1 KO (lane 1) and WT mice (lane 2) were incubated with BDNF-B (100 ng/ml) for 30 min at 37ºC. Culture media containing BDNF-B (lane 3) and cell lysates were incubated with strepavidin-sepharose beads and samples were used for SDS-PAGE and probed with streptavidin-HRP. BDNF-B can be detected in WT neurons but not HAP1 KO neurons. The high molecular weight in supernatant is the dimer of BDNF-B.

2.4.4 HAP1 is required for endocytosis of TrkB but not p75^{NTR} in mouse cortical neurons

Previous studies showed that TrkB internalisation is reduced in neurons form the brain stem in which HAP1 expression is suppressed (Sheng, Xu et al. 2008). Our data from the current study suggest that HAP1 may be also involved in the endocytosis of BDNF receptors TrkB and p75^{NTR}. To test the hypothesis, we determined the neuronal surface level of TrkB and p75^{NTR} after BDNF treatment by surface biotinylation. As the TrkB/BDNF complex gets endocytosed, we expected less surface TrkB will be detected in BDNF-treated cells compared to no treatment control in WT neurons. Cultured cortical neurons from WT and HAP1 KO mice were incubated with or without BDNF-containing culture medium, followed by cell surface biotinylation. We have found that the surface TrkB-FL (140 kDa) level was reduced (by 47%, $p < 0.01$ vs control) upon BDNF stimulation of HAP1 WT neurons while no difference was observed in HAP1 KO neurons after BDNF stimulation (Fig. 2-4, A and B). As TrkB-T1 (95 kDa) has been found to bind to BDNF and get endocytosed together with BDNF (Alderson, Curtis et al. 2000) and to inhibit BDNF signalling in Xenopus oocytes (Eide, Vining et al. 1996), we also examined whether surface TrkB-T1 levels altered on BDNF stimulation in HAP1 KO and WT and found that surface TrkB-T1 levels were not changed in HAP1 WT or KO on BDNF stimulation (Fig. 2-4, A and C). This result suggests that HAP1 KO neurons did not internalize cell surface TrkB and truncated TrkB was not internalized in response to BDNF. Surprisingly, no reduction of surface p75^{NTR} level was found on BDNF stimulation of HAP1 WT or KO cortical neurons (Fig. 2-4, A and D). Through these results, we suggest that HAP1 may play a critical role in BDNF endocytosis by regulating TrkB endocytosis but not p75^{NTR} in cortical neurons.

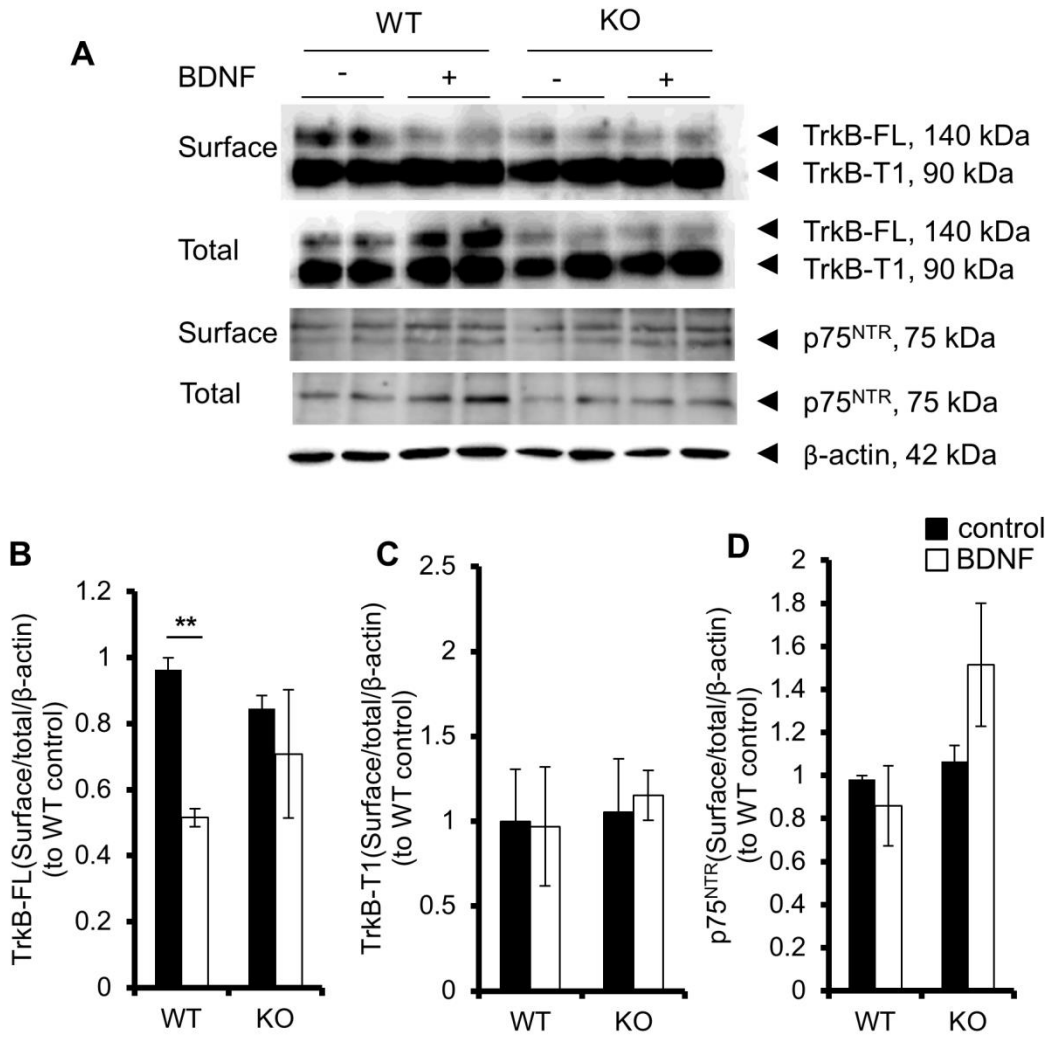


Figure 2-4 Endocytosis of TrkB and p75NTR in cortical neurons upon BDNF stimulation.

A) Western blots showing BDNF-induced endocytosis of TrkB and $p75^{NTR}$. HAP1 WT and KO cortical neurons, after 72 hour culture, were stimulated with 0 (control) or 100 ng/ml BDNF for 30 min. Surface TrkB-FL, TrkB-T1 and $p75^{NTR}$ were detected by Western blotting using anti-TrkB and anti- $p75^{NTR}$ antibodies, respectively, after surface protein biotinylation and fractionation of biotinylated proteins with streptavidin-agarose. Total TrkB-FL, TrkB-T1, $p75^{NTR}$ and β-actin were determined using whole cell lysates by Western blot with anti-TrkB, anti-$p75^{NTR}$ and anti-β-actin antibody, respectively. **B, C, D**) Densitometric ratios of surface/total TrkB-FL levels (B), surface/total TrkB-T1 levels (C) and surface/total $p75^{NTR}$ levels (C) assessed by biotinylation assay. Data presented as mean ± SEM (n=3 per group, student's t-test) . "WT control" was normalised to 1. TrkB-FL, full-length of TrkB; TrkB-T1, truncated TrkB.

2.4.5 TrkB expression is not reduced in cortex of HAP1 KO mice

It has been reported that TrkB protein expression level is reduced in cerebellum, hypothalamus and brainstem in HAP1 KO neonates (Sheng, Xu et al. 2008, Xiang, Yang et al. 2014). To investigate whether the impaired TrkB internalisation in HAP1 KO cortical neurons could be owing to the reduced TrkB expression level itself, the mRNA and protein levels of TrkB were examined in cortex and brainstem tissue of neonates (P 0) of HAP1 WT and KO, respectively. We have found that the TrkB mRNA level was reduced significantly in brainstem ($p < 0.05$) but not in the cortex of HAP1 KO compared to WT however there was no difference found in cortex (Fig.2-5, A and C). Similarly, the protein level of TrkB was also reduced in the brainstem ($P < 0.05$) but not in the cortex of HAP1 KO compared to WT (Fig. 2-5, B and D). These results confirm that impaired TrkB internalisation is caused by absence of HAP1 rather than decreased levels of TrkB, because HAP1 gene deletion does not affect TrkB mRNA and protein level in the cortex. However, HAP1 gene deletion reduced TrkB mRNA and protein levels in the brainstem, suggesting that HAP1 gene loss may affect the transcription level of TrkB in the brainstem.

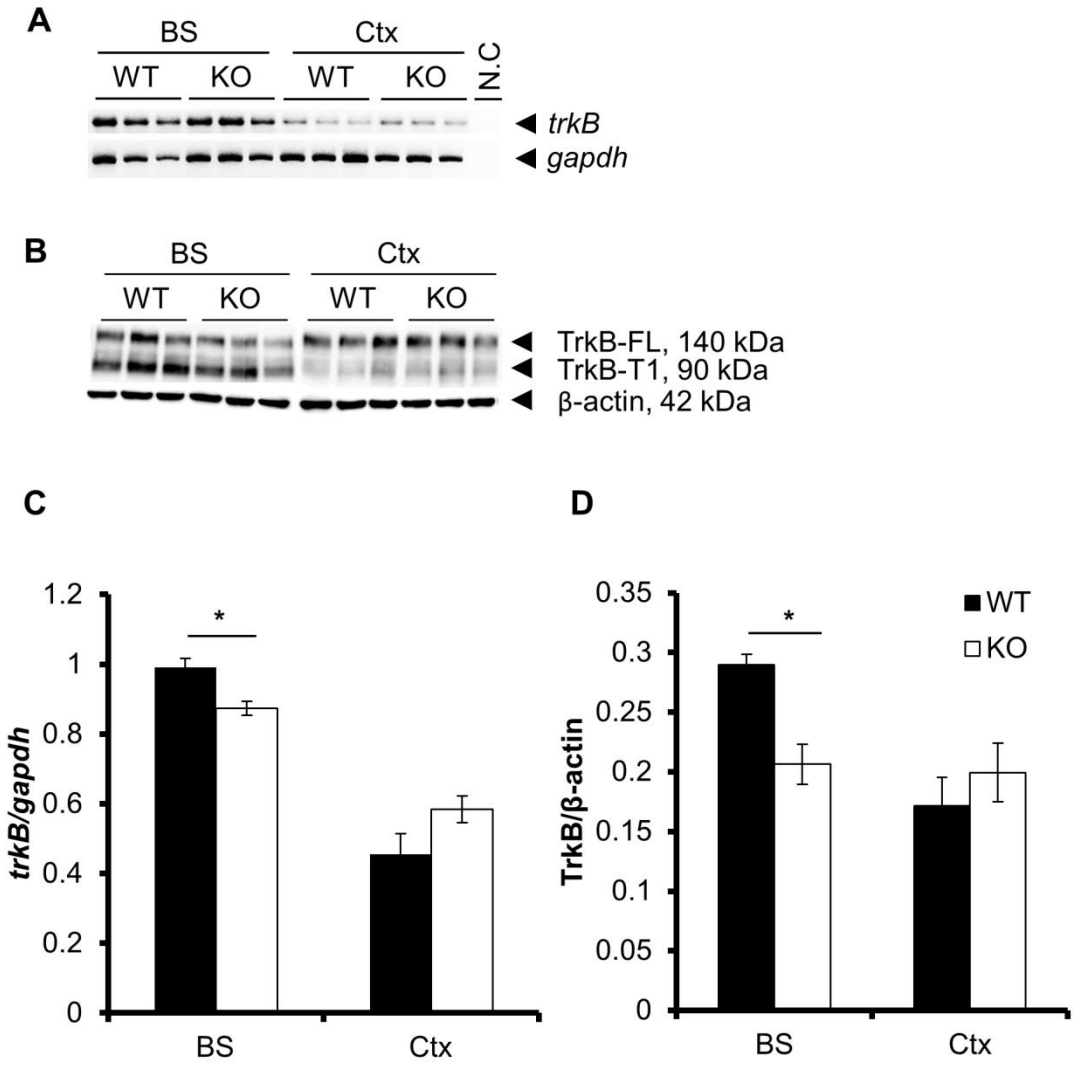


Figure 2-5 Semi-quantitative RT-PCR of trkB mRNA and TrkB protein level in the cortex and brainstem tissue.

The mRNA expression levels were determined in the cortex and brainstem of HAP1 WT and HAP1 KO. **A)** *trkB* and *gapdh* RT-PCR products were subjected to agarose gel electrophoresis. *Gapdh* was used as internal control. **B)** Western blot analysis of cortex and brainstem tissues from WT and HAP1 KO mice. **C, D)** Quantification of densitometry of the ratios of *trkB/gapdh (C) and* TrkB/ β-actin (D) in HAP1 WT and KO cortex or brainstem. Data are presented as mean ± SEM (n=3 per group, student's t-test, *$p < 0.05$). BS, brainstem; Ctx, cortex.

2.4.6 HAP1 is required for BDNF-induced downstream signalling

TrkB activation by BDNF binding triggers the down-stream signalling including phosphorylation of ERK, Akt and PLCγ-1(Huang and Reichardt 2003). The abolished endocytosis of BDNF and TrkB in cortical neurons from HAP1 KO mice led us to hypothesize that its downstream signal transduction pathways may also be affected. To test the hypothesis, we compared the phosphorylated ERK (pERK), Akt (pAkt) and PLCγ-1 (pPLCγ-1) under BDNF treatment between WT and HAP1 KO cortical neurons by Western blotting. We have demonstrated that the levels of pERK ($p < 0.01$), pAkt ($p < 0.001$) and pPLCγ-1 ($p < 0.05$) after BDNF treatment for 15 min in HAP1 WT neurons were significantly increased (1.53 fold) compared to WT control (0 ng/ml BDNF) neurons (Fig. 2-6, A-D). However, there was no increase of phosphorylation on ERK, Akt and PLCγ-1 in HAP1 KO cortical neurons in response to BDNF treatment (Fig. 2-6, A-D). These results suggest that HAP1 is required for the signal transduction of BDNF.

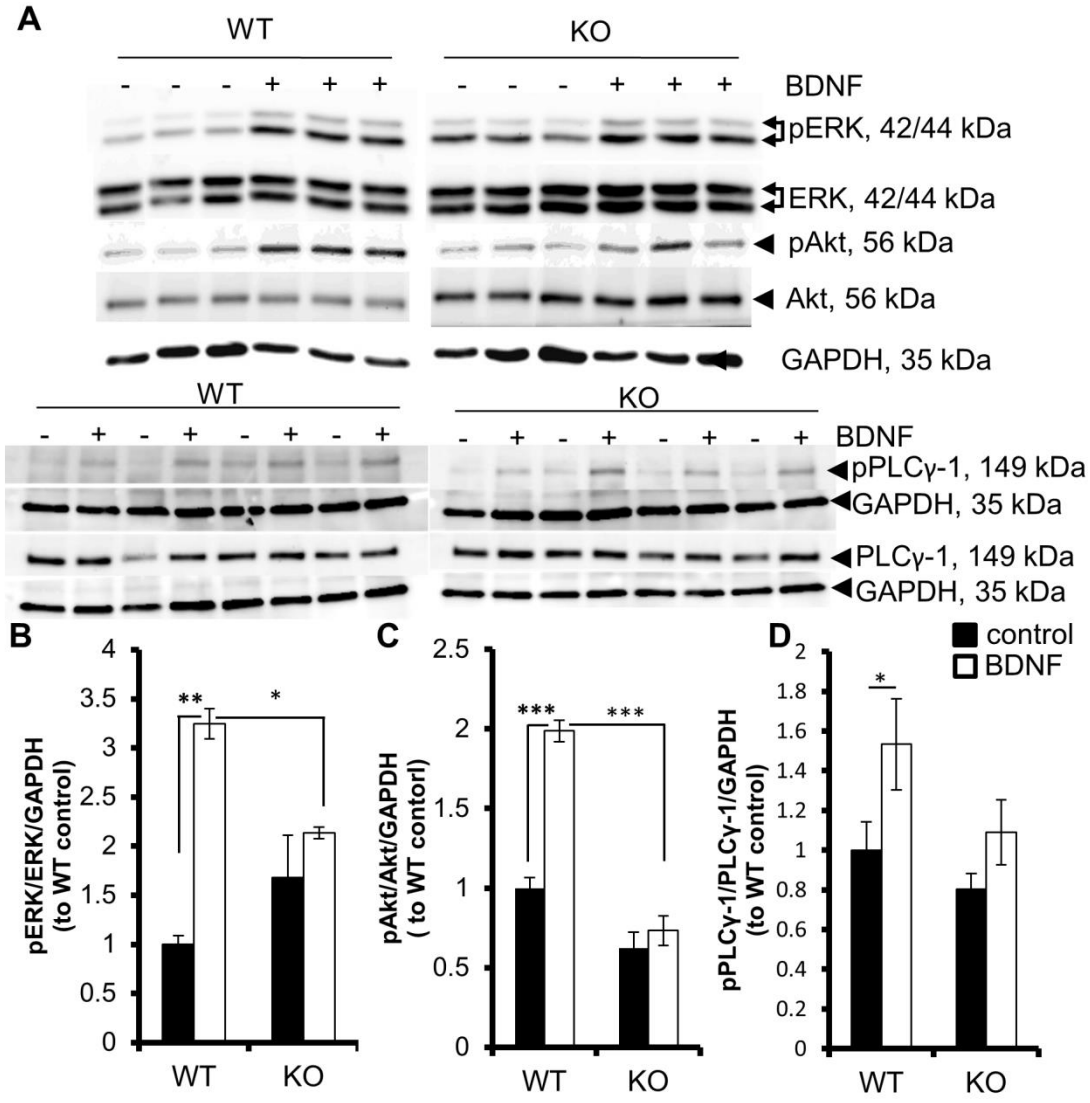


Figure 2-6 TrkB signalling is decreased in HAP1 KO cortical neurons.
A) Representative Western blots showing BDNF-induced TrkB down-stream signal transduction proteins. HAP1 WT and KO cortical neurons were stimulated with 100 ng/ml BDNF for 30 min and then subjected to Western blot analysis with antibodies to phosphorylated ERK (pERK), Akt (pAkt) and PLCγ-1 (pPLCγ-1) and total ERK, Akt and PLCγ-1. GAPDH serves as an internal control. **B,C,D)** Densitometric quantification of the ratios of pERK to total ERK (B), pAkt to total Akt (C) and pPLCγ-1 to total PLCγ-1 (D). Data are presented as mean ± SEM; n=3-4 per group. *p < 0.05, **p < 0.01, *** p < 0.001; one-way ANOVA with Tukey's post-hoc test). "WT control" was normalised to 1. All bands of Western blot were normalised with their corresponding GAPDH.

2.4.7 Proliferation of cerebellar granule cells is impaired in HAP1 KO mice *in vitro* and *in vivo*

As BDNF plays a critical role in cellular proliferation of cerebellar granule cells, we hypothesized that neuronal proliferation in response to BDNF could be impaired in HAP1 KO mice. To test this, we observed the proliferation of cerebellar granule cells *in vitro* and *in vivo* by BrdU incorporation experiment. As BrdU is an analogue of thymidine, during the 'S' phase of the cell cycle, it can be incorporated into DNA instead of thymidine and cab serve a proliferation marker. The *in vitro* experiment showed that the number of BrdU-positive cells increased 1.5 times relative to control upon BDNF treatment (at 10 and 100 ng/ml) in WT ($p < 0.05$ vs control) cells while there was no increased number of BrdU-positive cells in HAP1 KO cells. There was a significant difference in BDNF treated cerebellar granule cells between WT and HAP1 KO granule cells ($p < 0.01$ and $p < 0.001$ vs KO) (Fig. 2-7, A and B). The result of the *in vivo* experiment was similar to the *in vitro* experiment. The BrdU-positive cells (%) in BDNF-injected WT mice were increased (more than two fold) in comparison to PBS-injected ($p < 0.01$) or BDNF-injected KO ($p < 0.001$) granular cells (Fig. 2-7, C and D). However, there was no difference in BrdU-positive cells (%) between PBS and BDNF-injected KO mice (Fig. 2-7, C and D).

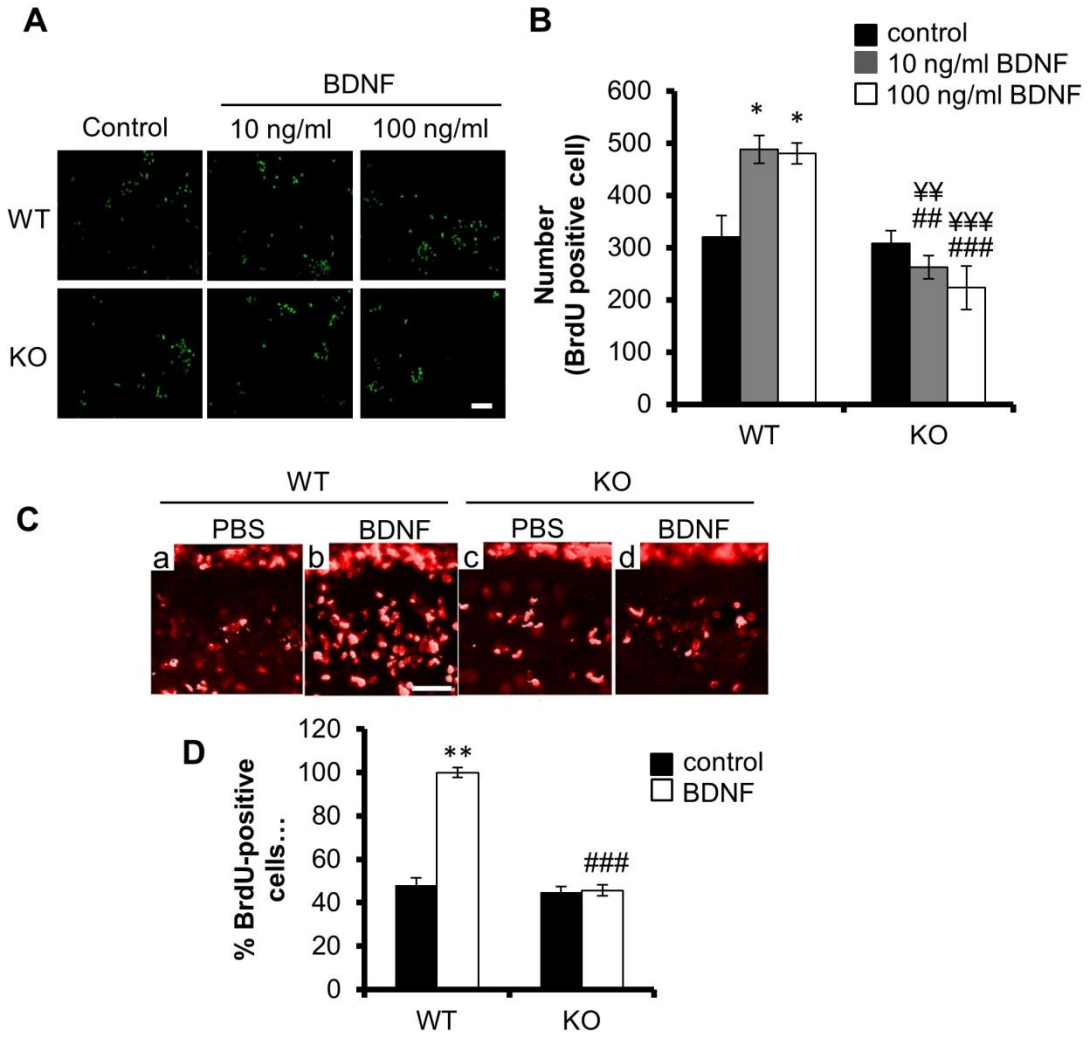


Figure 2-7 BDNF induces proliferation of cerebellar granule cells in HAP1 WT but not in HAP1 KO in vivo and in vitro.

A,B) Isolated cells from P0 cerebellum tissue of HAP1 WT and KO mice were cultured on glass coverslips coated with poly-D-lysine (PDL) overnight and for additional 24 hrs with 10 µg/ml BrdU and 0 (control), 10 or 100 ng/ml BDNF containing culture medium, respectively. The cells were then fixed and stained with anti-BrdU antibody according to the procedure described in Materials and Methods. Scale bar, 100 µm. BrdU-positive cells (green) in the presence or absence of BDNF in WT and KO granular cells are shown in (A). Quantification of of BrdU-positive cells is shown in (B). Data are presented as mean ± SEM; *$p < 0.05$ vs WT control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs WT 10 ng/ml BDNF; $^{¥¥}p < 0.01$, $^{¥¥¥}p < 0.001$ vs WT 100 ng/ml BDNF; n=3-4 coverslips per group; six areas were observed in each coverslip and the same locations were applied to all the observed coverslips for image taking; One-way ANOVA, Tukey's post-hoc test. **C, D)** Effect of BDNF on granule cell proliferation in WT and HAP1 KO neonatal mice *in vivo*. Sections were labelled with BrdU-positive cells (red colour) demonstrating proliferation of granule cells at 24 hour post-BrdU injection (C,a-d). (C, a-b) Representative microphotographs of BrdU-positive granule cells from HAP1 KO mice treated with PBS (a) or BDNF (b). (C, c-d) Representative microphotographs of BrdU-positive granule cells from WT mice treated with PBS (c) or BDNF (b) Quantification of BrdU-positive cells is shown in (D). Data are presented as mean ± SEM; **$p < 0.05$ vs WT control; $^{\#\#\#}p < 0.001$, WT BDNF vs HAP1 KO BDNF; n=6 per

group; one-way ANOVA, Tukey's post-hoc test. Scale bars, 50 μm. "HAP1 WT BDNF" was normalised as 100 %.

2.5 Discussion

In the present study, we found that HAP1 is co-localised with internalised mature BDNF but not mature BDNF synthesised within neurons. In addition, we also find that HAP1 is co-localised with BDNF receptors $p75^{NTR}$ and TrkB and the co-localisation is increased in response to BDNF stimulation in PC12 cells and neurons. In co-transfected cells, we found that HAP1 interacts with $p75^{NTR}$ or TrkB respectively as detected by co-IP and FRET analysis, and the interactions were dramatically increased in response to BDNF. Furthermore, we found that HAP1 is required for the endocytosis of BDNF and its receptors $p75^{NTR}$ and TrkB as the internalisation of these factors were abolished in HAP1 KO neurons. Further studies indicate that HAP1 appears essential for the activation and phosphorylation of Akt, Erk and PLCγ-1 in cortical neurons in response to BDNF. Lastly, we found that HAP1 is also required for proliferation of cerebellar granule cells in response to BDNF stimulation in vitro and in vivo. Based on these data, we conclude that HAP1 is an essential molecule for the endocytosis and signal transduction of BDNF/TrkB in neurons.

2.5.1 HAP1 interacts with TrkB and $p75^{NTR}$ and this interaction is stimulated by BDNF

We have found that exogenously added BDNF showed much higher co-localisation with HAP1A than intracellularly expressed BDNF pool in PC12 cells. Moreover, adding antibodies to BDNF and $p75^{NTR}$ with exogenous BDNF treatment reduced the co-localisation of HAP1A with BDNF. Immunostaining of endogenous HAP1 and $p75^{NTR}$ also showed that co-localisation between HAP1 and $p75^{NTR}$ increased by exogenous BDNF

addition. These findings suggest that HAP1 may have different roles for endocytosed and intracellular BDNF. It should be noted that PC12 cells express $p75^{NTR}$ but not TrkB (Lou, Yano et al. 2001, Saxena, Howe et al. 2004). Therefore, $p75^{NTR}$ could be the only receptor for BDNF endocytosis in PC12 cells.

Our Co-IP and FRET experiments in HEK293T cells demonstrated that the interaction of HAP1A/TrkB and HAP1A/$p75^{NTR}$ increased upon BDNF treatment. This result is consistent with a recent finding that TrkB is in a complex with HAP1 (Xiang, Yang et al. 2014). Taken together, our data suggest that HAP1 interacts with TrkB and $p75^{NTR}$ and the interaction can be stimulated by BDNF.

2.5.2 The role of HAP1 in endocytosis of BDNF and its receptors

We have found that endocytosis of BDNF was abolished in HAP1 KO cortical neurons and the defect was rescued by HAP1A gene delivery into HAP1 KO cortical neurons. This result was also confirmed by pull-down assay of internalised biotinylated BDNF. Our results clearly demonstrated that HAP1 is essential for BDNF endocytosis. Our finding is partially consistent with a previous study which showed that the endocytosis of biotinylated BDNF was reduced in the brainstem neurons from HAP1 KO mice (Sheng, Xu et al. 2008). A few studies have shown that TrkB protein levels are significantly lower in hypothalamus (Xiang, Yang et al. 2014), cerebellum and brainstem tissue (Sheng, Xu et al. 2008) in HAP1 KO mice than in WT mice. RT-PCR and Western blot of TrkB in the cortex and brainstem tissue in WT and HAP1 KO confirmed that the internalisation impairment of BDNF in HAP1 KO is resulted from absence of HAP1 but not from the TrkB expression level reduction.

We have found that BDNF treatment of WT cortical neurons significantly reduced cell surface TrkB but had no effect on cell surface TrkB in HAP1 KO cortical neurons. However, BDNF stimulation did not trigger endocytosis of truncated TrkB (TrkB-T1), which is

consistent with a previous report (Zheng, Shen et al. 2008). It has been reported that TrkB-T1 is able to bind and become endocytosed with BDNF (Alderson, Curtis et al. 2000) by possibly forming a dimer with full-length TrkB, which suggests that TrkB-T1 endocytosis may be controlled by different neuronal activity (Eide, Vining et al. 1996, Ninkina, Adu et al. 1996, Du, Feng et al. 2003). Moreover, it has been demonstrated that TrkB-T1 internalisation begins slowly after BDNF triggering and the internalisation is most active at 90 min (Fryer, Kaplan et al. 1997). It is possible that our study missed the optimal time for detection of TrkB-T1 in cortical neurons during BDNF stimulation time. Moreover, it could be worthwhile examining the binding sites between HAP1-TrkB and HAP1-TrkB-T1. If TrkB-T1 does not have a binding domain with HAP1 while TrkB does, it could explain why TrkB-T1 was not endocytosed on BDNF stimulation. Interestingly, in contrast to TrkB, we failed to detect any difference in cell surface $p75^{NTR}$ between WT and HAP1 KO cortical neurons in response to BDNF. One reason for this discrepancy could be due to the low level of $p75^{NTR}$ expression in cortical neurons (Kordower, Bartus et al. 1988, Mufson, Bothwell et al. 1989, Mufson and Kordower 1992).

2.5.3 The role of HAP1 in TrkB signalling and neuronal proliferation

Another significant finding of the present study is that HAP1 is required for the signal transduction through TrkB in response to BDNF. This study found that the phosphorylation of ERK, Akt and PLCγ-1 was increased in response to BDNF in WT cortical neurons as expected but the increase was not observed in HAP1 KO neurons. Once TrkB is stimulated by BDNF, the signal transduction occurs and the TrkB downstream pathways, MAPK/ERK, PI3K/Akt and PLCγ-1 are activated, through the phosphorylation of TrkB (Huang and Reichardt 2003, Chen, Nguyen et al. 2007, Chapleau and Pozzo-Miller 2012). It is widely accepted that BDNF/TrkB complex is critical for neuronal survival, growth and proliferation

(Barde 1989, Glass, Nye et al. 1991, Meyer-Franke, Kaplan et al. 1995, Cheng, Wang et al. 2003, Choi, Li et al. 2009). Our data indicate that HAP1 is a critical molecule involved in the signal transduction of BDNF/TrkB. As the endocytosis of BDNF and its receptors requires HAP1 and is apparently regulated by HAP1, we propose that HAP1 is a key molecule regulating trafficking and signal transduction of signalling endosomes containing BDNF. Our results also indirectly suggest that the endocytosis of neurotrophins and their receptors is an essential step for neurotrophin signalling and consistent with the signalling endosome hypothesis (Bronfman, Tcherpakov et al. 2003, Ye, Kuruvilla et al. 2003, Howe and Mobley 2004). Our data, however, argues against the alternative retrograde signalling models suggested by several studies using immobilised NGF and TrkA in chamber cultures (MacInnis and Campenot 2002). It has been reported that the immobilised NGF to the beads can promote neuronal survival without NGF internalisation (MacInnis and Campenot 2002, Heerssen, Pazyra et al. 2004). This theory is reasonable as TrkA can be autophosphorylated without NGF stimulation when it is at high concentrations in PC12 cells (Hempstead, Rabin et al. 1992) and highly concentrated TrkA could exist in the vesicles transported retrogradely. In Campenot chambers, which is a device to isolate dendrites and single axons in neuron culture, retrograde signal could be carried by spreading TrkA phosphorylation waves on the plasma membranes within 1 minute after NGF treatment on distal part of axons (Senger and Campenot 1997, MacInnis and Campenot 2002). However, NGF treatment to proximal axons and cell bodies does not show phosphorylated TrkA in distal axons (Ye, Kuruvilla et al. 2003). Although our findings do not exclude the possible alternative retrograde signalling mechanisms, the findings that abolishing BDNF endocytosis by HAP1 deletion diminishes TrkB downstream signalling indicate the BDNF/TrkB signalling endosome is a main mode of signal transduction for BDNF.

We have also found that proliferation of cerebellar granule cells with BDNF treatment was impaired in HAP1 KO mice while BDNF treatment triggered the increase in proliferation of

cerebellar granule cells both *in vitro* and *in vivo*. This result suggests that HAP1 is an essential intracellular molecule regulating the signal transduction of BDNF in the development of the nervous system. Our data support previous findings that the phenotype of HAP1 KO mice shares many features of BDNF KO such as postnatal lethality, and abnormal energy metabolism (Ernfors, Lee et al. 1994, Jones, Farinas et al. 1994, Li, Yu et al. 2003) as the neurotrophic function of BDNF could be defected in HAP1 KO mice. However, the finding that HAP1 is required for BDNF-induced proliferation of granule cells is in contrast to that of the recent study showing BDNF can rescue the deficit of progenitor cell proliferation in the hypothalamus in conditional HAP1 KO mice (Xiang, Yang et al. 2014). This discrepancy may be related to regions of the brain. In the present study, we have used new born mice as a model and focused on cerebellar granule cells.

In addition, the findings that HAP1 is required for the endocytosis and signal transduction of BDNF and TrkB support the concept that the biological functions of BDNF requires HAP1. Retrolinkin binds endophilnin A1 and those proteins are involved in early endocytic trafficking of BDNF/TrkB (Fu, Yang et al. 2011). The authors showed that suppressed Retrolinkin expression prevents TrkB internalisation under BDNF stimulation and activation of ERK, resulting in reduced dendrites growth of CNS neurons. It has been also reported that inhibition of clathrin-mediated endocytosis interferes with BDNF/TrkB internalisation and dendrites outgrowth and survival of cortical neurons through Akt activation (Zheng, Shen et al. 2008). BDNF also plays a role in cellular proliferation through Akt activation (Zhang, Liu et al. 2011). Moreover, HAP1 plays a role in retro- and anterograde trafficking to transport vesicles (Li, Gutekunst et al. 1998, McGuire, Rong et al. 2006, Rong, Li et al. 2007). Taken together, it may be reasonable to conclude that HAP1 gene deletion impairs BDNF/TrkB internalisation and further endosomal trafficking intracellularly to the cell body to facilitate TrkB downstream signal cascade, leading abolished neuronal proliferation.

As BDNF/TrkB endocytosis is critical for its neurotrophic function, our study warrants further investigation into roles of HAP1 in neurodegenerative disorders such as Huntington's disease and Alzheimer's disease and endocytosis of other growth factors.

Chapter 3: Development of mature BDNF-specific sandwich ELISA

STATEMENT OF AUTHORSHIP

Chapter 3 -- (2015) ***"Development of mature BDNF-specific sandwich ELISA." J Neurochem 134(1): 75-85.***

Yoon Lim (Candidate)

Designed and performed experiments, analysed data, interpreted the results of the experiments, prepared figures, drafted, edited and revised the manuscript.

I hereby certify that the statement of contribution is accurate

Signed: Date: 26/07/2015

Jin-Hua Zhong

Contributed to the antibody purification.

I hereby certify that the statement of contribution is accurate

Signed: Date: 29/07/2015

Xin-Fu Zhou

Conceived and designed the experiments, contributed to the critical manuscript revision and acted as the corresponding author.

I hereby certify that the statement of contribution is accurate

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ORIGINAL ARTICLE

Development of mature BDNF-specific sandwich ELISA

Yoon Lim,*·† Jin-Hua Zhong* and Xin-Fu Zhou*

*School of Pharmacy and Medical Sciences, Sansom Institute, Division of Health Sciences, University of South Australia, Adelaide, SA, Australia

†Department of Human Physiology, Centre for Neuroscience, University of Flinders, Adelaide, SA, Australia

Abstract

Mature brain-derived neurotrophic factor (mBDNF) plays a vital role in the nervous system, whereas proBDNF elicits neurodegeneration and neuronal apoptosis. Although current enzyme-linked immunosorbent assay (ELISA) has been widely used to measure BDNF levels, it cannot differentiate mBDNF from proBDNF. As the function of proBDNF differs from mBDNF, it is necessary to establish an ELISA assay specific for the detection of mBDNF. Therefore, we aimed to establish a new mBDNF-specific sandwich ELISA. In this study, we have screened and found a combination of antibodies for a sandwich ELISA. A monoclonal antibody and sheep anti-BDNF were chosen as capture and detection antibody for sandwich ELISA respectively. The new ELISA showed no cross-reactivity to human recombinant NT-3, NT-4, nerve growth factor and negligible cross-reactivity (0.99–4.99%) for proBDNF compared to commercial ELISA kits (33.18–91.09%). The application of the new mBDNF ELISA was shown through the measurement of mBDNF levels in different brain regions of rats and in the brain of β-site amyloid precursor protein cleaving enzyme 1 $(BACE1)^{-/-}$ and WT mice and compared to western blot. Overall, this new ELISA will be useful for the measurement of mBDNF levels with high specificity.

Keywords: ELISA and cross-reactivity, mature BDNF (mBDNF), proBDNF.

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Mature brain-derived neurotrophic factor (mBDNF) has a critical role in the survival, differentiation and proliferation of neurons in the peripheral (PNS) and the central nervous system (CNS) (Davies 1994; Huang and Reichardt 2001). Like other neurotrophins, mBDNF is originally synthesized as a precursor protein, called pre-proBDNF. The signal peptide is then cleaved, releasing proBDNF (~34 kDa), followed by intracellular or extracellular processing by proteases such as furin, tissue plasminogen activator (tPA) and metalloproteases to generate mBDNF (13 kDa) (Seidah *et al.* 1996; Mowla *et al.* 2001; Pang *et al.* 2004; Nagappan *et al.* 2009). Recent studies have reported that proBDNF elicits long-term depression and cellular apoptosis, whereas mBDNF triggers long-term potentiation and cell survival. This strongly suggests that although both mBDNF and proBDNF are derived from the same gene, they have opposing roles (Lee *et al.* 2001; Pang and Lu 2004; Woo *et al.* 2005; Sun *et al.* 2012).

A growing body of evidence has demonstrated that BDNF levels are altered in neurological disorders such as Alzheimer's disease (AD) (Laske *et al.* 2007; Angelucci *et al.* 2010) and depressive disorders (Cunha *et al.* 2006; Yoshimura *et al.* 2006; Zhu *et al.* 2013). More specifically, it has been shown that blood BDNF levels increase in major depressive disorder (MDD) patients treated with pharmacological treatment (Yoshimura *et al.* 2007; Huang *et al.* 2008), suggesting that BDNF levels in blood could be a biomarker in MDD and an anti-depressant (Yoshimura *et al.* 2007; Lin 2009; Hashimoto 2010). In contrast to mBDNF, which is reduced in MDD, proBDNF levels in the blood and lymphocytes have shown to be increased (Zhou *et al.* 2013). In addition, a few recent studies have proved that BDNF levels in the brain and blood are firmly connected (Karege *et al.* 2002; Sartorius *et al.* 2009; Klein *et al.* 2011).

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Address correspondence and reprint requests to Prof. Xin-Fu Zhou, School of Pharmacy and Health Sciences, Sansom Institute, University of South Australia, Adelaide, SA 5000, Australia. E-mail: xin-fu.zhou@unisa.edu.au

Abbreviations used: BSA, bovine serum albumin; HRP, horseradish peroxidase; mBDNF, mature brain-derived neurotrophic factor; MDD, major depressive disorder; NGF, nerve growth factor; PBS, phosphate-buffered saline.

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As mBDNF and proBDNF show such different biological activities, there is growing importance in being able to distinguish and measure their levels accordingly (Hashimoto 2010; Teche *et al.* 2013). BDNF ELISA was initially developed in 1990s based on two polyclonal antibodies for sandwich ELISA (Nawa *et al.* 1995) or one-site ELISA using single monoclonal antibody (Kolbeck *et al.* 1999). However, current ELISA techniques utilized in many major studies that measure BDNF levels are performed with commercially available kits which cannot distinguish proBDNF from mBDNF (Teche *et al.* 2013). More recently, a BDNF ELISA kit from Adipo Bioscience (changed to Aviscera Bioscience, Santa Clara, CA, USA) (Yoshida *et al.* 2012a,b; Sodersten *et al.* 2014) has claimed to possess this ability to measure the specific levels of mBDNF. However, there is still a lack of studies with clear data on the cross-reactivity of proBDNF. Therefore, with its questionable specificity against mBDNF, there is still a need for the development of an accurate, reliable and reproducible mBDNF-specific ELISA kit.

Our recent studies have used an in-house mBDNF ELISA (Xiong *et al.* 2013; Zhou *et al.* 2013). In this study, we show the screening of mono and polyclonal antibodies against mBDNF, evaluation of mBDNF-specific antibodies and the mBDNF-specific sandwich ELISA. Commercial BDNF ELISA kits were compared to our new ELISA in regards to cross-reactivity for human proBDNF. Furthermore, we also present validations and applications of our new mBDNF sandwich ELISA.

Materials and methods

Antibody production and purification

Antibody production

All animal work carried out during the production of antibodies were ethically approved by the AWC of Flinders University. Recombinant human mBDNF produced from *E.coli* (Amgen Inc., Thousand Oaks, CA, USA) was used as an antigen for the production of polyclonal and monoclonal antibodies. Briefly, for polyclonal antibody production, an emulsion was produced when 0.5 mg of mBDNF in 2 mL phosphate-buffered saline (PBS) containing 0.4% glutaraldehyde (Sigma-Aldrich, St Louis, MO, USA) was mixed with 2 mL of complete Freund's adjuvant (Sigma-Aldrich). The emulsion was subcutaneously injected into an adult sheep at multiple locations within the back and groin regions. Subsequent injections of half the amount of antigen and incomplete Freund's adjuvant were repeated every 2 weeks until antibody titres reached a desirable level. Similarly, for monoclonal antibody production, 50 μg of antigen was injected into BALB/c mice using the same injection protocol as previously described. All procedures were conducted in duplicate ($n = 3$). Once titres reached a desirable level (1/50 000), the best responding mouse in each group was culled. Following sacrifice, spleens were harvested, minced and lymphocytes isolated and fused with × 63 myeloma cells to generate hybridoma cells. Fused cells were cultured in two 24-well plates and screened against mBDNF as described. Selected clones were expanded for further characterization.

Antibody purification (Protein G beads)

IgG purification was performed using Protein G Beads (P-7700, Sigma, St. Louis, MO, USA). Briefly, protein G beads were hydrated in 50 mL of filtered water for 30 min under gentle rotation. The resin was poured into a column and washed with 50 mL of deionized water, followed by equilibration with 50 mL of binding buffer (50 mM $NaPO_4$, 500 mM NaCl, pH6.8). Serum was mixed with binding buffer with 1 : 2 of volume ratio, followed by filtration through 0.45 μm syringe filter (Sartorius, Adelaide, SA, Australia). The serum mixture was then loaded into the column and binding buffer (10 mL) was added to wash the column. This was repeated three times until the serum mixture flow through the column completely and the optical density (Sodersten *et al.* 2014) at 280 nm reached the baseline. Then, 50 μL of 1 M Tris-HCl (pH 8.0) was added to the elution collection tubes. The final elution step was performed by adding 1 mL of 100 mM glycine (pH2.7) into the column. Each fraction was collected and the concentration was measured using Nanodrop (Thermo Fisher Scientific, Rockford, IL, USA). All elutes were stored at −80°C until needed and were subjected to SDS-PAGE and Coomassie Blue staining to check the purity of proteins prior to use.

Affinity purification of IgG using mBDNF affinity column

Sheep anti-BDNF IgG was affinity purified through mBDNF affinity column. First, the mBDNF affinity column was made according to the manufacture's instruction. Briefly, 0.15 g of cyanogen bromide-activated Sepharose 4B beads (GE Healthcare Australia, Silverwater, NSW, Australia) was swollen in excess with 1 mM HCl for 1 h with rotation. The swollen beads were equilibrated in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). After equilibration, the beads were mixed with 1 mg of mBDNF in coupling buffer for 2 h at 25°C with gentle rotation. Subsequently, the beads were washed with 10 bed volumes of coupling buffer and 0.1 M Tris-HCl pH8.0 was added into the column for blocking any remaining active groups. The beads were then washed for four cycles with 0.1 M sodium acetate, pH 4.0 containing 0.5 M NaCl followed by a second wash with 0.1 M Tris-HCl pH8.0 containing 0.5 M NaCl.

To purify IgG anti-mBDNF, sheep anti-BDNF serum was mixed with PBS with 1 : 2 ratio and filtered through 0.4 μm. The mixture was applied to the column and passed through, followed by washing with 10 column volumes of PBST (PBS, 0.1% Tween-20) and this was followed by elution with 0.1 M Glycine pH2.5.

Biotinylation

Biotinylation of antibody was performed according to manufacturer's instruction. Briefly, antibody was dialysed against PBS and then incubated with Sulfo-NHS-SS-biotin (Pierce, Thermo Fisher Scientific) for 2 h on ice. Free biotin was removed by dialysis against PBS.

Recombinant protein production

Cloning of His-proBDNF and GST-proBDNF

For human proBDNF expression, proBDNF-His or Glutathione S-transferase (GST) were constructed. ProBDNF open reading frame

(ORF) was amplified by PCR with primers (proBDNF-His set: 5′-GCGAATTCATGGCCCCCATGAAAG AAGC-3′ (Forward) and 5′-GCCTCGAGGTCTTCCCCTTTTAAT GGTC-3′ (Reverse); proBDNF-GST primer set: 5′-GCGAATTCGC CCCCATGAAA-GAAGC-3′ (Forward) and 5′-ATCTCGAG TTAGTCTTCC CCTTTTAATGGTC-3′ (Reverse) using AAproBDNF-EGFP (enhanced green fluorescent protein (EGFP)) (a furin-resistant construct with amino acids 125 and 127, R to A mutations kindly donated by Dr Masami Kojima) as a PCR template. The PCR product and vectors, pET-21a vector (Promega, Madison, WI, USA) or pGEX-4T-1 (GE Healthcare Australia), were cut with *EcoRI* and *XhoI* (Promega). The proBDNF inserts and vectors were then ligated by T4-DNA ligase (Promega) and then transformed into One Shot Top10 competent cells (Life Technology, Mulgrave, Vic., Australia). The final constructs were verified by DNA sequencing.

Expression of His-proBDNF and GST-proBDNF

The plasmids pET-proBDNF and pGEX-proBDNF were transformed into *E. coli* BL21 and proBDNF expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). The induced recombinant proteins were affinity purified with Ni^{++} resin (Qiagen, Doncaster, Vic., Australia) for proBDNF-His and GST beads (GE Healthcare Australia) for proBDNF-GST, respectively, according to the manufacturer's instructions.

Cloning of proBDNF-pcDNA3.1-Myc-His

Human proBDNF (Furin-resistant) was cloned into pcDNA3.1-myc-his vector. Briefly, the cDNA sequences of human proBDNF were amplified by PCR using AAproBDNF-EGFP as PCR template, which was performed for 30 cycles at; 94°C for 1 min, 60°C for 1 min, and 72°C for 60 s. The primers used were as follows: 5′-AGCAAGCTTGCCACCATGACCATCCTTTTCC TTA C-3′ (Forward) and 5′-GCCTCGAGGTCTTCCCCTTTTAATGGT C-3 (Reverse). The PCR product obtained was cut with *HindIII* and *XhoI* and ligated into pcDNA3.1-myc-his vector (Invitrogen, Carlsbad, CA, USA). DNA restriction enzyme digestion and DNA sequencing were performed to confirm cloning.

Antigen

Human mature BDNF was kindly donated by Amgen. Human recombinant neurotrophin-3 (NT-3, NP_001096124.1, aa 152–270), neurotrophin-4 (NT-4, XP_005259019.1, aa 86–209) and nerve growth factor (NGF, NP_002497.2, aa 121–241) were expressed and purified from *E.coli* in the laboratory previously by past researchers. Furin-resistant proBDNF [ProBDNF (Sf9)] was expressed in an insect cell line and affinity purified (Virovek, Hayward, CA, USA). ProBDNF (R&D) was purchased from R&D (Minneapolis, MN, USA). PreBDNF, the recombinant human pro-domain (aa 19–128) of full-length proBDNF (1–247) was expressed in BL-21.

Human and animal tissues

All human and animal tissues used in this study were approved by University of South Australia Human Research Ethics Committee and the Animal Ethic Committee of SA Pathology/AHS and all the procedures were undertaken according to the guidelines of the National Health and Medical Research Council of Australia. Half brain tissues of WT and precursor protein cleaving enzyme 1 ($BACE1^{-/-}$) mice (WT, 4–6-month-old, 2 females and 3 males; $BACE1^{-/-}$, 4–5-month-old, 4 females and 1 male) were kindly donated by Professor Robert Vassar (Northwestern University, IL, USA). Five 4-week-old female Sprague–Dawley rats were purchased (University of South Australia, Australia). Human blood samples were kindly donated by Dr Ralph Martin (Edith Cowan University, Australia) and rat serum (8-week-old Sprague-Dawley rat) was kindly donated by Dr Fiona Zhou (University of South Australia, Australia).

General western blot

Western blot experiments were conducted as follows. Crude cell lysate or purified protein was loaded and separated on 10–12% gel by SDS-PAGE. The proteins were transferred to Hybond-c membrane (GE Healthcare Australia) and blocked with 5% skim milk in PBS for 1.5 h at 25°C under gentle rocking. Membranes were cut and incubated with primary antibody with proper dilution overnight at 4°C. After washing three times with PBST (PBS, 0.1% Tween-20), the membranes were incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibody at 25°C for 1 h. The membrane was washed again with PBST four times and incubated with enhanced chemiluminescence (GE Healthcare Australia) and the signals were detected with Kodak film or Image Quant LAS 4000 (GE Healthcare, Silverwater, NSW, Australia). Image J software (Research Service Branch; National Institute of Health, http://rsbweb.nih.gov/ij/index.html) was used for densitometry.

For western blot with brain homogenates from WT and $BACE1^{-/-}$ mice, 100 μg of homogenate of each mice was separated in 12% denaturing SDS-PAGE gel. The transferred membrane was incubated with rabbit anit-BDNF (1 : 100, SC-546, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and followed by secondary antibody (goat anti-rabbit IgG-HRP, Sigma-Aldrich, Australia).

Elisa

Indirect ELISA

mBDNF, His-/GST-proBDNF, preBDNF, proBDNF(Sf9) were diluted at 1 μg/mL (proBDNF-Myc-His at 0.12 μg/mL) in coating buffer (50 mM Carbonate, pH9.6) and 100 μL of this mixture was added into each well of a 96-well microplate and then incubated for 1 h at 37°C. The plate was then washed three times with PBS. Following the wash, 150 μL of 3% bovine serum albumin (BSA) in PBS was added and incubated for 1 h at 37°C. Polyclonal or monoclonal antibodies against mBDNF or proBDNF were diluted in sample diluent (1% BSA, PBST) and 100 μL was applied to each well and the plate was incubated at 37°C for 1 h. For competition assay, proBDNF(Sf9) was added at 0–10 μg/mL concentration on the primary antibody step. The plate was then washed four times with wash buffer. HRP-conjugated secondary antibodies were diluted (1 : 5000) in sample diluent and added to each well and incubated at 37°C for 1 h. Following washing, 100 μL of freshly prepared 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma-Aldrich) substrate was applied to every well to allow the colour to develop for 10–15 min At last, 0.1 N HCl was used to stop the colour reaction. Using a microplate reader Sunrise (TECAN, Mainz-Kastel, Germany), multimode plate reader DTX-880 (Beckman Coulter, Miami, FL, USA) or Benchmark plate reader (Bio-Rad Laboratories, Hercules, CA, USA), absorbance readings at 450 nm were taken.

Sandwich ELISA

For mBDNF sandwich ELISA, microplates (96-well) were coated with the capture antibody, B34D10 at 1 μg/mL in 50 mM carbonate buffer pH9.6 and incubated overnight at 4°C. The wells were then washed with PBS and blocked with 170 μL of 3% BSA in PBS at 37°C for 1 h or alternatively at 25°C for 2 h. Dilutions of standard mBDNF were made in a range from 750 to 11.7 pg/mL and test samples were diluted in sample diluent (1% BSA, 0.125% Tween-20 in PBS). After washing with wash buffer (PBS, 0.1% Tween-20) the detection antibody, biotinylated sheep a-mBDNF IgG at 0.15 μg/mL was added to each well and incubated at 37°C for 1 h. Streptavidin-HRP (1 : 5000) was then added and incubated at 37°C for 1 h. The plate was washed thoroughly and TMB substrate was added to each well, and incubated at 25°C for 10 min in the absence of light. Finally, to stop the colour development, 0.1 N HCl was added and absorbance readings at 450 nm were taken using a micro plate reader (Sunrise, TECAN) to determine OD values. For proBDNF sandwich ELISA, sheep anti-proBDNF IgG (5 μg/mL) and sheep anti-BDNF IgG-biotin (0.15 μg/mL) were used as a capture and detection antibodies respectively.

Brain tissue samples tested in this study were homogenized in RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% Sodium deoxycholate, pH7.4) containing the protease inhibitors (Roche, Castle Hill, NSW, Australia). The homogenates were sonicated and then centrifuged at 16,000 × g for 30 min at 4°C and total protein concentration of supernatant was determined using BCA protein assay Kit (Thermo Scientific, Rockford, IL, USA).

Cross-reactivity test for proBDNF with commercial BDNF ELISA kits

The following commercial human BDNF ELISA kits were purchased: BDNF Emax Immunoassay System (Cat. #. G7610, Promega); Quantikine ELISA (Cat. #. DBD00, R&D Systems); Chemikine BDNF Sandwich ELISA Kit (Cat. #. CYT306, Millipore Corporation, Bedford, MA, USA); Human BDNF ELISA Kit (Cat. #. SK00752-01, Aviscera Bioscience). ELISA performed according to manufacturer's instruction. To avoid any discrepancy, same mBDNF (Amgen) molecule was used in different ELISA kits. Recombinant human proBDNF in the human proBDNF ELISA system (Cat. #.DY3175, R&D Systems) was applied at 2000 pg/mL in triplicate.

Validation assay of mBDNF sandwich ELISA

The intra-assay and inter-assay variability was decided with several different concentration points of highly purified mBDNF. Seven different standard concentration points, 750, 375, 187.5, 93.75, 46.88, 23.44 and 0 pg/mL, were measured 3–5 times within the same ELISA run. This procedure was repeated three times independently and each run was performed on different days. The coefficient of variation was determined [%CV = (SD (standard deviation)/average) × 100]. The limit of detection was determined from 14 independent measurement of blank as described previously (Feinberg *et al.* 2009). Dilution parallelism was determined by two-fold serial dilution of human serum, human plasma, rat serum and mouse brain homogenate in sample diluent. Dilution parallelism was described as change in concentration from previous dilution (%) = (the current dilution/the previous dilution) × 100. Spiking recovery test was performed by the addition of 10 ng/mL (human serum) and 3 ng/mL (human plasma) and calculated [Recovery % = (Observed /Expected) × 100].

Statistical analysis

All the statistical analyses were performed with Microsoft Excel 2010 software. Statistical significance was assessed by the use of two-tailed Student's *t*-test and $p < 0.05$ was considered significant. All plot data present mean ± SEM (standard error of the mean).

Result

Antibody screening

All the monoclonal and polyclonal antibodies against mBDNF or proBDNF used for this study were developed in our laboratory. To screen mBDNF-specific antibody, western blot and ELISA were performed. For western blot, *E.coli*-expressed proBDNF and mBDNF were used as a pair for incubation with each antibody (Fig. 1a). B19-2-C1 and B34D10 are monoclonal antibodies and both of them recognized mBNDF and proBDNF weakly. Interestingly, sheep anti-BDNF serum specifically detected mBDNF but not proBDNF. Sheep anti-proBDNF serum and PB17A monoclonal antibody only detected proBDNF but not mBNDF. PB19B2E2 monoclonal antibody selected against the *E coli*-expressed recombinant preBDNF (immunizing antigen) did not recognize either proBDNF or mBDNF. In ELISA, B34D10 showed higher OD value than B12-2-C1 against mBDNF but showed similar OD with blank against two proBDNF proteins. Interestingly, sheep anti-BDNF showed maximum OD value (above the threshold of plate reader capacity) to mBDNF but did not show reactivity to proBDNF similar to western blot result. On the other hand, sheep anti-proBDNF showed high reactivity to His- and GST-proBDNF. All these biochemical assay results suggest that sheep anti-BDNF antibody recognizes mBDNF with high specificity and B34D10 also has potential to be mBDNF-specific antibody although the overall signals on western blot and ELISA were not as strong as the reactivity of sheep anti-BDNF (Table 1).

mBDNF antibody–specificity test with proBDNF expressed in mammalian cells

Although sheep anti-BDNF and B34D10 showed highly specific reactivity to mBDNF not proBDNF, it could be possible that eukaryote-expressed proBDNF might show different reactivity to antibodies owing to post-translational modification including glycosylation (Mowla *et al.* 2001) and hindrance of epitope resulting from the tertiary structure of protein. To remove that possibility, proBDNF-Myc-His was cloned, transiently expressed in mammalian-derived HEK293T cells and purified. The expression and purification were observed by Coomassie blue staining (Fig. S1A) and western blot (Fig. S1B) with sheep anti-proBDNF respec-

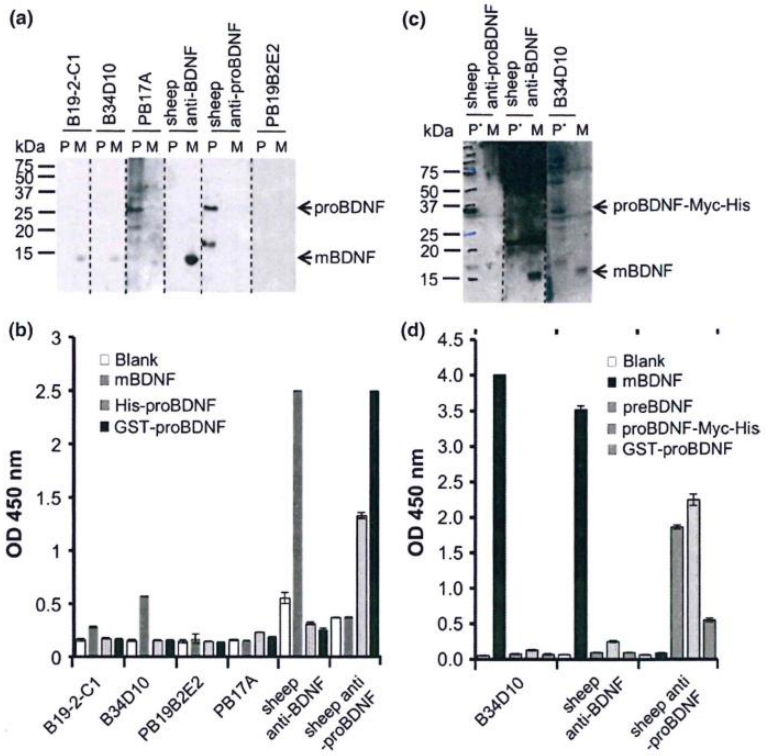


Fig. 1 Antibody screening against mature brain-derived neurotrophic factor (mBDNF) and proBDNF by western blot and ELISA. (a) 100 ng of mBDNF (M) and proBDNF (P) were separated by SDS-PAGE and their immunoreactivity was assayed with different antibodies as shown. (b) Antigens (mBDNF, 1 μg/mL; His-proBDNF and GST-proBDNF, 1 μg/mL and blank) were coated onto the 96-well plate and indirect ELISA was performed with different antibodies. (c) 10 μg of ProBDNF-Myc-His (P*) and 100 ng of mBDNF (M) were separated by SDS-PAGE and their immunoreactivity was assayed with different antibodies. (d) Antigens (mBDNF, 1 μg/mL; preBDNF, 1 μg/mL; proBDNF-Myc-His 0.12 μg/mL; GST-proBDNF, 1 μg/mL and blank) were coated for indirect ELISA as shown (b) and different antibodies were tested. Arrows indicate proBDNF, mBDNF and proBDNF-Myc-His in (a) and (c). Bar graphs show the mean ± SEM for triplicate determinations.

Table 1 Score of antibody properties determined by western blot and ELISA. The more + indicates the more activities. n.d: not determined

Antibody	Test	mBDNF	His-proBDNF	GST-proBDNF	Source
B19-2-C1	ELISA	+	–	–	Mouse
	WB	+	±	n.d	
B34D10	ELISA	+	–	–	Mouse
	WB	+	±	n.d	
PB19B2E2	ELISA	–	–	–	Mouse
	WB	–	–	n.d	
PB17A2	ELISA	+	–	–	Mouse
	WB	–	+	n.d	
sheep anti-BDNF	ELISA	+++	–	–	Sheep
	WB	+++	–	n.d	
sheep anti-proBDNF	ELISA	–	++	+++	Sheep
	WB	–	+++	n.d	

tively. As a result of western blot, sheep anti-proBDNF only recognized proBDNF-Myc-His as expected. Interestingly, sheep anti-BDNF just recognized mBDNF specifically but not proBDNF-Myc-His. However, B34D10 recognized both proBDNF-Myc-His or mBDNF. It seems long exposure (10 min) caused high background and resulted in non-specific bands on proBDNF-Myc-His lysate (Fig. 1c). On indirect ELISA, sheep anti-proBDNF showed reactivity to preBDNF, proBDNF-Myc-His and GST-proBDNF but not mBDNF (Fig. 1d). Like western blot, sheep anti-BDNF showed high reactivity to mBDNF, whereas it showed very low or negligible reactivity to proBDNF-Myc-His or preBDNF and GST-proBDNF respectively (Fig. 1d). Interestingly, B34D10 showed dramatically high reactivity to mBDNF, whereas it showed extremely low reactivity to proBDNF-Myc-His, preBDNF and GST-proBDNF (Fig. 1d). As a result, sheep anti-BDNF antibody showed high specificity to mBDNF and almost did not recognize mammalian-expressed proBDNF on western blot and ELISA. In addition, B34D10 also showed dramatically specific reactivity to mBDNF on ELISA although it showed weak reactivity on western blot. These results propose that both of sheep anti-BDNF and B34D10 are highly specific to mBDNF and not proBDNF.

Comparison of in-house-made antibodies to commercial antibodies and competitive ELISA: specificity of sheep anti-BDNF antibody

Following the specificity test, B34D10 and sheep anti-BDNF antibodies were further compared to other commercial anti-BDNF antibodies, which have been widely used by researchers. To do that, insect cell (Sf9)-expressed purified proBDNF (furin-resistant mutant) (Fig. S2) and mBDNF were used.

As for sheep anti-BDNF and B34D10, those antibodies showed similar results with previous ones (Fig. 1b and d). As shown in Fig. 2a, sheep anti-BDNF and B34D10 showed

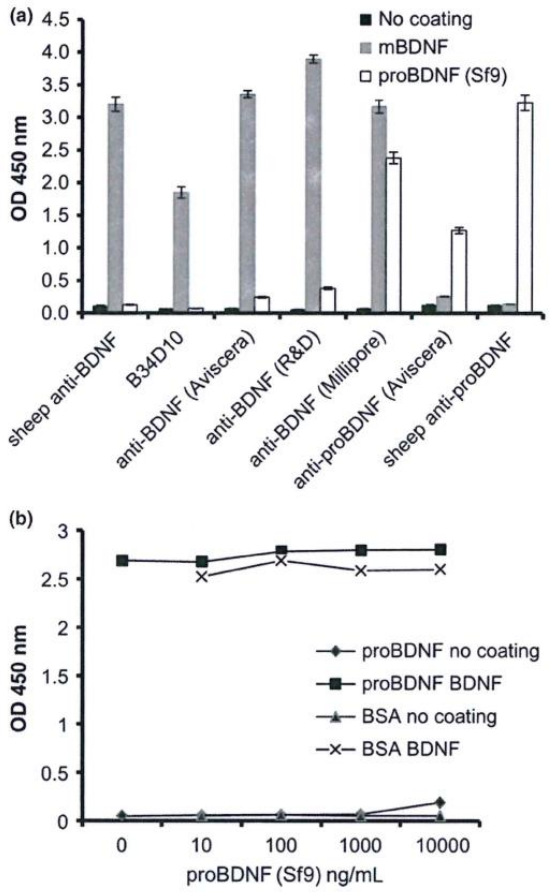


Fig. 2 Comparison of newly developed ELISA kit with commercial brain-derived neurotrophic factor (BDNF) ELISA kits against mature and proBDNF (Sf9). (a) Indirect ELISA was performed to compare assay specificity against proBDNF (Sf9) and mBDNF. Antigens (mBDNF and proBDNF (Sf9), 1 μg/mL and no coating) were coated into 96-well plate and different antibodies were added; B34D10-biotin (1 μg/mL), sh anti-BDNF-biotin (1 μg/mL) and sh anti-proBDNF-biotin (1 μg/mL), anti-BDNF (Product #35928, R&D, 2 μg/mL) anti-BDNF-biotin (CYT306 Part #60583, Millipore, 1 : 1000), anti-BDNF-biotin (SK00572-01, Aviscera Bioscience), anti-proBDNF–biotin (SK00752-06, Aviscera Bioscience). Bar graphs show the mean ± SEM for triplicate determinations. (b) The mBDNF (1 μg/mL) was coated onto 96-well plate and biotinylated sheep anti-BDNF (1 μg/mL) was added with proBDNF (Sf9) at various concentrations. Bovine serum albumin (BSA) was added with same concentration with proBDNF (Sf9) as negative control. The plots show the mean value of triplicate determinations.

very high reactivity to mBDNF, however they did not show any reactivity to proBDNF(Sf9) as there was no OD value difference between blank and proBDNF(Sf9). On the other hand, the anti-BDNF from Millipore showed high reactivity to both of mBDNF and proBDNF(Sf9) suggesting the antibody is not specific to mBDNF. The anti-BDNF from Aviscera Bioscience and R&D recognized mBDNF with high reactivity and it also recognized proBDNF(Sf9) with low reactivity. Sheep anti-proBDNF was used as control for proBDNF(Sf9) and it only recognized proBDNF(Sf9) but not mBDNF. These results confirm that sheep anti-BDNF and B34D10 are highly specific to mBDNF only but not to proBDNF, whereas Aviscera Bioscience, R&D and Millipore anti-BDNF antibodies showed cross-reactivity to proBDNF (Sf9).

To confirm the specificity of antibody to mBDNF, competitive ELISA was conducted using proBDNF(Sf9) as a competitor. As a result, proBDNF(Sf9) did not show any competition effect up to 10 μg/mL compared to BSA as a control molecule (Fig. 2b). This competitive ELISA result shows that the sheep anti-BDNF antibody does not recognize proBDNF and supports previous results.

Setup of mBDNF sandwich ELISA: Standard curve and cross-reactivity test

To setup sandwich ELISA, B34D10 was used as capture antibody and sheep anti-BDNF was used as detection antibody. The antibody pair was then optimized with different capture and detection antibody concentrations (Fig. S3). In all subsequent experiments, the concentration of capture and detection antibody is 1 and 0.15 μg/mL respectively.

A standard curve of mBDNF was constructed with B34D10 and biotinylated sheep anti-mBDNF as capture and detection antibody respectively (Fig. 3a). The limit of detection was 1–3 pg/mL based on 14 independent determinations of blank.

As human BDNF has ~ 55% sequence homology with other neurotrophins such as NGF, NT-3 and NT-4 (Ullrich *et al.* 1983), it is necessary to test the cross-reactivity of those molecules on mBDNF ELISA . The recombinant human (Rh) NT-3, NT-4, NGF, proBDNF(Sf9) and another recombinant human proBDNF, expressed and purified in Chinese hamster ovary (CHO) cells, supplied by R&D company [proBDNF(R&D)]. As shown in Fig. 3b, RhNT-3, NT-4, NGF and proBDNF(Sf9) did not show any cross-reactivity up to 50 ng/mL. proBDNF(R&D) showed 0.99% at 50 ng/mL concentration. Next, we compared new mBDNF ELISA with a few commercial BDNF ELISA kits including Promega, R&D, Millipore and Aviscera Bioscience to evaluate the cross-reactivity for proBDNF (R&D) at 2000 pg/mL. R&D, Aviscera Bioscience and Millipore showed 33.18%, 91.09% and 53.52% cross-reactivity, respectively, however, our new mBDNF ELISA showed only 4.99% cross-reactivity for proBDNF (R&D) (Fig. 3c). We failed to measure both mature and proBDNF by Promega kit owing to poor standard curve generated (Fig. S5). These results suggest that new mBDNF ELISA is highly specific against mBDNF but not proBDNF and other neurotrophins.

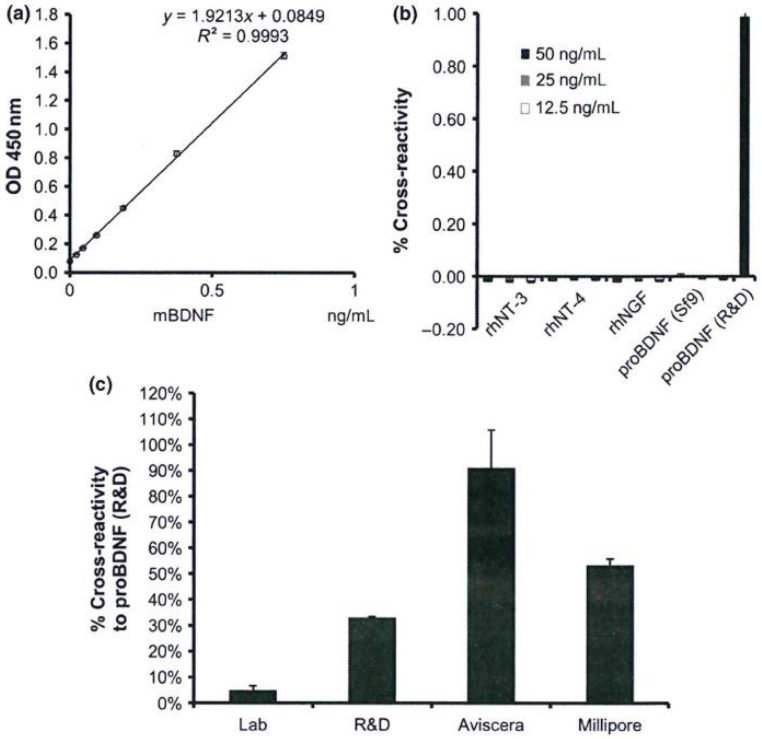


Fig. 3 Representative standard curve of mature brain-derived neurotrophic factor (mBDNF) sandwich ELISA, cross-reactivity test of other neurotrophins and comparison of cross-reactivity to proBDNF (R&D) with other commercial ELISA kits. (a) Representative mBDNF standard curve with dynamic range 0.023–0.75 ng/mL of mBDNF protein; Linear regression is presented with the corresponding equation and the squared correlation coefficient R^2. (b) Bar graphs presenting cross-reactivity (%) of various neurotrophic factors. (c) Bar graphs presenting cross-reactivity (%) for proBDNF (R&D) at 2000 pg/mL in new mBDNF ELISA and a few commercial BDNF ELISA kits. The plots show the mean ± SEM for triplicate determinations. Aviscera; Aviscera Bioscience

Moreover, our new ELISA has much higher specificity to proBDNF than commercial BDNF ELISA kits.

Validation of mBDNF sandwich ELISA for measurement of mBDNF in different tissues

Precision data for intra- and inter-assay were assessed using seven different concentration of mBDNF and the variation of coefficiency for intra-assay precision ranged from 1.18% to 7.70% and inter-assay precision from 1.90% to 4.73% (Table S1).

To see how efficiently mBDNF ELISA can be applied for different types of tissue samples, the dilution parallelism and spiking recovery were determined. The dilution 1 : 80–1 : 320 showed 103.43–110.19% of dilution parallelism and 100.56–98.62% of recovery in pooled sera and spiking sample (Table S2A). Similarly, single human serum also showed acceptable dilution parallelism and recovery. As for human plasma, 1 : 10–1 : 40 showed acceptable dilution parallelism and recovery (Table S2B).

As it has been previously reported that BDNF presents substantially in rat blood (Karege *et al.* 2002; Klein *et al.* 2011), rat serum and mouse brain tissues were also assessed for the dilution parallelism. Two rat sera showed 99.51–112.16% of dilution parallelism within dilution of 1 : 10–1 : 80 (Table S3A). Mouse brain tissue homogenate concentration range 500–125 μg/mL showed 90.61–114.58% dilution parallelism (Table S3B).

Application of mBDNF sandwich ELISA

The cleavage of amyloid-beta (Aβ) from amyloid precursor protein by β-Site amyloid precursor protein cleaving enzyme 1 (BACE1) is critical in the pathophygiology of AD (Kimura *et al.* 2010). Recently, it has been demonstrated that partial deletion of BACE1 gene with AD background mice expresses more BDNF than BACE1 WT with AD background mice. To see whether complete BACE1 gene deletion alters BDNF expression level and show the application ability of our novel mBDNF sandwich ELISA, mBDNF levels were measured in WT and $BACE1^{-/-}$ mice brain tissues by ELISA and western blot. ELISA (Fig. 4a) and western blot (Fig. 4c and d) showed that there was no difference in mBDNF levels between WT and BACE1. ProBDNF levels of WT and $BACE1^{-/-}$ were also measured by proBDNF ELISA (Fig. 4b) and no difference was observed. Our result suggests that the complete BACE1 deletion may not affect BDNF metabolism.

The levels of mBDNF (Fig. 5a) and proBDNF (Fig. 5c) in various rat brain regions were determined by ELISA and the ratio of mBDNF and proBDNF was analysed (Fig. 5d). The ratio of mBDNF and proBDNF represents the conversion rate of proBDNF to mBDNF which is critical process *in vivo*. The highest mBDNF concentration was found in the hypothalamus, followed by septum and hippocampus and the highest proBDNF concentration was found in hypothalamus, followed by thalamus and septum. Interestingly, the ratio of mBDNF/

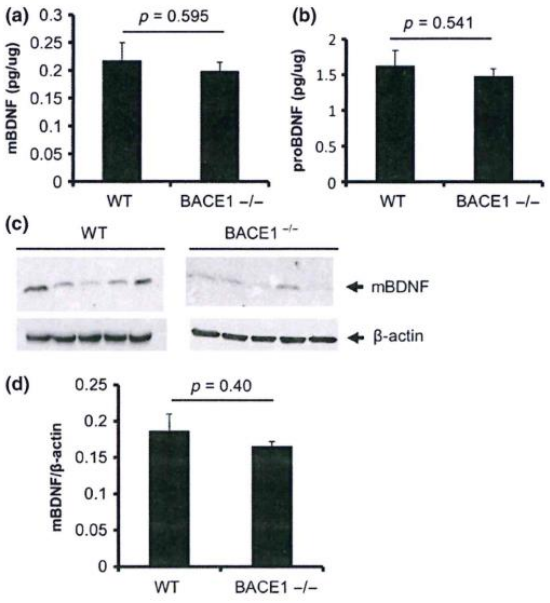


Fig. 4 Determination of mature brain-derived neurotrophic factor (mBDNF) and proBDNF levels by ELISA and western blot in WT and BACE1 $^{-/-}$ mice brain homogenates. (a) mBDNF ELISA. (b) proBDNF ELISA. (c) mBDNF was detected by commercially available rabbit anti-BDNF antibody (sc-546) and β-actin was detected for loading control. (d) The ratio of mBDNF to β-actin. All bar graphs are presented as mean ± SEM (n = 5 per group)

proBDNF in the hippocampus, hypothalamus and septum was two times higher than that in other brain regions. This result suggests that in a physiological condition, the processing rate of proBDNF to mBDNF in different brain regions are different, likely depending on neuronal activities and functional status.

Discussion

In this study, we screened mono and polyclonal BDNF antibodies by western blot and ELISA. Sheep anti-BDNF and B34D10 showed high specificity to mBDNF but not to proBDNF expressed in *E.coli*, HEK293 and Sf9 cells. Moreover, Sheep anti-BDNF antibody proved its specificity to mBDNF by indirect competitive ELISA applying proBDNF (Sf9). A sandwich ELISA was developed using B34D10 as capture antibody and sheep anti-BDNF antibody as detection antibody. The sandwich ELISA did not show any cross-reactivity with other neurotrophins including NT-3, NT-4, NGF and proBDNF (Sf9) or < 1% of cross-reactivity with proBDNF(R&D) respectively. Through direct comparison with other commercial BDNF ELISA kits, it has been also shown that our new mBDNF sandwich ELISA has negligible cross-reactivity, whereas others have 6–18 times higher cross-reactivity. The sandwich ELISA showed good precision data and CV's of intra- and inter-assay. In addition to that, the sandwich ELISA also showed very sharp linearity of dilution and recovery with spiking in human serum and plasma and linearity of dilutions in rat serum and mouse brain tissues. We have presented that our new mBDNF ELISA showed similar result with western blot. We have also measured and compared the ratios of mBDNF and proBDNF in various rat brain regions.

Antibody specificity to proBDNF and cross-reactivity

In this study, we have validated the antigenic specificity of sheep anti-BDNF and B34D10 against proBDNF expressed from various sources including *E.coli*, insect cell, HEK293T cell and CHO cell. Our finding is that B34D10 and sheep anti-BDNF almost do not recognize either native or linearized proBDNF. This feature contributes to the specificity of new sandwich ELISA.

Although we have demonstrated the specificity of the new ELISA in this study, it is still not clear why the antibodies, especially ELISA shows better performance than other tested commercial ELISA kits. According to the manufacturer's instructions, the Chemikine BDNF Sandwich ELISA kit (Millipore) includes two monoclonal antibodies against human mBDNF. The Quantikine ELISA kit (R&D) also two monoclonal antibodies to human recombinant mBDNF and shows about 13% cross-reactivity to proBDNF. The BDNF Emax Immuno Assay System (Promega) includes an antibody to the C-terminal of mBDNF (Chen *et al.* 2006; Yoshida *et al.* 2012a,b). The human BDNF ELISA kit (Aviscera Bioscience) does not supply the antibody information details. The kit instruction supplies cross-reactivity data for NT-3, NT-4, NGF and pro-domain part of proBDNF (aa 19–128); however, the cross-reactivity with the full-length proBDNF (aa 19–247) is not supplied. One possible reason could be that B34D10 and sheep anti-BDNF may recognize preferably native form of mBDNF as B34D10 failed to detect denatured mBDNF or proBDNF on western blot in mouse brain, spinal cord or dorsal root ganglion homogenates (data not shown). Another possible reason is that the epitope of B34D10 may be hidden in native proBDNF and it could contribute to the antigenic specificity. As the whole mBDNF protein was injected on immunization, it could be interesting to identify the epitope of B34D10. It is notable that all the commercial BDNF ELISA kits were used with their own sample diluent supplied with the kits, therefore the reaction condition should be optimal. To our knowledge, this is the first study to show direct comparison of cross-reactivity for proBDNF in commercial BDNF ELISA kits. In case of R&D kit, the cross-reactivity for proBDNF is claimed at 13%, although it showed over 30% cross-reactivity in our hands. Moreover, the human BDNF ELISA kit supplied from Aviscera Bioscience has been used in a few recent publications which reported mBDNF and proBDNF levels separately (Yoshida *et al.* 2012, Sodersten *et al.* 2014), showing over 90% cross-reactivity for proB-

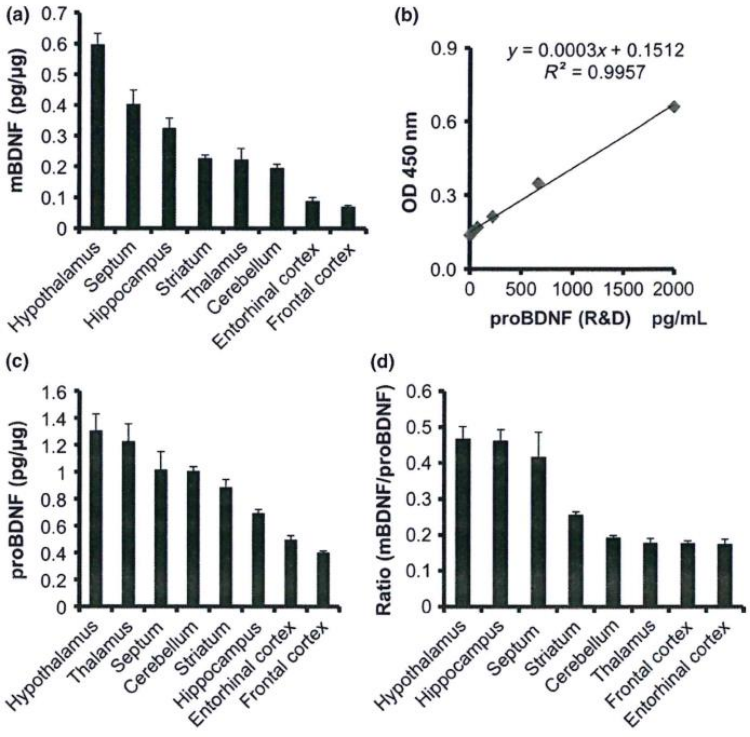


Fig. 5 Measurement of mature brain-derived neurotrophic factor (mBDNF) and proBDNF levels in various rat brain regions and the ratio of mBDNF and proBDNF. (a) mBNDF concentrations determined by new mBDNF ELISA (b) Representative proBDNF ELISA standard curve using human recombinant proBDNF (R&D). The plot shows the mean value for triplicate determination. Linear regression is presented with the corresponding equation and the square correlation coefficient R^2. (c) proBDNF concentrations determined by proBDNF ELISA. (d) Bar graphs presenting the ratio of mBDNF and proBDNF. 4-week-old female Sprague–Dawley rats ($n = 5$). All bar graphs are presented as mean ± SEM.

DNF(R&D). It is worth mentioning that the proBDNF (R&D) had been stored at −20°C for a couple of months until thawed for cross-reactivity test after initial protein reconstitution. The freeze-and-thaw may cause partial cleavage of proBDNF molecule, which generates mBDNF or reactive peptides. This hypothesis could explain the discrepancy of 0.99 versus 4.99% in our ELISA and 13.0 versus 30.18% in R&D's ELISA. Nevertheless, the cross-reactivity percentage of our new ELISA is lower than other commercially available kits and highly specific and acceptable indeed. Overall, our findings suggest that this sandwich ELISA for mBDNF can detect mBDNF levels with negligible interference by proBDNF. Promega ELISA kit did not generate acceptable standard curve (Fig. S4); therefore, we could not test the specificity for proBDNF(R&D) using the Promaga kit. However, Promega BDNF ELISA kit has been used widely by researchers (Teche *et al.* 2013). Therefore, it is likely we used a faulty kit in this study. It is also notable that mBDNF standard molecules supplied by different manufacturers show differences in terms of concentration (Fig. S5). Promega's standard molecule showed relatively lowest concentration. Millipore, R&D and Aviscera Bioscience showed twice, three times and seven times higher concentration of mBDNF standard molecule respectively. It is clear that the inconsistent concentration of standard mBDNF molecule among different suppliers can cause high variation in BDNF levels.

Validation of ELISA

There is no fixed performance standard required for immune assay, although it is generally acceptable when the %CV is 10–15%. With our newly developed ELISA, the CVs for intra- and inter-assay variability showed that this assay is highly reproducible for all of the working concentration range.

In general, dilution parallelism and recovery after spiking are acceptable when the variation is no more than 80–120% between doubling dilutions. In our new ELISA, human serum and plasma, rat serum and mouse brain tissue homogenate were subjected to the dilution parallelism and spiking recovery test (human serum and plasma only). Our data showed consistent dilution parallelism of all samples assayed and got almost 100% recovery after spiking with exogenous BDNF in these samples. Taken together, based on these preliminary data, it is suggested that 1 : 40–1 : 320 for human serum, 1 : 10–1 : 40 for human plasma, 1 : 10–1 : 40 for rat serum and 500–125 µg/mL for mouse brain tissue homogenate could be used to measure mBDNF levels by our new sandwich ELISA.

Application and review of previous report

We found that the levels of mBDNF and proBDNF in the brain of $BACE1^{-/-}$ were not different from those of WT mice as determined by ELISA and western blot. Our results suggest that BACE1 may not affect BDNF levels in the

brain. Nevertheless, the mBDNF levels determined by ELISA and western blot were consistent, suggesting that new mBDNF ELISA can be useful for mBDNF measurement.

The new mBDND ELISA can be applied for the measurement of biological samples. We have measured mBDNF and proBDNF levels in various rat brain regions as described by Nawa *et al.* (1995). Consistent with the results from Nawa's study, we found that BDNF levels in the hippocampus, hypothalamus and septum were higher than those in other brain regions including cerebellum and frontal cortex. In contrast, the BDNF levels in some brain regions such as thalamus and striatum by the present assay were less consistent with those by Nawa' assay. In addition to the specificity of the assay we show here, it should be noted that the gender and age of the rats may account for the inconsistency. Furthermore, different protein extraction methods and different standards could also affect the measurement.

As proBDNF opposes the functions of mature BDNF and the level of BDNF is altered in various neurological disorders, it is essential to measure mBDNF specifically without cross-reactivity with proBDNF. It could supply a new window to monitor the progress of the disease or the efficacy of drugs to treat diseases. This new sandwich ELISA for mBDNF would allow the re-evaluation of the previous findings on BDNF studies and also serve as a good tool for BDNF studies in the future. Indeed, one of our main findings is that the ratio of mBDNF to proBDNF changes in different brain regions and is not always consistent with the mature BDNF levels. The beneficial effects of BDNF may depend on the ratio of mBDNF versus proBDNF. Measurement of the ratio of mBDNF/proBDNF in ageing population and in diseased conditions could be important for understanding the role of BDNF in neurological and mental disorders.

Acknowledgements and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Expression of proBDNF-Myc-His in mammalian cells.

Figure S2. Expression of proBDNF(Sf9) in insect cells.

Figure S3. Representative data for the optimization of capture and detection antibody concentration for mBDNF sandwich ELISA.

Figure S4. Standard curves used for proBDNF cross-reactivity test.

Figure S5. Comparison of relative concentration of mBDNF standard molecules supplied with ELISA kits.

Table S1. Intra- and inter-assay variability of mBDNF sandwich ELISA.

Table S2. Dilution parallelism and spiking recovery of mBDNF sandwich ELISA with human serum and plasma.

Table S3. Dilution parallelism of mBDNF sandwich ELISA with rat serum and mouse brain tissue.

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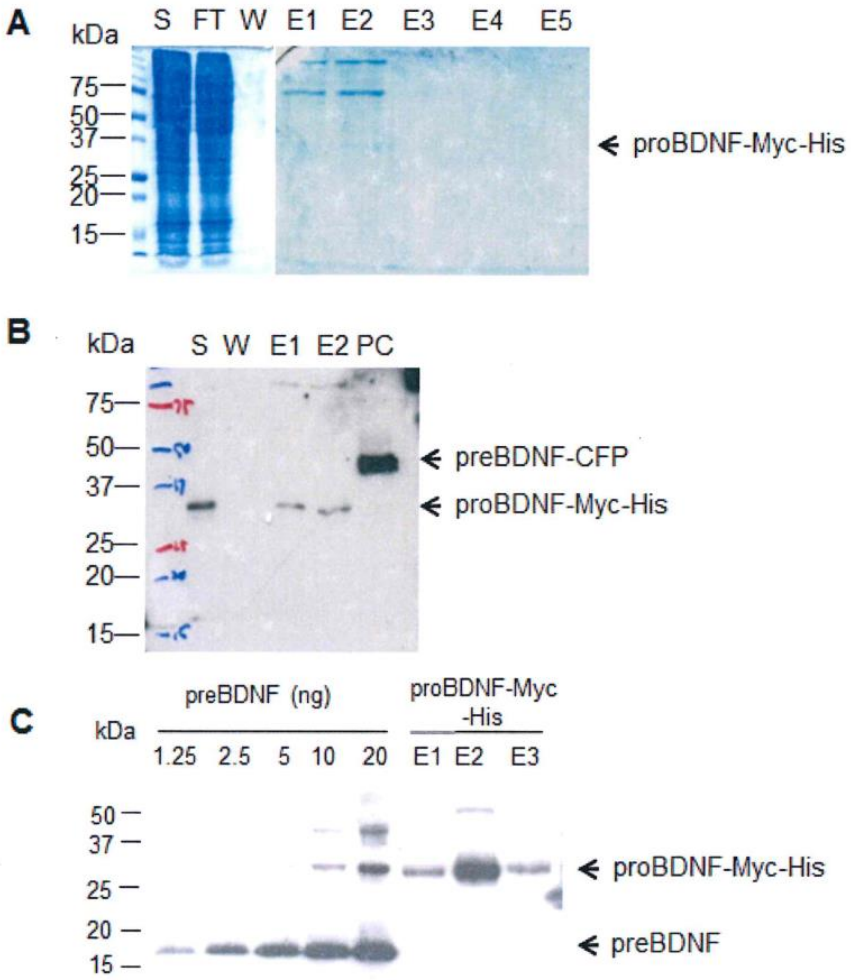
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Supplementary Information

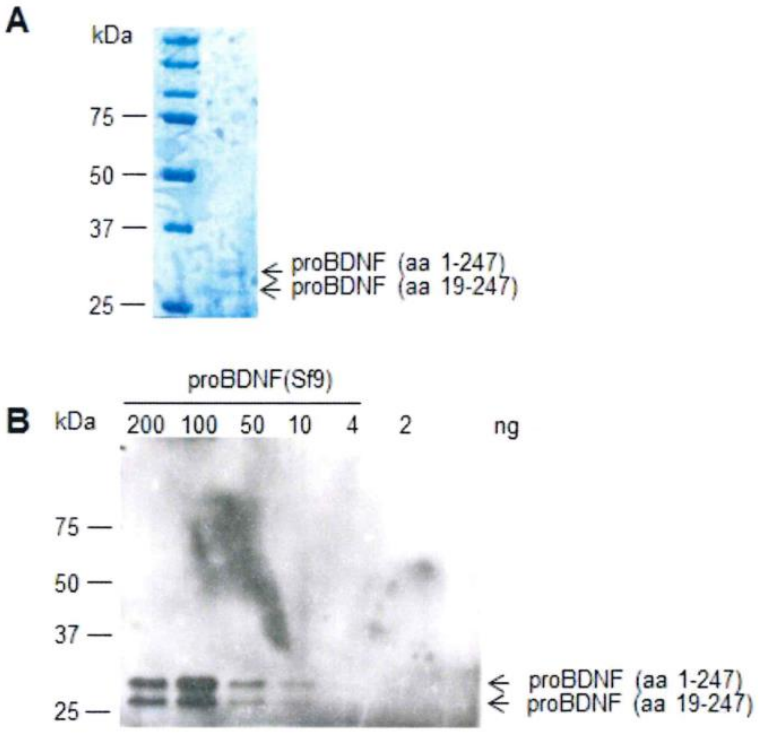


Supplementary Figure 1. Expression of proBDNF-Myc-His in mammalian cells. **(A)** Coomassie blue staining showing proBDNF-Mys-His. S (start), FT (flow through), W (washing), E1 (elute 1), E2 (elute 2), E3 (elute 3), E4 (elute 4), E5 (elute 5). **(B)** Western blot analysis of proBDNF-Myc-His. S (start), W (wash), E1 (elute 1), E2 (elute 2), PC (positive control; HEK293T cell lysate expressing preBDNF-CFP). Western blot with biotinylated sheep anti-proBDNF IgG. **(C)** Quantification of purified proBDNF-Myc-His using purified

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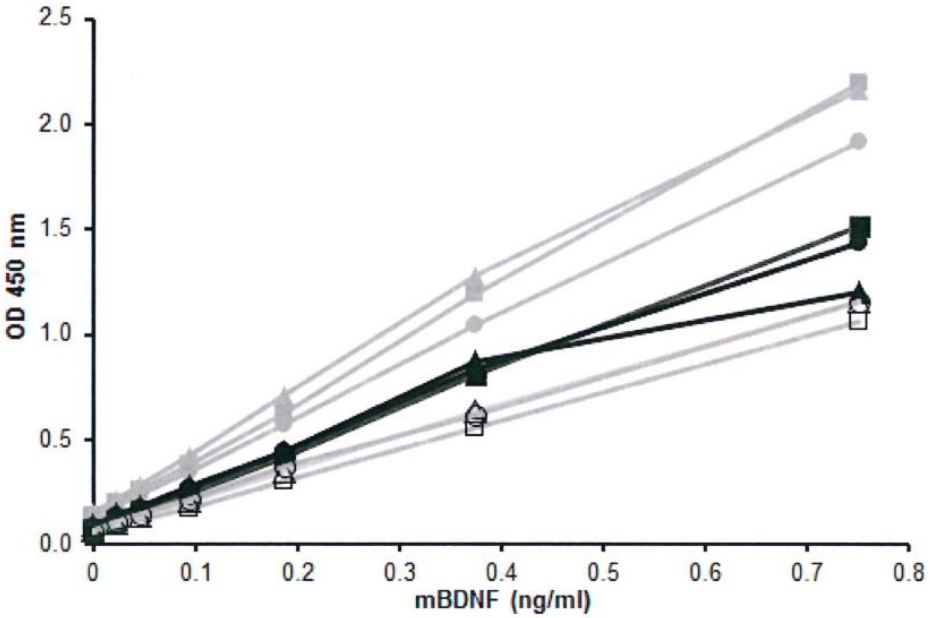
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preBNDF (aa 19-128 of proBDNF) expressed in *E.coli* as standard reference by Western blot. Western blot with biotinylated sheep anti-proBDNF IgG.



Supplementary Figure 2. Expression of proBDNF(Sf9) in insect cells. ProBDNF(Sf9) was expressed and purified in Sf9 cells by Virovek company (Hayward, CA, USA) and checked by Coomassie blue staining and Western blot. **(A)** Coomassie blue staining, purified 1 μg of proBDNF(Sf9). **(B)** Western blot of ProBDNF (Sf9) Biotinylated sheep anti-

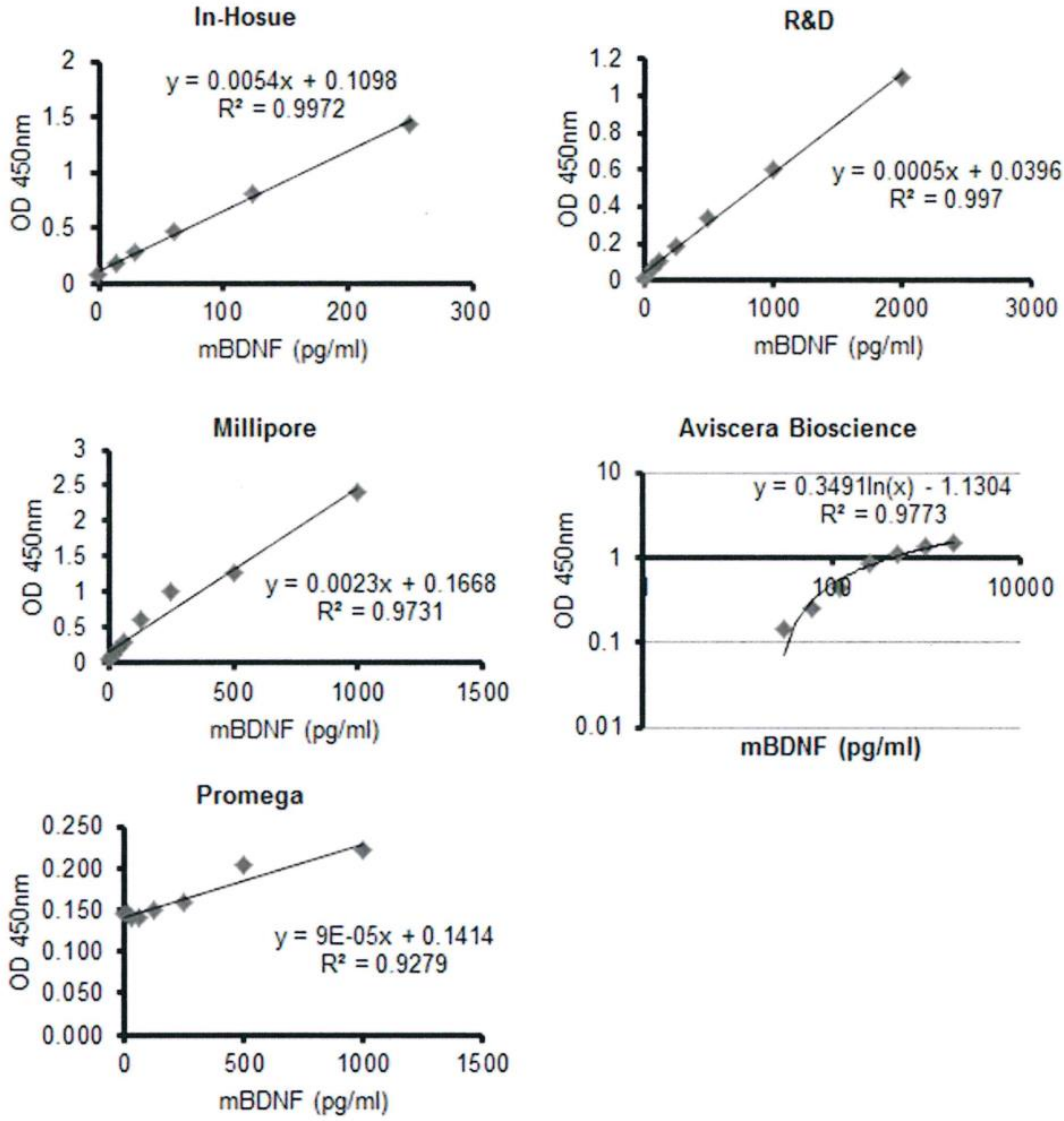
proBDNF (2 μg/ml) was used to detect proBDNF (Sf9).



	Detection antibody 0.4 μg/ml			Detection antibody 0.2 μg/ml			Detection antibody 0.1 μg/ml		
Capture antibody	1 μg/ml	2 μg/ml	4 μg/ml	1 μg/ml	2 μg/ml	4 μg/ml	1 μg/ml	2 μg/ml	4 μg/ml
Equation	y = 2.3985x + 0.1268	y = 2.7152x + 0.1754	y = 2.7152x + 0.1754	y = 1.9331x + 0.0717	y = 1.8166x + 0.0997	y = 1.5339x + 0.1357	y = 1.3396x + 0.0577	y = 1.4615x + 0.0709	y = 1.4442x + 0.0809
R^2	0.9998	0.9961	0.9961	0.9995	0.997	0.9649	0.9998	0.9994	0.9993
Shape	Grey circle	Grey square	Grey triangle	Black square	Black circle	Black triangle	Open square	Open triangle	Open circle

Supplementary Figure 3. Representative data for the optimization of capture and detection antibody concentration for mBDNF sandwich ELISA. Capture antibody (B34D10) was coated at 1, 2, or 4 μg/ml concentration into 96-well plate. mBDNF was added with the concentration of 0.75, 0.375, 0.188, 0.094, 0.047, 0.023 and 0 ng/ml. Detection antibody (biotinylated sheep anti-BDNF) was then added with 0.1, 0.2 or 0.4 μg/ml concentration. A plot was made according to each condition **(top)**. All OD values were mean

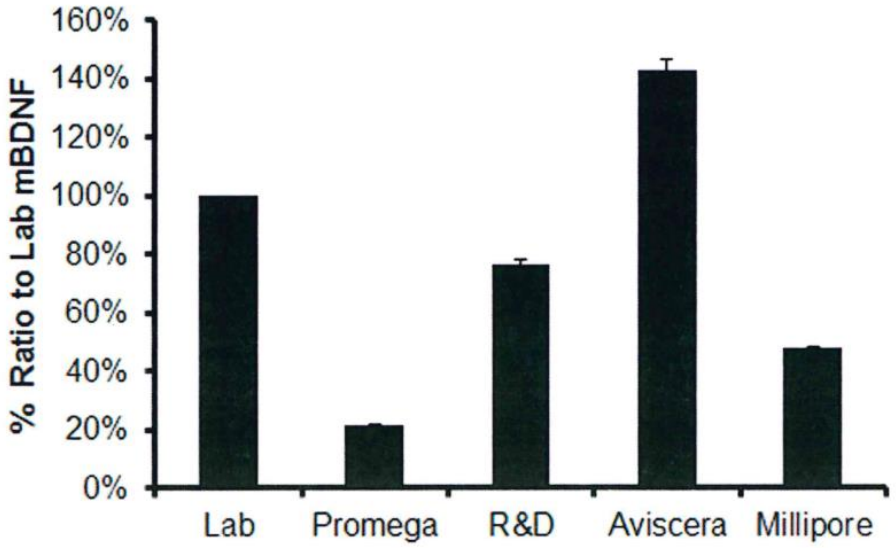
of triplicate determinations. All the equations and the squared correlation coefficient R^2 value details of plots were sorted in the table **(bottom)**.



Supplementary Figure 4. Standard curves used for proBDNF cross-reactivity test. Plots are representative standard curves of ELISA kits from different manufacturer. The equations of the regression straight line are indicated in each plot.

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Supplementary Figure 5. Comparison of relative concentration of mBDNF standard molecules supplied with ELISA kits. mBDNF standard molecules were prepared according to the manufacturer's instruction and measured at 200 pg/ml in new mBDNF sandwich ELISA. Results are shown as the mean ± SEM (duplicate, two separate sets). Aviscera; Aviscera Bioscience. The measure of lab mBDNF standard molecule was normalised to 100%.

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		Intra Assay			
Assay No.		Replicate	Mean	SD	%CV
1	Std point 1	6	0.0784	0.0009	1.20
	Std point 2	6	0.1262	0.0040	3.19
	Std point 3	6	0.1708	0.0068	4.00
	Std point 4	6	0.2608	0.0101	3.87
	Std point 5	6	0.4507	0.0204	4.53
	Std point 6	6	0.8325	0.0321	3.85
	Std point 7	6	1.5119	0.0484	3.20
2	Std point 1	3	0.0784	0.0021	2.68
	Std point 2	3	0.1214	0.0035	2.89
	Std point 3	3	0.1664	0.0035	2.10
	Std point 4	3	0.2438	0.0116	4.77
	Std point 5	3	0.4140	0.0259	6.26
	Std point 6	3	0.8134	0.0401	4.94
	Std point 7	3	1.5189	0.1170	7.70
3	Std point 1	5	0.0765	0.0009	1.18
	Std point 2	5	0.1173	0.0044	3.75
	Std point 3	5	0.1603	0.0047	2.95
	Std point 4	5	0.2492	0.0098	3.95
	Std point 5	5	0.4293	0.0190	4.42
	Std point 6	5	0.8125	0.0228	2.80
	Std point 7	5	1.4918	0.0624	4.18

Inter-Assay	Mean	SD	%CV
Std point 1	0.0778	0.0015	1.90
Std point 2	0.1220	0.0055	4.54
Std point 3	0.1661	0.0070	4.24
Std point 4	0.2530	0.0120	4.73
Std point 5	0.4352	0.0246	5.64
Std point 6	0.8213	0.0301	3.66
Std point 7	1.5062	0.0658	4.37

Supplementary Table 1. Intra and inter-assay variability of mBDNF sandwich ELISA. Seven different concentrations of human recombinant mBDNF were utilized for mBDNF ELISA. For intra – and inter-assay, 6, 3 and 5 replicates for each standard point were measured in three independent assays, SD: standard deviation, %CV: (% of coefficient of variation) = (SD/Mean) X 100

A	No spike	Spike level (10 ng/ml)

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Sample	Dilution Factor (DF)	Observed (ng/ml) x DF	% Change in concentration from previous	Observed (ng/ml) x DF	Expected (ng/ml)	% Recovery
Human serum pool (n = 12)	20	21.78	NA	26.25	31.78	82.59
	40	27.73	127.36	34.96	37.73	92.64
	80	28.69	103.43	38.90	38.69	100.56
	160	29.74	103.68	40.43	39.74	101.73
	320	32.77	110.19	42.18	42.77	98.62
Human serum (n = 1)	40	9.70	NA	17.79	19.70	90.30
	80	9.99	103.00	21.06	19.99	105.35
	160	10.73	107.43	21.34	20.73	102.91
	320	11.63	108.35	22.90	21.63	105.89

		No spike		Spike level (3 ng/ml)		
Sample	Dilution Factor (DF)	Observed (ng/ml) x DF	% Change in concentration from previous dilution	Observed (ng/ml) x DF	Expected (ng/ml)	% Recovery
Human plasma (n = 1)	10	2.00	NA	4.69	5.00	93.84
	20	2.74	137.36	6.01	5.74	104.60
	40	2.66	97.16	6.00	5.66	105.91

Supplementary Table 2. Dilution parallelism and spiking recovery of mBDNF sandwich ELISA with human serum and plasma. (A, B) Human serum pool (n=12), human serum (n=1) and human plasma (n=1) were used to test the recovery of dilution with or without adding exogenous mBDNF (spiking). Human serum and plasma were diluted in sample diluent (see materials and method). For spiking, 10 ng/ml and 3 ng/ml of mBDNF were added into human serum and plasma, respectively, followed by serial dilution. %Recovery = (Observed /Expected) x 100

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A

Sample	Dilution Factor (DF)	Observed (ng/ml) x DF	% Change in concentration from previous dilution
Rat serum 1	10	1.84	N/A
	20	2.03	110.03
	40	2.09	103.17
	80	2.08	99.51
Rat serum 2	10	1.78	N/A
	20	2.00	112.16
	40	2.01	100.50
	80	2.02	100.48

B

Sample	Observed (pg/ml)	Mouse brain homogenate (µg/ml)	Observed /Mouse brain homogenate (pg/µg)	% Change in concentration from previous dilution
Mouse brain 1	206.54	500	0.41	N/A
	93.58	250	0.37	90.61
	50.77	125	0.41	108.51
	30.67	62.5	0.49	120.83
Mouse brain 2	169.81	500	0.35	N/A
	83.49	250	0.33	95.58
	46.30	125	0.38	114.58
	28.73	62.5	0.50	131.18
Mouse brain 3	339.21	500	0.68	N/A
	114.33	250	0.46	67.41
	51.95	125	0.42	90.87
	34.31	62.5	0.55	132.11

Supplementary Table 3. Dilution parallelism of mBDNF sandwich ELISA with rat serum and mouse brain tissue. **(A)** Two rat serum and **(B)** three mouse brains were used to test the linearity of dilution. Rat serum and mouse brain homogenates were diluted in sample diluent . In case of mouse brain homogenates, 500 µg/ml was the initial concentration and two-fold diluted serially. Change in concentration from previous dilution (%) = (the current dilution / the previous dilution) x 100.

Chapter 4: Upregulation of blood proBDNF and its receptors in major depression

STATEMENT OF AUTHORSHIP

Chapter 4 --- (2013) "*Upregulation of blood proBDNF and its receptors in major depression." J Affect Disord 150(3): 776-784.*

Yoon Lim (Candidate)

Developed mature BDNF-specific ELISA, measured mBDNF level, analysed data, prepared figures, wrote the manuscript, contributed to trouble shooting in Western blotting, performed the statistical correlation analyses in Fig.5 B,C to confirm the data obtained by Chinese collaborators.

I hereby certify that the statement of contribution is accurate

Signed: Date: 26/ Jul/ 2015

Li Zhou

Designed and performed experiments, analysed data, prepared figures, wrote and revised the manuscript.

I hereby certify that the statement of contribution is accurate

Signed: Date: 26/Jul/2015

Jing Xiong

Designed and performed experiments, analysed data, prepared figures, wrote and revised the manuscript.

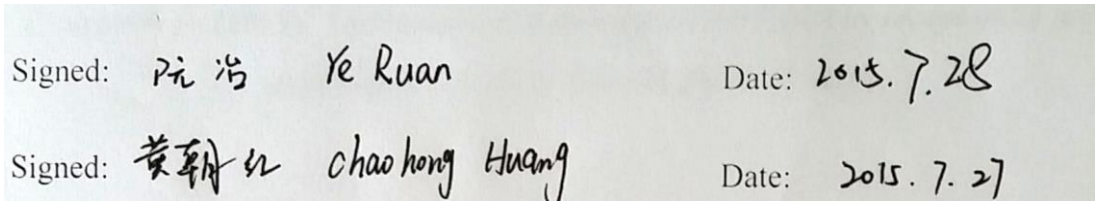
I hereby certify that the statement of contribution is accurate

Signed: Date: 28/Jul/2015

Ye Ruan, Chaohong Huang, Yuhong Zhu, Jin-Hua Zhong

Contributed to the sample and reagents preparation.

I hereby certify that the statement of contribution is accurate



Signed: Date: 28/Jul/2015

Signed: Date: 29/Jul/2015

Zhicheng Xiao

Contributed to the conceptualisation and data interpretation, critical manuscript evaluation and acted as the corresponding author.

I hereby certify that the statement of contribution is accurate



Xin-Fu Zhou

Contributed to the conceptualisation and data interpretation, critical manuscript evaluation and acted as the corresponding author.

I hereby certify that the statement of contribution is accurate

Signed: Date: 27/Jul/2015

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Research report

Upregulation of blood proBDNF and its receptors in major depression

Li Zhou [a,c,1], Jing Xiong [a,f,1], Yoon Lim [b,1], Ye Ruan [c], Chaohong Huang [e], Yuhong Zhu [f], Jin-hua Zhong [b], Zhicheng Xiao [a,d,*], Xin-Fu Zhou [a,b,*]

[a] Key Laboratory of Stem Cells and Regenerative Medicine, Institute of Molecular and Clinical Medicine, Kunming Medical University, Kunming, Yunnan Province, PR China
[b] School of Pharmacology and Medical Sciences, University of South Australia, Adelaide, 5000, Australia
[c] Kunming Psychological Crisis Intervention and Research Center, Kunming City, Yunnan Province, PR China
[d] Department of Immunology and Stem Cell laboratories, Monash University, Clayton, Australia
[e] Yunnan Mental Hospital, Kunming City, Yunnan Province, PR China
[f] Department of Neurology, 2nd Affiliated Hospital, Kunming Medical University, Yunnan Province, PR China

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ABSTRACT

Background: In recent decades, the role of brain-derived neurotrophic factor (BDNF) in depression has received intensive attention. However, the relationship between proBDNF and depression has not been clearly elucidated.

Methods: Forty drug-free women patients diagnosed with major depression and 50 healthy female controls were enrolled in our study. Peripheral blood was sampled from all the subjects. With the blood samples, we assessed the relationship between BDNF and major depression from following aspects: the levels of BDNF, proBDNF and their receptors in the sera and lymphocytes. The mRNA levels of these factors in lymphocytes were also examined. Furthermore, the correlations between each factor and the severity of major depression were tested.

Results: It was found that: (a) the protein and serum levels of proBDNF, sortilin and p75NTR were higher in major depressive patients than in healthy controls while mature BDNF and TrkB levels were lower; (b) the BDNF, TrkB, sortilin and p75NTR mRNA levels changed in line with their protein levels; (c) The levels of mature BDNF and TrkB had negative correlations with the major depression severity, and the levels of proBDNF, p75NTR and sortilin were positively correlated with the scores of HRSD-21; (d) the ratio of proBDNF and mBDNF was imbalanced in major depressive patients.

Conclusion: The balance between the proBDNF/p75NTR/sortilin and mBDNF/TrkB signaling pathways appears dysregulated in major depression and both pathways should be considered as biomarkers for the major depression

Limitations: More cases on both genders should be enrolled in our study. And further works on the mechanisms of how BDNF and its receptors are regulated in depression should also be carried out.

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1. Introduction

Depression is a complex disease. The World Health Organization suggests that by 2030, depression will be a leading cause of loss of ability to work and mortality (Colin and Mathers, 2006). Especially, the incidence of major depression is high with a lifetime prevalence of 16%, and annual prevalence of 6.6% (Kessler et al., 2003).

Neurotrophins play important roles in neuron survival, plasticity, neurogenesis and synaptogenesis (Thoenen, 1995; Bartrup et al., 1997). Among the family, BDNF is the one most widely expressed. BDNF is originally synthesized as a glycosylation precursor protein, proBDNF (precursor for brain-derived neurotrophic factor) with 32–36 KDa molecular mass. In the secretion process, the precursor protein is converted into two fragments, mature BDNF (mBDNF) and precursor peptide (propeptide of proBDNF, preBDNF) by precursor protein convertase, subtilisin/kexin 1ike convertase family members, such as furin, pro-convertases 1/3 (PC1/3), PACE4, PC5/6-B and other proteases, such as tissue plasminogen activator (tPA), matrix metalloproteinases-3 (MMP-3) and MMP-7 (Meyer et al., 1996; Mowla et al., 1999; Lee et al., 2001; Matsumoto et al., 2008; Nagappan et al., 2009; Yang et al., 2009A). The "neurotrophin hypothesis" of depression holds that BDNF is a pivotal factor in the pathophysiology of depression. The down-regulation of BDNF is

* Corresponding authors at: Key Laboratory of Stem Cells and Regenerative Medicine, Institute of Molecular and Clinical Medicine, Kunming Medical University, Kunming, Yunnan Province, PR China. Tel.: +61883022329; fax: +61433403266.
E-mail addresses: zhicheng.xiao@monash.edu (Z. Xiao), Xin-Fu.Zhou@unisa.edu.au (X.-F. Zhou).
[1] These authors contribute equally to the work.

0165-0327/$ - see front matter Crown Copyright © 2013 Published by Elsevier B.V. All rights reserved.
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involved in the pathophysiology of depression and antidepressants can restore the BDNF level (Castrén et al., 2007). Many studies show that mBDNF and its high affinity receptor, the tyrosine kinase receptor B (TrkB), can lead to anti-depressive effects. And its precursor (proBDNF) binds with the receptors p75 neurotrophin receptor (p75NTR) and sortilin preferentially, but its role is not clear (Lee et al., 2001; Chao, 2003). Some reports show that proBDNF induces neuronal atrophy and apoptosis and dendritic pruning, which was a pro-depressant response (Lee et al., 2001; Woo et al., 2005; Zagrebelsky et al., 2005). As proBDNF and mBDNF have opposing biological effects (Lu et al., 2005; Xu et al., 2011; Sun et al., 2012), the regulation on the cleavage of proBDNF becomes critical for controlling BDNF functions (Deppmann et al., 2008). Although the BDNF hypothesis of depression is intriguing, we still do not know the expression level of proBDNF and its effect on depression. We hypothesize that proBDNF signaling is altered in human depression and the signaling changes may participate in the pathogenesis of depression. In the present study, we first examined the levels of proBDNF and its receptors p75NTR and sortilin from lymphocytes and serum in Chinese female patients with major depression. And we also described the role of another form of BDNF, mBDNF, and its receptor-TrkB in female patients with major depression.

2. Materials and methods

2.1. Subjects

Forty female patients with major depression who were diagnosed according to the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) criteria participated in the study after providing written consent (age range: 21–57 years; mean±SD age=42.67±9.58 years). All the major depressive disorder (MDD) patients were from Yunnan Mental Hospital inpatients. All the patients were Han population. We declare that our study was carried out in accordance with the latest version of the Declaration of Helsinki. The Ethics Committee of Yunnan Mental Hospital approved all procedures. The patients were all drug-naïve. All the patients met the selection criteria as follows: (a) the inpatients of Yunnan Mental Hospital from September 2010 to March 2011 met the ICD-10 diagnostic criteria for depression; (b) the 21-item Hamilton Rating Scale for Depression (HRSD-21) score≥35 points; (c) routine blood test, blood glucose, blood lipids and kidney function were normal; (d) no treatments with an antidepressant medication or recently antidepressants medication before being enrolled into our study; (e) to have obtained the informed consent of patients or their families. Exclusion criteria were (a) lifetime diagnosis of dementia, schizophrenia or schizoaffective disorder, or bipolar disorder according to ICD-10; (b) current diagnosis of alcohol dependency (ICD-10) requiring acute detoxification; (c) depression due to organic factors including Parkinson's disease or Multiple Sclerosis; (d) pregnancy or breast-feeding. The HRSD scores of major depressive groups were range 34–67, mean±SD=41.775±7.231.

Fifty control individuals in our studies were in general good health but were not screened for medical illness; they were age (age range: 25–60 years; mean±SD age=44.3±10.04 years), gender and ethnicity matched with the depression patients. And all the participants were born in the same place (Kunming City). Control group criteria were (a) to match depression patient group in age, gender, ethnicity and place of birth; (b) the HRSD-21 score<8 points; (c) routine blood test, blood glucose, blood lipids and kidney function were normal; (d) without histories of schizophrenia, depression, alcohol dependence and drug dependence. In all cases, assessments were conducted by a well-trained psychiatrist. The HRSD scores of healthy control group were range 2–7, mean±SD=3.7±1.035.

2.2. Study design

All patients and controls were subjected for the analysis of serum mBDNF ELISA assays. The levels of proBDNF, sortilin, p75NTR and TrkB in the serum and lymphocytes were determined by Western blots and their mRNA levels were assayed by quantitative RT-PCR. The correlation between these factors and depression scores was statistically analyzed. The details of the materials and methods for these studies were given in the supplement materials.

2.3. Statistical methods

All statistical analyses were performed using Statistical Product and Service Solutions 13.0 Version (SPSS). The age was expressed as mean±standard deviation. Results were given as mean±standard error of mean. Chi-square was used for categorical comparisons, and Student's t-test was employed for age difference. The significance was evaluated by Mann–Whitney U test. A P-value of <0.05 (2-tailed) was considered as statistically significant. The nonparametric correlation was used for assay of correlations of HRSD scores with proBDNF, mature BDNF, p75NTR, sortilin and TrkB.

3. Results

3.1. Western blotting analysis of the proBDNF, p75NTR, sortilin and TrkB

With the Western blots, we found that the protein levels of proBDNF, p75NTR and sortilin from both lymphocytes (Fig. 1A–D) and sera (Fig. 2A–D) were significantly increased in patients when compared with healthy controls (the results of lymphocytes in Fig. 1: proBDNF: $u=-2.078$, $P=0.038$; p75NTR: $u=-2.147$, $P=0.032$; sortilin: $u=-2.024$, $P=0.043$. The results of sera in Fig. 2: proBDNF: $u=-2.182$, $P=0.029$; p75NTR: $u=-2.544$, $P=0.011$; sortilin: $u=-2.569$, $P=0.010$). The protein level of TrkB in patients was reduced when compared with healthy controls (lymphocytes in Fig. 1D: $u=-2.048$, $P=0.041$; sera in Fig. 2D: $u=-1.988$, $P=0.047$).

The analysis of correlations of protein levels of proBDNF, TrkB, p75NTR and sortilin in lymphocytes and sera with HRSD scores in patients were shown in Fig. 3A–H. There were significant positive correlations between proBDNF (Fig. 3A, $r=0.418$, $P=0.012$), p75NTR (Fig. 3 B, $r=0.415$, $P=0.013$) and HRSD scores. And there was no correlation between sortilin and HRSD scores (Fig. 3 C, $r=0.342$, $P=0.057$). TrkB level had a significant negative correlation with HRSD scores ($r=-0.356$, $P=0.036$) (Fig. 3D). In the serum, the correlation between proBDNF and its receptors with HRSD was shown in Fig. 3E–H. The proBDNF, p75NTR and sortilin had significant positive correlation with HRSD scores (in Fig. 3E: $r=0.530$, $P=0.003$; in Fig. 3F: $r=0.521$, $P=0.001$; in Fig. 3G: $r=0.323$, $P=0.042$). TrkB had a significant negative correlation with HRSD scores (in Fig. 3H: $r=-0.328$, $P=0.039$).

We attempted to perform Western blotting analysis of lymphocytes and sera in all cases, however in some cases the proteins of interest were not measurable with the western blot method, due to the interference of high levels of serum proteins such as albumin or hemoglobulin, thus not all the samples were included for the analysis. The data from Western blots and RT-PCR assays represent all measurable samples without any selection or bias.

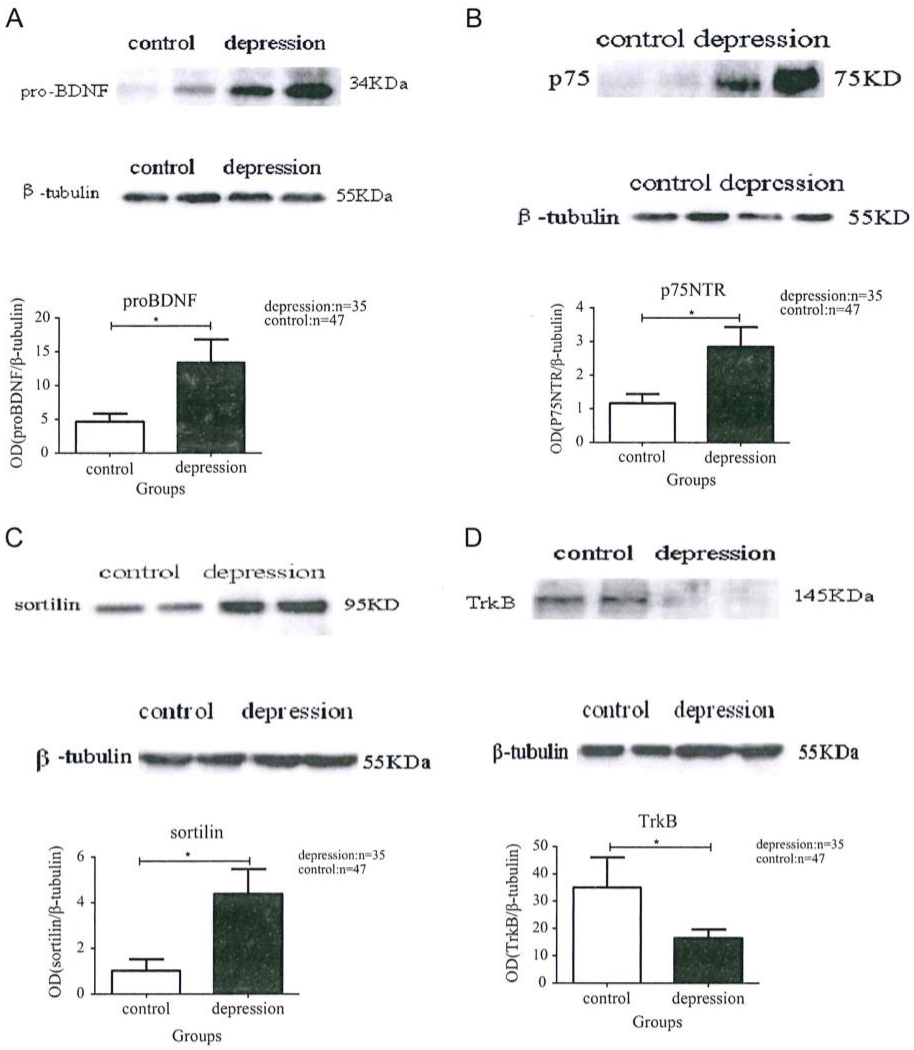


Fig. 1. Representative Western blot images and relative levels of proBDNF, p75NTR, sortilin and TrkB in lymphocytes. Densitometric values from all western blots were normalized to β-tubulin. The lymphocytes protein levels in depression and in controls were compared. (A) proBDNF ($n=35$ in each group, $u=-2.078$, $P=0.038$), (B) p75NTR ($n=35$, $u=-2.147$, $P=0.032$), (C) sortilin ($n=35$, $u=-2.024$, $P=0.043$) were significantly increased in major depressive patients when compared with healthy controls. And the lymphocytes protein level of (D) TrkB ($n=35$, $u=-2.048$, $P=0.041$) was significantly decreased in major depressive patients compared with healthy controls (independent-test, $^{*}P<0.05$).

3.2. *Real time RT-PCR analysis of BDNF, p75NTR, sortilin and TrkB mRNA expressions*

Real time RT-PCR revealed differences in BDNF, p75NTR, sortilin and TrkB mRNA levels in lymphocytes between the major depressive patients and the healthy controls (Fig. 4A–D). The BDNF (Fig. 4A, $u=-2.367$, $P=0.018$) and TrkB (Fig. 4D, $u=-3.084$, $P=0.002$) mRNA levels in major depressive patients were lower than in control group. In contrast, the p75NTR (Fig. 4B, $u=-2.284$, $P=0.022$) and sortilin (Fig. 4C, $u=-1.986$, $P=0.047$) mRNA levels in lymphocytes in patients were higher than in controls.

The correlations between mRNA levels and HRSD scores were also been assayed (Fig. 4E–H). There were significant negative correlations between BDNF and TrkB levels (BDNF: Fig. 4E, $r=-0.415$, $P=0.011$; TrkB: Fig. 4H, $r=-0.351$, $P=0.033$) with HRSD scores. p75NTR (Fig. 4F, $r=0.324$, $P=0.050$) and sortilin (Fig. 4G, $r=0.386$, $P=0.018$) had significant positive correlations with HRSD scores.

3.3. *ELISA*

As shown in Fig. S1, to confirm the specificity of mBDNF ELISA, NGF, NT-3, NT-4 and proBDNF were measured at 1 ng/ml and mBDNF at various concentrations (0–2 ng/ml; Fig. S1A). NGF, NT-3, NT-4 and proBDNF did not show any OD value higher than background level while mBDNF showed 0.67 of OD value at 1 ng/ml (Fig. S1 A). As it is critical to distinguish proBDNF and mBDNF, proBDNF was added at various concentrations (0–20 ng/ml; Fig. S1B). No OD value increase was observed on mBDNF ELISA at different concentration of proBDNF (Fig. S1B). This result

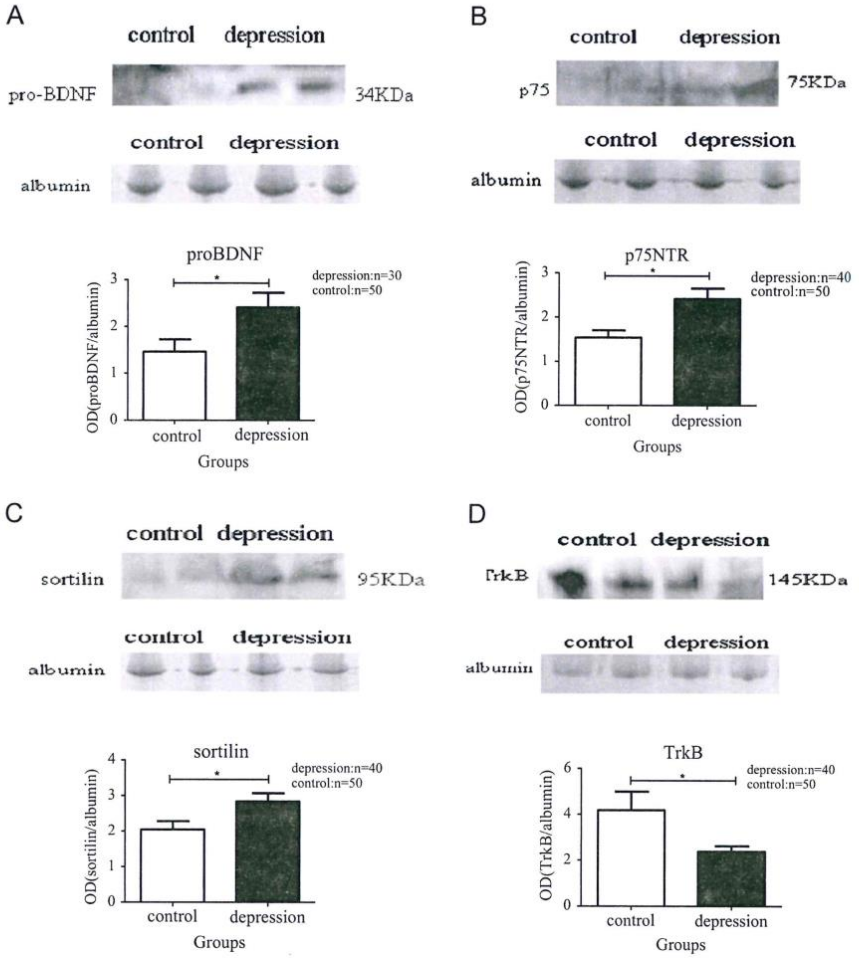


Fig. 2. Representative Western blot images and relative levels of serum proBDNF, p75NTR, sortilin and TrkB. Densitometric values from all western blots were normalized to *serum albumin*. The serum levels of (A) proBDNF ($n=30$ in each group, $u=-2.182$, $P=0.029$), (B) p75NTR ($n=40$ in each group, $u=-2.544$, $P=0.011$), (C) sortilin ($n=40$ in each group, $u=-2.569$, $P=0.010$) were significantly increased in major depressive patients when compared with healthy controls. And the serum level of (D) TrkB ($n=40$ in each group, $u=-1.988$, $P=0.047$) was significantly decreased in major depressive patients compared with healthy controls (independent *t*-test, $^{*}P<0.05$).

indicates that mBDNF ELISA is highly specific to only mBDNF and does not detect proBDNF and the other neurotrophins (NT-3, NT-4 and NGF).

The ELISA results of mBDNF indicated that the mBDNF levels in major depressive patients ($n=40$, mean±SEM=13.041±1.237) were significantly lower than in healthy controls ($n=50$, mean ±SEM=18.369±1.585), (Fig. 5A, $u=-2.347$, $P=0.019$). We also analyzed the relationship between proBDNF and mature BDNF in the serum. The results showed that the proBDNF level had a negative correlation with the level of mBDNF ($n=30$, $r=-0.394$, $P=0.031$), which revealed the imbalance of proBDNF and mBDNF in major depressive patients (Fig. 5B). The correlation between mBDNF and HRSD scores revealed that mBDNF had negative correlation with the HRSD scores (Fig. 5C, $n=40$, $r=-0.451$, $P=0.004$). And the TrkB ELISA level showed the results of TrkB ($n=40$, mean±SEM=488.916±35.550) in major depressive patients and in health controls ($n=40$, mean±SEM=617.772±6.965). The patients group was lower than healthy controls ($n=40$, $u=-1.786$, $P=0.074$, Fig. 5D). And TrkB had no significant correlation with HRSD scores, showed in Fig. 5E ($n=40$, $r=-0.198$, $P=0.220$).

4. Discussion

4.1. The expression of BDNF, p75NTR, sortilin and TrkB

Under the chronic and acute stimuli, the genes that make individuals vulnerable or resistant to the depression are all changed. Dwivedi et al. (2003) showed decreased BDNF mRNA and protein levels in postmortem hippocampus and frontal cortex of suicide victims and most of them were diagnosed as major depression. Numerous studies also have demonstrated decreased levels of BDNF mRNA and protein in the brain in depression (Smith et al., 1995; Vaidya et al., 1999; Hashimoto et al., 2004). BDNF levels in the blood of depression patients are also significantly reduced (Dwivedi et al., 2003; Molendijk et al., 2011). Especially, one recent paper reported lower serum mBDNF in MDD patients (Yoshida et al., 2012). Our results on BDNF mRNA levels and mBDNF levels in serum were consistent with these studies. We found that in major depressive patients, the mRNA expressions of BDNF and TrkB were down-regulated while p75NTR and sortilin were up-regulated.

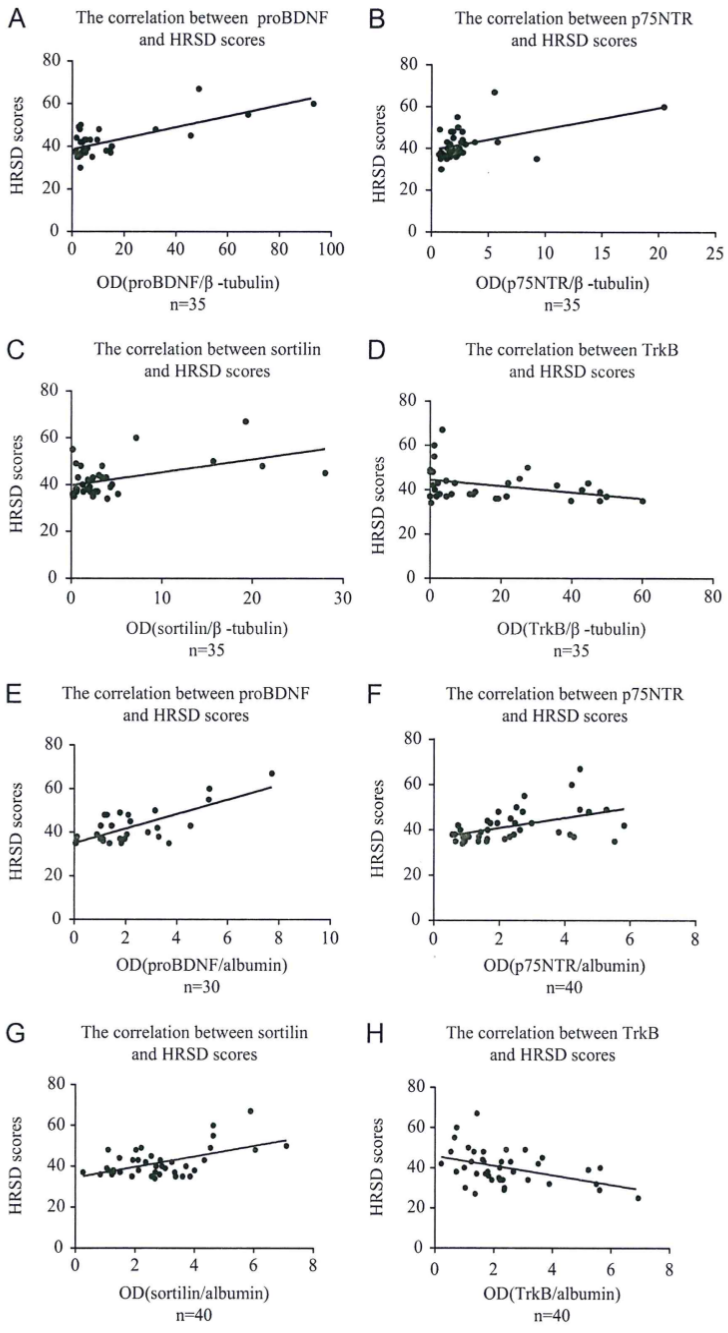


Fig. 3. Correlations of protein levels of proBDNF. TrkB, p75NTR and sortilin in lymphocytes and serum with HRSD scores in the major depression patients (A–H). Statistical analysis was performed with Spearman's correlation test. Of the lymphocytes, there were significant positive correlations between (A) proBDNF ($n=35$, $r=0.418$, $P=0.012$) and (B) p75NTR ($n=35$, $r=0.415$, $P=0.013$) with HRSD-21 scores. And there was no significant correlation between sortilin and HRSD scores ($n=35$, $r=0.342$, $P=0.057$) (C). TrkB levels had a significant negative correlation with HRSD-21 scores ($n=35$, $r=-0.356$, $P=0.036$) (D). And of the serum, there were significant positive correlations between (E) proBDNF ($n=30$, $r=0.530$, $P=0.003$), (F) p75NTR ($n=40$, $r=0.521$, $P=0.001$) and sortilin ($n=40$, $r=0.323$, $P=0.042$) (G) with HRSD-21 scores. TrkB levels had a significant negative correlation with HRSD-21 scores ($n=40$, $r=-0.328$, $P=0.039$) (H).

The functions of proBDNF are different from that of mature BDNF, and proBDNF has often an opposing role to mature BDNF (Lu et al., 2005; Xu et al., 2011; Sun et al., 2012). Thus, it is essential to differentiate mature BDNF from proBDNF in the major depression. In the present study, we have aimed to determine levels of BDNF and its receptors at both mRNA and protein levels, emphasizing the level of proBDNF in depressed patients (Lee et al., 2001; Lu et al., 2005; Woo et al., 2005; Zagrebelsky et al., 2005).

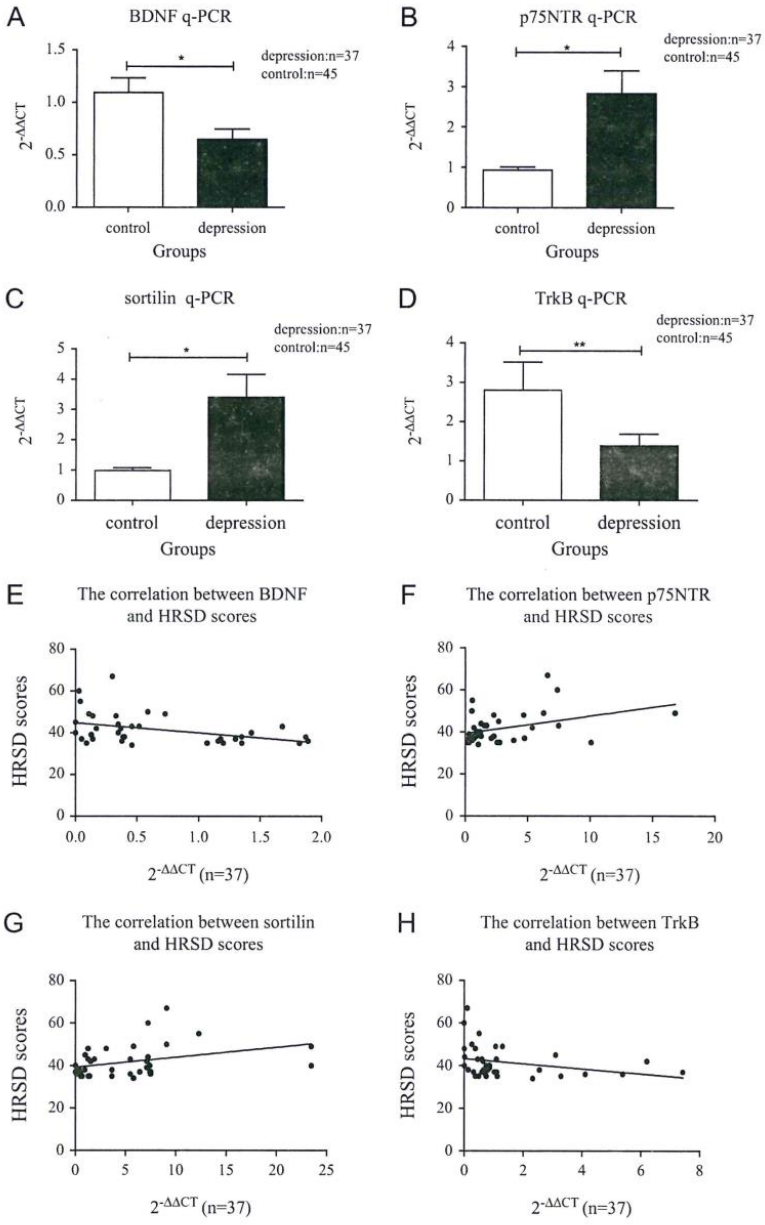


Fig. 4. mRNA expressions of BDNF, TrkB, p75NTR and sortilin in lymphocytes of major depressive patients and healthy controls (A-D). Histograms depict relative levels of (A) BDNF ($n=37$, $u=-2.367$, $P=0.018$), (B) p75NTR (n $=37$, $u=-2.284$, $P=0.022$), (C) sortilin ($n=37$, $u=-1.986$, $P=0.047$) and (D) TrkB ($n=37$, $u=-3.084$, $P=0.002$) mRNA in the lymphocytes. All samples were run in triplicates and normalize to β-actin (independent t-test, $^{*}P<0.05$, $^{**}P<0.01$). Correlations of mRNA expression levels of BNDF, TrkB, p75NTR and sortilin in lymphocytes with HRSD scores in the major depressive patients were in Fig. 3E–H. Statistical analysis was performed using Spearman's correlation test. There were significant negative correlations between (E) BDNF ($n=37$, $r=-0.415$, $P=0.011$) and TrkB levels ($n=37$, $r=-0.351$, $P=0.033$) (H) with HRSD scores. (F) p75NTR ($n=37$, $r=0.324$, $P=0.050$), (G) sortilin ($n=37$, $r=0.386$, $P=0.018$) had positive correlations with HRSD-21 scores.

In addition to the changes of mRNAs, we found that the protein expressions of proBDNF, p75NTR, and sortilin were increased while the TrkB was decreased in major depressive patients. Using the western blot method or ELISA, we got consistent results of TrkB expression, indicating our Western results are reliable. Meanwhile the balance of proBDNF and mBDNF appears tipping towards proBDNF signaling as demonstrated by increased expressions of proBDNF, p75NTR and sortilin. ProBDNF binds predominantly to p75NTR and induces functionally and morphologically opposite effects to those induced by the activation of mature BDNF/TrkB signaling pathway (Lee et al., 2001; Lu et al., 2005). The activation of p75NTR by proBDNF has been shown to induce neuronal atrophy and apoptosis, dendritic pruning and the induction of long-term depression (LTD) (Lee et al., 2001; Lu et al., 2005; Woo et al., 2005; Zagrebelsky et al., 2005). Despite a significant literature shows reduction in mature BDNF levels in the blood

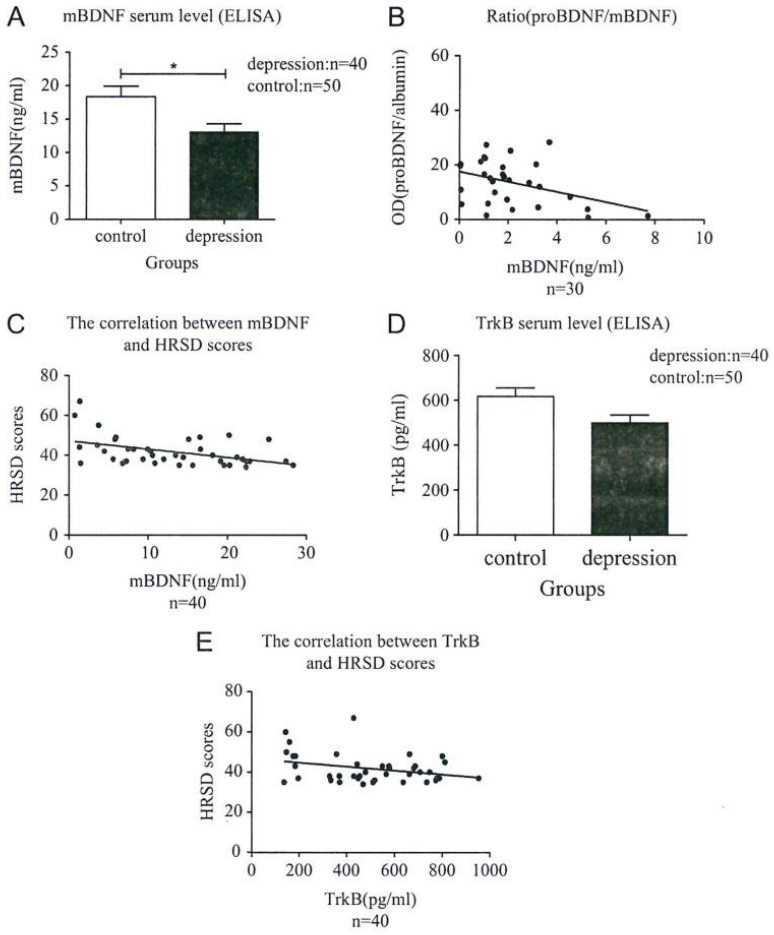


Fig. 5. The mature BDNF level in the serum is reduced in depression. (A) The ELISA result of mBDNF ($n=40$, mean±SEM=13.041±1.237) in major depressive patients and health controls ($n=50$, mean±SEM=18.369±1.585; $u=-2.347$, $P=0.019$). (B) The correlation between of relative levels of proBDNF (the results of serum) and the ELISA results of mBDNF ($n=30$, $r=-0.394$, $P=0.031$). (C) The correlation between mBDNF and HRSD scores ($n=40$, $r=-0.451$, $P=0.004$). And (D) the ELISA result of TrkB ($n=40$, mean±SEM=488.916±35.550) in major depressive patients and health controls ($n=40$, mean±SEM=617.772±6.965; $u=-1.786$, $P=0.074$). (E) The correlation between TrkB and HRSD scores ($n=40$, $r=-0.198$, $P=0.220$).

and brain plays an important role in the development of depression, the functional roles of proBDNF/p75NTR/sortilin signaling pathway in the depression are not known. Our data suggest that the mBDNF/TrkB signaling pathway and proBDNF/p75NTR/sortilin signaling pathway could be equally important and are all involved in the pathogenesis of depression. So, the proBDNF cleavage becomes critical for maintenance of the balance between mature BDNF and proBDNF and their functional responses (Lu et al., 2005; Martinowich et al., 2007; Yang et al., 2009B). In the pathological conditions, such as AIDS brain disease (Bachis et al., 2012) and seizures (Le and Friedman, 2012), proneurotrophins are increased due to the down-regulation of processing enzymes. These studies suggest that the intracellular and extracellular enzymes which cleave proBDNF into mBDNF were crucial for the neurodegenerative diseases, including depression. A recent paper reported no significant difference in serum proBDNF levels between MDD patients and healthy controls (Yoshida et al., 2012). However, the detection sensitivity of the proBDNF ELISA kit (Aviscera Bioscience) is very low with a claimed sensitivity around 10 ng/ml and many samples were below the detection limit. Using this kit, we found that the detection sensitivity is around 60 ng/ml (data not shown). In contrast, we are able to detect proBDNF by Western blot in most of samples studied, supporting our conclusion that the proBDNF level in the serum of MDD is increased.

4.2. Correlation between BDNF signaling molecules and depression

Several reports demonstrate that the serum BDNF level, which is likely reflective of BDNF levels in the brain, is significantly decreased in depressed patients (Karegea et al., 2002; Shimizu et al., 2003). This decline in serum BDNF levels is negatively correlated with the Montgomery Asberg Depression Rating Scale, that is, with the severity of depressive symptoms (Karegea et al., 2002). A meta-analysis of 11 clinical studies showed that the mean serum BDNF concentration in acutely depressed patients was significantly lower than healthy controls (Sen et al., 2008). Another meta-analysis (Brunoni et al., 2008) confirmed these results and found a significant correlation between BDNF concentration and changes in depression severity. In the present study, we found that TrkB and mBDNF had negative correlation with HRSD scores, consistent with the literature. Furthermore, we also found that the proBDNF and p75NTR levels were positively correlated with

the HRSD scores, raising the possibility of using their levels as biomarkers for major depression.

4.3. The balance between proBDNF and mBDNF in the blood is disrupted in depression

Almost all studies for detecting the serum BDNF level in the literature have used commercial kits which do not differentiate mature BDNF from proBDNF. As the functions of proBDNF are different from mature BDNF, the measurement of "total BDNF" or "BDNF" does not reflect the functions and status of *BDNF* gene products in patients. It is therefore essential to develop ELISA kits which can specifically detect mature BDNF and proBDNF respectively in order to understand their functions. Using a mouse monoclonal antibody as capture and affinity purified polyclonal antibodies as detection, we have developed an ELISA assay for specific measurement of mBDNF. Our mBDNF ELISA kit does not recognize proBDNF, or other mature neurotrophins. Thus, our study represents the first ELISA assay that specifically measures mature BDNF. For the detection of proBDNF from lymphocytes and serum we used sheep polyclonal antibodies which specifically recognize proBDNF by Western blots but not other neurotrophins. The specificity of this antibody has been well described in our previous studies (Fan et al., 2008). We found that in major depressive patients, the serum level of proBDNF was higher than in the healthy controls, while the mature BDNF level (from ELISA results) and BDNF mRNA levels were significantly lower than in the healthy controls. We also compared the proBDNF (the results from western blotting in the serum), the mBDNF levels and the ratio of proBDNF and mBDNF (Fig. 5B, $n=30$, $r=-0.394$, $P=0.031$) in the major depressive patients. We found that the level of proBDNF was negatively correlated with mature BDNF, indicating the dysfunction of proBDNF converting enzymes in depression. The precise reason why the proBDNF level was not properly processed in MDD patients is still not clear. We also assayed the correlation between mBDNF and HRSD scores. The serum mBDNF had negative relationship with the HRSD scores (Fig. 5C, $n=40$, $r=-0.451$, $P=0.004$). The results were consistent with our assumptions that proBDNF was increased while the mBDNF was reduced in depression. The changes of proBDNF and mBDNF were correlated with the severity of depression. Although the mechanism of this change is not clearly understood, we still hold that the proBDNF in serum and lymphocytes can be a biomarker for the depression. We postulate that under chronic stress, the enzymes that can convert proBDNF into mBDNF, like furin, tPA and MMP-7 (Bachis et al., 2012;Le and Friedman, 2012), were changed, so the balance between proBDNF and mBDNF was broken. Future studies require further elucidation of the mechanism of why proBDNF is unable to be processed in depression.

4.4. Limitation of the study

Many factors regarding sample handling could affect the level of proBDNF and BDNF. A non-fasting state of blood sampling, longer sample storage and being a binge drinker all result in attenuated BDNF levels. Smoking and living in an urban area can increase BDNF levels (Bus et al., 2011). This is one of the reasons why we only include females in the present study. Because in China over 50% males are smokers. In a few cases of males we recruited, most were confounded with either smoking or drinking or both. As a result, we only included females in order to make our subjects more homogenous and consistent in the present study. However, our conclusion may not be restricted to females as BDNF changes in depression always occur in both genders (Ozana et al., 2010; Bocchio-Chiavetto et al., 2010).

In addition to gender limitation, the overall number of cases in the present study is small. Despite we took a great effort to include all samples in the analysis, some cases could not be included due to sensitivity of the assays. Furthermore, current report is preliminary and descriptive without any mechanistic investigations. One should bear in mind of these limitations to interpret the findings. Future studies should extend to a larger number of cases of both genders and exclude confounders such as drinking and smoking.

5. Conclusions

Our preliminary finding shows that proBDNF and its receptors p75NTR and sortilin are up-regulated in the serum and lymphocytes of major depressive patients. We found the levels of mRNAs for TrkB and BDNF are down-regulated. Our data suggest that the balance between the proBDNF/p75/sortilin and mBDNF/TrkB pathways is disrupted. Despite of limitations, such as sample availability and heterogeneity of the patient population and a small number of cases in our study, our results suggest a potential role of the proBDNF/p75NTR/sortilin signaling in the disease. But with this beginning, further work should also be done alongside to know more about the depression.

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Conflict of Interest

All authors declare no conflict of interest.

Author disclosure:

We acknowledge that the manuscript cited above represents original work by the authors and that the material in this manuscript has not been previously published nor will be submitted for publication elsewhere until final action has been taken by editor.

All named authors have made substantial contributions to the intellectual content of the manuscript and have participated sufficiently in the work to take public responsibility for the content.

We confirm that the research was approved by the Ethics Committee of Yunnan Mental Hospital.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jad.2013.03.002.

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Supplements

Materials and Methods

Laboratory Assays

Blood samples collecting: after having obtained the permission, the coagulant vacuum blood collection tubes and anticoagulation blood collection vacuum tubes with Complete Proteinase Inhibitor Cocktail (cat.#04693116001, Roche Diagnostics, Indianapolis, IN, USA), plasminogen activator inhibitor 1 (PAI-1) (cat.A8111, Sigma-Aldrich) and the furin inhibitor I (Lot.#D00078005, Calbiochem) were used to get 2-5 ml peripheral blood individually.

Preparation for serum samples

Human blood obtained from the antecubital vein was collected in anticoagulant-free tubes after overnight fasting and kept on ice for 4 hours, and then centrifuged for 10 min at 13000 rpm, 4°C. Serum was carefully collected and kept at -80°C before assaying.

Preparation for the lymphocytes

Blood was obtained from the antecubital vein after overnight fasting in anti-coagulant tubes and kept at 4°C. Then the lymphocytes were isolated with the lymphocytes isolation medium and kept in -80°C before total RNA and protein extraction.

Real time RT-PCR

The total RNA was extracted from the lymphocytes using the Trizol reagent (Invitrogen, USA) according to the manufacturer instruction. The RNA purity was determined by A260nm/A280nm absorption ratio and the integrity was determined by the 1% denaturing agarose gel electrophoresis. The quantification was determined by spectrophotometry using the NanoDrop ND-1000 UV-Vis Spectrophotometer (Nano Drop Technologies, Wilmington DE, USA). cDNA synthesis was through the PrimeScript® RT reagent Kit Perfect Real Time (Code: DRR037A TaKaRa Biotechnology, Dalian, China) according to the manufacturer instruction. cDNA was then stored at -20°C before usage. Expression levels of BDNF, TrkB, p75NTR and

sortilin genes were determined by ABI 7300 (ABI Applied Biosystems, USA) and SYBRGreen. Each SYBR Green reaction (20ul total volume) contained 1× FastStart Universal SYBR Green master (cat.04913850001, Roche diagnostic), 0.5uM primers and 0.5ug cDNA. The β-actin gene expression was used as an internal control. The following primers were used:

BDNF forward: TACTTTGGTTGCATGAAGGCTGCC
reverse: ACTTGACTACTGAGCATCACCCTG

TrkB forward: AGGGCAACCCGCCCACGGAA
reverse: GGATCGGTCTGGGGAAAAG

p75NTR forward: GTGGGACAGAGTCTGGGTGT
reverse: AAGGAGGGGAGGTGATAGGA

sortilin forward: TTGATGATCTCAGAGGCTCAG
reverse: TGAAGATTCTTCCTCCACGAC

β-actin forward: CGGGAAATCGTGCGTGAC
reverse: TGGAAGGTGGACAGCGAGG

The information on primers of BDNF, TrkB, and p75NTR were cited from Anne-Laure Fauchais *et al.* and the sortilin primers were designed by us. The PCR cycling conditions were: 95°C for 10 min, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Samples were processed in technical duplicates and a melting analyzes was performed in each sample at the end of PCR. The Ct value was determined for target genes (BDNF, TrkB, p75NTR and sortilin) and the endogenous control β-actin gene in each sample. The differences between target genes and the control Ct were determined for each sample, resulting in the ΔCt value. To determine relative differences in mRNA expression between different target genes, the $2^{-\Delta\Delta Ct}$ was applied.

Western blotting

The protein extraction from lymphocytes is for the western blotting. A part of lymphocytes was used for protein extraction. The lymphocytes were homogenized in the RIPA solution (including 50mM Tris, 150mM NaCl, 1.0% Triton x-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with Complete Proteinase Inhibitor

Cocktail (cat.#04693116001, Roche Diagnostics, Indianapolis, IN, USA), Plasminogen Activator Inhibitor 1(PAI-1) (cat.A8111, Sigma-Aldrich) and furin inhibitor I (Lot.#D00078005, Calbiochem). Following centrifugation at 13000 rpm for 30 min, the supernatant was collected and a small part was used for protein determination using the Micro BCA Protein Assay kit (cat.CW0014, Cwbiotech). The remaining part was mixed immediately with sample loading buffer (four volumes proteins with one volume loading buffer) then heated at 95°C for 15 min. And 50ug of protein was loaded onto sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and then electroblotted onto Immunblot PVDF membranes (400mA, 2 hours) for the determination of pro-BDNF, p75NTR, sortilin and TrkB. The membranes were blocked with 5% non-fat milk dissolved in 0.1% Tween-20/Tris-buffered saline (TBST) at room temperature for 1 hour. Then, the membranes were incubated overnight at 4 °C with primary antibodies (anti-proBDNF 1:200; anti-p75NTR 1:250, #sc-6188, Santa Cruz Biotechnology; anti-sortilin 1:1,000, cat.ab16640, Abcam; anti-TrkB 1:1000, cat.#07-225, Millipore). After rinsing the membranes in TBST, blots were incubated with horseradish peroxidase conjugated anti-goat secondary antibody (cat.NO: sc-2020, donkey anti-goat IgG-HRP, Santa Cruz Biotechnology) for pro-BDNF and p75NTR (1:1,000) and horseradish peroxidase conjugated anti-rabbit secondary antibody (cat.CW0103, goat anti-rabbit IgG, HRP conjugated, Cwbiotech) for anti-sortilin and anti-TrkB (1:1,000) at room temperature for 1 hour. After rinsing with TBST, the membranes were incubated with enhanced chemiluminescent (ECL) substrate (cat.cw0049, Cwbiotech) and captured with the Molecular Imager ChemiDocTM XRS+ Imaging System (BIO-RAD, USA). Following stripping, membranes were incubated overnight with an anti-β-tubulin antibody 1:1,000 (cat.CW0098A, Cwbiotech) and then incubated with peroxidase conjugated anti-mouse secondary antibody (cat.CW0102, goat anti-mouse IgG, HRP conjugated, Cwbiotech) and processed as described above. The intensity of bands was determined with the Image J software (NIH, Bethesda, MD, USA), and data were expressed as the ratio between the band intensity of the pro-BDNF, p75NTR, sortilin and TrkB to that of the β-tubulin.

The serum western blotting

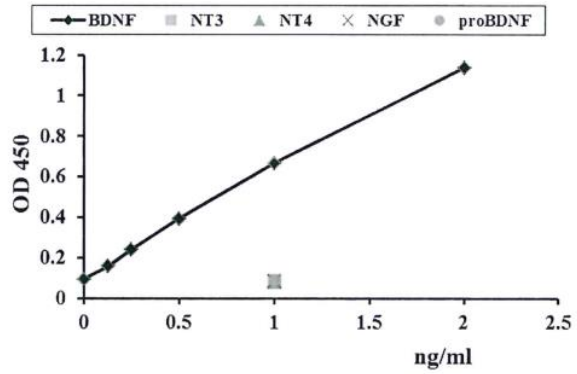
The total serum protein was determined by the BCA Protein Assay kit (cat.CW0014, Cwbiotech). The serum samples were diluted 1:4 in PBS containing 1% Triton x-100 and 1×complete protease inhibitor mixture (cat.#04693116001, Roche Diagnostics). Samples were mixed with SDS sample buffer (125 mM Tris-Hcl ph 6.8, 20% glycerol, 4% SDS, 0.02% Bromphenol Blue, and 125 mM dithiothreitol) then incubated at 50 °C for 30 min. After the treatments with the sera, 200 ug protein in the serum was loaded onto SDS-PAGE and the western blotting process was as described above. The serum albumin levels on Coomassie Blue staining on SDS-PAGE were used as internal loading controls and references for calculation of specific band intensity.

Determination of mBDNF concentration with ELISA

Serum was assayed for mBDNF in duplicate, using an ELISA assay kit developed in-house. The steps were as following: Dilute the capture antibody (protein G – purified mouse anti-mBDNF monoclonal antibody, B34D10) 2 ug/ml in coating buffer (50 mM Carbonate) and immediately add 100 ul to every well in the microplate, incubate for 1 hour at 37 °C. Then wash the plate 3 times with PBS. Next, add 150 ul of blocking buffer (PBS-BSA) per well and incubate 1 hour at 37 °C. Dilutions of standard mBDNF are made in a range from 2 ng/ml – 0.125 ng/ml. Apply 100 ul diluted serum per well and incubate plate at 37 °C for 1 hour. Then wash the plate 4 times with wash buffer. The next step is to dilute the detecting antibody 2.5 ug/ml in sample diluent and apply 100 ul to every well. Then incubate the plate at 37 °C for 1 hour. Wash the plate for 4 times with wash buffer. The optimal dilution factor was also tested. Incubate the plate at 37 °C for 1 hour then wash the plate 4 times with wash buffer. And then apply 100 ul of freshly prepared TMB substrate to every well. Allow colour to develop for 10 -15 minutes. At last, use 1 N sulfuric acid to stop the colour reaction. The absorbance at 450 nm was measured with a microplate reader (Model Sunrise, TECAN, Germany) to determine BDNF concentration according to the standard curve. In order to determine the specificity of the kit for mBDNF, proBDNF, nerve growth factor (NGF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4) at 1

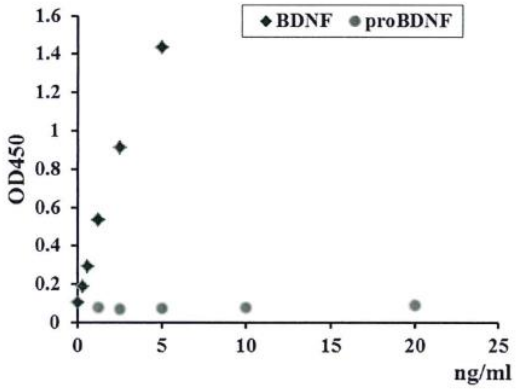
ng/ml or 0 – 20 ng/ml were added in the incubation step.

Figure S1. Standard curve and specificity of mBDNF ELISA. (A) Recombinant human mature BDNF (rhBDNF) standard curves (from 0 to 2 ng/ml) were in a dose-dependent manner. However, the other neurotrophins at 1 ng concentration did not show any reactivity. (B) Various proBDNF concentrations (from 0 to 20 ng/ml) were tested on mBDNF ELISA and proBDNF was not detected.



A

B



Reference

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Chapter 5: Serum mature BDNF level in Alzheimer's disease and its correlation with cognitive decline

Yoon Lim: Designed and performed experiments, statistical analyses, interpreted the results of the experiments and statistical analyses, prepared figures, drafted the manuscript.

Veer Gupta and Dr. Stephanie R. Smith: organised serum samples, performed statistical analysis (part of Table 5-1)

Larisa Bobrovskaya: contributed to the critical reading and revision

Xin-Fu Zhou: Conceived and designed experiments, statistical analyses, contributed to the critical revision

5.1 Abstract

Cognitive decline and amyloid burden are hallmarks of Alzheimer's disease (AD). The associations of blood BDNF level with AD or cognitive function have been controversial. In the present study we aimed to determine mature BDNF (mBDNF) serum levels in the Australian Imaging, Biomarkers and Lifestyle (AIBL) cohort (a large well characterised cohort of AD patients) using the mBDNF-specific ELISA developed in Chapter 3. We also aimed to examine correlations between serum mBDNF and cognitive function/decline or amyloid burden in this cohort. We analysed serum samples from Perth and Melbourne and determined serum mBDNF levels of 753 healthy controls (HC) (age, 70.52 ± 7.0; female, 58 %), 126 patients with mild cognition impairment (MCI) (age 76.31 ± 7.6; female, 57 %) and 202 AD patients (age 78.83 ± 8.7; female, 61 %) at baseline (BL) and of 687 HC (age 71.78 ± 6.8; female, 59 %), 79 MCI (age 76.57 ± 7.6; female, 52 %) and 192 AD (age 79.21 ± 7.9; female, 59 %) patients at 18 month follow-ups (18M) of the AIBL cohort. The serum mBDNF levels were analysed in association with gender, clinical categories, Mini-Mental State Examination (MMSE) and cerebral amyloid burden. The inter-assay coefficient of variation (%CV) of the ELISA performed on the Melbourne site samples showed very high %CV (18.15 ± 1.09) while that of the Perth site samples showed acceptable %CV (7.85 ± 1.09). Therefore, the mBDNF ELISA data from the Perth sites were analysed separately from the Melbourne site. For the Perth site, no significant difference in mBDNF serum concentration among HC, MCI and AD at either BL, 18M or over the 18 month period was found. The females showed significantly higher mBDNF serum levels than the males at BL and 18M. A negative correlation was observed between mBDNF serum levels and MMSE score in the entire cohort and in the males. We did not observe correlations between serum mBDNF and cognitive decline or amyloid burden.

For the Melbourne site, the male AD patients showed a significantly higher mBDNF serum levels than HC at 18M. The mBDNF serum levels also increased significantly over the period of 18 months in the males. The female HC showed significantly increased mBDNF levels over the 18 month period. However, we did not find any correlations between serum mBDNF and MMSE or amyloid burden.

Overall, our findings suggest that mBDNF serum concentration could be associated with cognitive performance however it may not be associated with cognitive decline over time.

5.2 Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder which represents the most frequent cause of the cognitive decline with a progressive memory loss (McKhann, Drachman et al. 1984, Grossman, Bergmann et al. 2006). The neuropathological hallmark of AD is the accumulation of amyloid beta (Aβ) plaques, neurofibrillary tangles and neuronal cell loss in the brain (Braak and Braak 1991, Blasko and Grubeck-Loebenstein 2003, Masters and Selkoe 2012). Recently, a growing body of evidence has suggested that the reduction of neurotrophins such as brain-derived neurotrophic factor (BDNF) plays an important role in the AD pathogenesis (Mattson, Maudsley et al. 2004). BDNF has important functions in neuronal differentiation, survival and synaptic plasticity in the central nervous system (CNS) (Huang and Reichardt 2001). A few recent *in vitro* studies have suggested that BDNF mitigates Aβ neurotoxicity. Although BDNF and its preferred receptor tropomyosin-related kinase B (TrkB) are not directly related to amyloidogenesis, it may support neurons to endure Aβ-caused neuronal death and increase neuronal plasticity and synaptic excitation (Hariri, Goldberg et al. 2003, Fahnestock 2011, Lu, Nagappan et al. 2013). It has been shown that BDNF can cross the blood-brain barrier in both directions (Poduslo and Curran 1996, Pan, Banks et al. 1998), suggesting blood BDNF could reflect the brain BDNF. Additionally, a few recent animal studies have demonstrated that the brain and blood BDNF levels are tightly connected (Karege, Schwald et al. 2002, Sartorius, Hellweg et al. 2009, Klein, Williamson et al. 2011), suggesting that blood BDNF levels could pinpoint the BDNF levels in brain in humans.

Several studies have demonstrated that BDNF expression is altered in the post-mortem brains of AD, however the results are not consistent. Most studies report a decrease of BDNF mRNA and protein including mature and proBDNF in brains of both of preclinical (Peng, Wuu et al. 2005) and last phases of AD (Connor, Young et al. 1997, Hock, Heese et al. 2000).

On the other hand, some studies have shown an increase of BDNF expression level in the hippocampus of AD (Durany, Michel et al. 2000, Savaskan, Muller-Spahn et al. 2000). Likewise, the data in regards to BDNF levels in serum of AD are also controversial. Some studies have demonstrated that serum BDNF levels are decreased in patients with mild cognition impairment (MCI) (Yu, Zhang et al. 2008) and in AD patients having mild (Laske, Stransky et al. 2007) and moderate to severe dementia (Yasutake, Kuroda et al. 2006) while other studies have shown that the serum BDNF levels did not alter in AD patients having mild to moderate dementia (O'Bryant, Waring et al. 2010). Furthermore, some studies have reported that serum BDNF concentrations even increase significantly in early phase of AD patients compared to age-matched healthy controls and more severe phases of AD patients (Laske, Stransky et al. 2006, Angelucci, Spalletta et al. 2010). The Mini-Mental State Examination (MMSE) is a method generally used for the cognitive mental status assessment (Folstein, Folstein et al. 1975, Tombaugh and McIntyre 1992, Crum, Anthony et al. 1993) to detect cognitive impairment as an initial step (Tangalos, Smith et al. 1996, Tierney, Szalai et al. 2000) although it cannot make formal diagnoses (O'Connor, Pollitt et al. 1989) and has a ceiling effect problem (Lonie, Tierney et al. 2009). Mixed results have been reported in regards to the correlation between serum BDNF and MMSE. Some studies have shown that serum BDNF level is positively correlated with MMSE in AD (Laske, Stransky et al. 2006, Konukoglu, Andican et al. 2012) or aged healthy subjects (Gunstad, Benitez et al. 2008) or negatively correlated with MMSE in AD group treated with cholinesterase inhibitors (CEI) (Konukoglu, Andican et al. 2012). Other studies reported no correlation between serum BDNF level and MMSE (Laske, Stransky et al. 2007, O'Bryant, Hobson et al. 2009).

As mentioned earlier, the accumulated amyloid plaque is a hallmark of AD. Positron emission tomography (PET) with $[11]^{C}$-Pittsburgh compound B (PiB) (PiB-PET) makes possible to visualize amyloid deposition *in vivo* (Klunk, Engler et al. 2004). A recent study showed that BDNF plasma levels have a significant negative correlation with PiB

standardized uptake value ratio (SUVR) in multiple brain areas. The authors also demonstrated that PiB SUVR has negative correlation with MMSE (Hwang, Lazaris et al. 2015).

It has been reported that proBDNF stimulates long term depression while mBDNF triggers long term potentiation. ProBDNF also elicits cellular apoptosis, strongly suggesting that both of mBDNF and proBDNF play critical but opposite roles in neurons (Lee, Kermani et al. 2001, Pang and Lu 2004, Woo, Teng et al. 2005, Sun, Lim et al. 2012). As mBDNF and proBDNF have different biological functions, it is critical that mBDNF and proBDNF levels should be determined separately. However, commercially available ELISA kits cannot distinguish proBDNF from mBDNF (Hashimoto 2010, Teche, Nuernberg et al. 2013). Recently, we have developed mBDNF specific sandwich ELISA which detects mBDNF with high specificity (< 5% cross-reactivity with proBDNF) (Zhou, Xiong et al. 2013, Lim, Zhong et al. 2015); thus allowing use to specifically determine mBDNF in a large number of patient samples.

Recently, the Australian Imaging, Biomarkers and Lifestyle (AIBL) cohort has been established and has recruited over 1000 participants (Ellis, Bush et al. 2009). As the relationships of BDNF serum levels with AD and MMSE have been contradictory we performed this study to examine whether serum mBDNF levels correlate with AD or cognitive decline. We also examined any possible correlation between serum mBDNF and amyloid burden using the AIBL cohort (Ellis, Bush et al. 2009).

5.3 Materials and Methods

5.3.1 The Australian Imaging, Biomarkers and Lifestyle (AIBL) Cohort

The cohort recruitment process and mood, cognitive and neuropsychological assessments have been described previously (Ellis, Bush et al. 2009). The baseline (BL) cohort consisted of 1,112 volunteers; healthy controls (HC, n=768) and patients with mild cognitive impairment (MCI, n=133) and Alzheimer's disease (AD, n=211) and the participants were assessed at 18 month follow-up (18M). Diagnoses were made according to the criteria internationally agreed in a consistent manner, which was ensured by a clinical review panel meeting for discussing BL classification (McKhann, Drachman et al. 1984, Petersen, Smith et al. 1999, Winblad, Palmer et al. 2004). The final clinical classification of individuals, who were diagnosed as having AD or MCI at BL or were healthy controls who required further investigation, was based on the panels consensus diagnosis and was determined prior to obtaining various other data including positron emission tomography (PET) with $[11]^{C}$-Pittsburgh compound B (PiB) (PiB-PET) imaging. MMSE was performed as described previously (Folstein, Folstein et al. 1975). Briefly, eleven questions covered five areas of cognitive function including orientation, registration, attention and calculation, recall, and language. The AIBL study was approved by the institutional ethics committees of Austin Health, St Vincent's Health, Hollywood Private Hospital and Edith Cowan University. All volunteers gave written informed consent before participating in the study.

5.3.2 Serum samples

All the blood samples for baseline (BL) testing and 18 months follow-up (18M) were collected in the mornings after overnight fast as described previously (Ellis, Bush et al. 2009). Isolated serum samples were aliquoted and stored in liquid nitrogen. In this study, the serum samples were transferred from two different sites, Melbourne (n=1206 (BL, n=641; 18M,

n=565)) and Perth (n=833 (BL, n=440; 18M, n=393)) on dry ice and stored at -80 °C freezer on arrival and until use. All human serum samples used in this study were approved by University of South Australia Human Research Ethics Committee (approved project number: 0000030671).

5.3.3 mBDNF sandwich ELISA

mBDNF sandwich ELISA was performed in a similar way as previously described (Zhou, Xiong et al. 2013, Lim, Zhong et al. 2015). Ninety-six-well microplate (Thermo Fisher Scientific, Rockford, IL, USA) was coated with capture antibody, B34D10 with 1 μg/ml in coating buffer (50 mM carbonate pH 9.6), incubated overnight at 4 °C, washed with PBS and blocked with 170 μl of 3% BSA in PBS at 37 °C for 1hour or at 25°C e for 2 hours. Dilutions of standard mBDNF were made in a range of 0-750 pg/ml and serum was diluted 1:80 in sample diluent (1% BSA, 0.125% Tween-20 in PBS) and followed by incubation at 37 °C for 2 hours. Before serum dilution, serum samples were thawed and centrifuged at 2,000 x g for 3 min to precipitate any debris in serum. After washing with wash buffer (PBS, 0.1% Tween-20), detection antibody, biotinylated sheep anti-mBDNF IgG (0.15 μg/ml) was added to each well and the plate was incubated at 37 °C for 1 hour. After 4 times washing with the wash buffer, streptavidin-HRP (1:5000; Sigma-Aldrich, St Louis, MO, USA) was added and incubated at 37 °C for 1 hour. The plate was washed 4 times with the wash buffer and 3,3′,5,5′-Tetramethylbenzidine (TMB) (S Sigma-Aldrich, St Louis, MO, USA) substrate was added to each well, followed by incubation at 25 °C for 10 min in the dark. At last, to stop the colour development, 0.1 N HCl was added. The absorbance at 450 nm was measured with a microplate reader (VICTOR, Perkin-Elmer, Waltham, Massachusetts, USA) to determine optical density (OD) values. All standards and samples were run in triplicates and duplicates, respectively, and the mean values of the duplicate samples were used for further

statistical analysis. If there was bigger than 10% OD value difference between singlets in the duplicate, the sample was determined again. For the Perth site serum samples, baseline and its matching 18M samples were loaded on same plate to reduce inter-plate variance; however, this sample loading method was not applied to Melbourne site samples because the samples were provided in random order and there were no written labels (only machine readable labels) on the sample tubes. All the measurements were blind tested.

5.3.4 Inter-assay variation test

Inter-assay coefficient of variation (%CV) is widely used to test plate-to-plate variation in ELISA (Schultheiss 2009, Wang, Song et al. 2011, Thomsson, Strom-Holst et al. 2014). In general, the inter-assay %CV is calculated (%CV = (SD/mean) x 100) based on the mean of a few replicates on one plate and the same assay is repeated several times on other plates. In this study, inter-assay %CV was calculated to evaluate a plate-to-plate variation during a series of mBDNF ELISA of the Melbourne and Perth serum samples. The concentration of mBDNF of Melbourune and Perth sites serum samples were determined in duplicates in the two independent runs. %CV was calculated for each sample and the average of %CV was calculated with 96 (Melbourne) and 186 (Perth) serum samples.

5.3.5 Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS version 17; SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad Software). Continuous variables were analysed by one-way Analysis of Variance (ANOVA) for comparison by statistical means. Statistical analysis of age (in years), MMSE score and mBDNF serum level were performed using one-way Analysis of Variance (ANOVA) with Tukey’s honestly significant differences (HSD) post hoc analysis. Statistical analysis of

gender was performed using $\chi 2$ test. Linear regression was carried out to assess associations between serum mBDNF level and MMSE score or PiB SUVR. Two-tailed Student's t-test was undertaken to compare means between two groups. $P < 0.05$ was considered significant. All the plots are presented as mean ± SEM.

5.4 Results

The BL and 18M demographic characteristics of healthy controls (HC), patients with mild cognitive impairment (MCI) and Alzheimer's disease (AD) are given in Table 5-1. Mean age was significantly higher amongst participants with MCI and AD when compared to HC clinical categories ($p < 0.0001$) at BL and 18M. Mini–mental state examination (MMSE), a part of dementia assessment (Ellis, Bush et al. 2009), was different significantly across groups ($p < 0.0001$). Serum mBDNF levels were measured by mBDNF-specific sandwich ELISA which is described in Chapter 3. At BL, serum mBDNF levels were significantly altered across clinical category groups (ANOVA, $p < 0.01$) although mBDNF levels for MCI (18.55 ± 5.92 ng/ml) tended to be higher than HC (17.56 ± 6.00 ng/ml) and lower than AD (18.96 ± 6.47 ng/ml) at BL. mBDNF levels were significantly increased in AD compared to HC ($p < 0.05$) however, there was no statistical difference between HC and MCI groups ($p = 0.219$) or MCI and AD groups ($p = 0.826$), respectively. Similarly, serum mBDNF levels of the subjects who followed up at 18M were significantly altered across clinical category groups (ANOVA, $p < 0.01$). mBDNF levels were significantly increased in AD compared to HC ($p < 0.05$) however, there was no statistical difference between HC and MCI ($p = 0.253$) or MCI and AD ($p = 0.865$). Serum mBDNF levels were significantly different between male and female through the entire cohort including HC, MCI and AD (Fig. 5-1). At BL, mBDNF levels for males (17.18 ± 0.28 ng/ml) were significantly lower than females (18.47 ± 0.24 ng/ml, $p < 0.001$). Likewise, mBDNF levels for male (18.28 ± 0.29 ng/ml)

were significantly lower than in females (19.82 ± 0.29 ng/ml, $p < 0.001$) at 18M. Interestingly, mBDNF levels of HC at BL are much higher than at 18M and the change of mBDNF levels between BL and 18M in HC is larger than that of MCI or AD at either time point.

	BL			
	HC (n=753)	MCI(n=126)	AD(n=202)	p
Age (years)	70.52 ± 7.0	76.31 ± 7.6	78.83 ± 8.7	< 0.0001
Gender (female %)	58	57	61	0.73
MMSE Score	29 ± 2	26 ± 4	20 ± 6	< 0.0001
mBDNF (ng/ml)	17.56 ± 6.00	18.55 ± 5.92	18.96 ± 6.47	< 0.01
mBDNF (ng/ml) followed-up	n=687, 17.52 ± 4.58	n=79, 18.25 ± 4.43	n=192, 18.91 ± 4.58	<0.01

	18M			
	HC(n=687)	MCI(n=79)	AD(n=192)	p
Age (years)	71.78 ± 6.8	76.57 ± 7.6	79.21 ± 7.9	< 0.0001
Gender (female %)	59	52	59	0.482
MMSE Score	29 ± 2	27 ± 3	19 ± 9	< 0.0001
mBDNF (ng/ml)	19.13 ± 6.40	19.03 ± 6.59	19.60 ± 6.93	0.644

Table 5-1 Comparison of demographic characteristics and serum mBDNF levels among HC, MCI and AD at BL and 18M.
Statistical analysis of age, MMSE score, mBDNF concentration were performed with one-way ANOVA without covariates except gender ($\chi 2$ test). BL, base line; 18M, 18 month follow-ups; HC, healthy control; MCI, mild cognitive impairment; AD, Alzheimer's disease; MMSE, Mini-Mental State Examination. Values are mean ± SD.

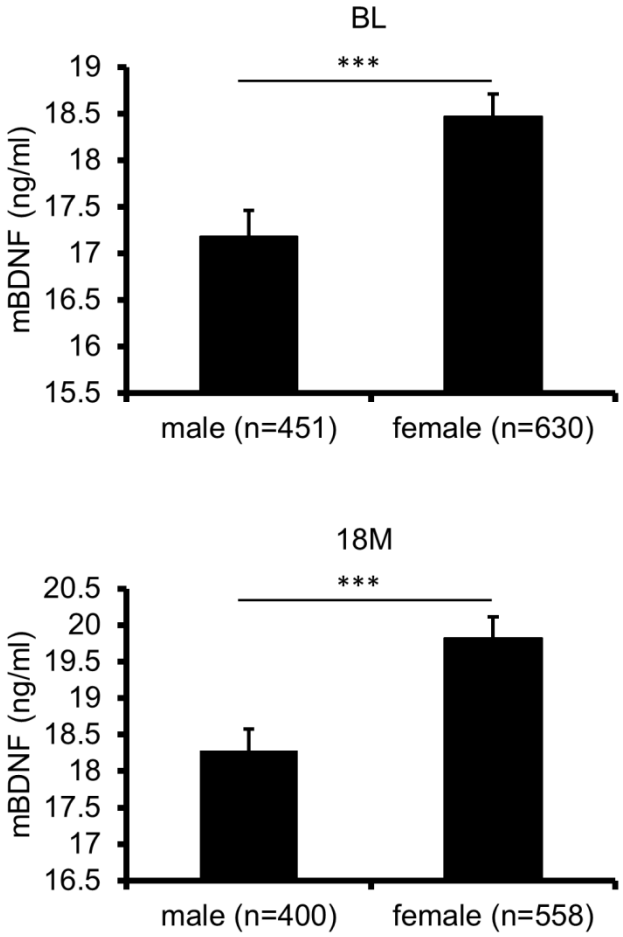


Figure 5-1 mBDNF serum levels in male and female of entire cohort.
Serum mBDNF levels of male and female in the entire cohort at BL and 18M were compared. Bar graphs present mean ± SEM (ANOVA, ***$p < 0.001$). BL, baseline; 18M, 18 month follow-up.

Interestingly, we found that the overall mBDNF levels are higher in either clinical category including HC, MCI and AD, especially, in HC at 18M when compared to BL. These results were unlikely a true change and biologically meaningful as previous reports demonstrated that BDNF serum levels decrease with increasing age in both sexes (Shimada, Makizako et al. 2014). As the serum samples from Perth and Melbourne were analysed separately, we decided to check the two different sites separately.

In the males of Melbourne site, mBDNF serum levels of AD increased significantly (BL, 18.41 ± 0.86 ng/ml; 18M, 25.02 ± 0.61 ng/ml; $p < 0.0001$) over the period of 18 months and are significantly higher than in HC (at 20.29 ± 0.46 ng/ml, $p < 0.01$) at 18M (Fig. 5-2, A). In the females of Melbourne site, mBDNF serum levels of HC increased significantly (BL, 19.08 ± 0.40 ng/ml; 18M, 22.33 ± 0.46 ng/ml; $p < 0.0001$) over the period of 18 months, however there were no significant changes of mBDNF levels between other groups at either time point (Fig. 5-2, B). In the samples obtained from Perth site, no significant mBDNF level changes were observed among HC, MCI and AD at either time point although mBDNF levels of AD and MCI showed decreasing trends over the period of 18 months in the males and females, respectively (Fig. 5-2, C and D). mBDNF levels of females and males were also analysed (Fig. 5-3). In the Perth site samples, mBDNF serum levels of the females are significantly higher than those of male at both BL and 18M, however no mBDNF level changes were observed over the 18 month period in the males or females (Fig. 5-3, B). Interestingly, in the Melbourne site samples, there was no mBDNF level difference between the males and females at either BL and 18M, however mBDNF increased significantly over 18 months in the males and females (Fig. 5-3, A). As these two analyses have shown very inconsistent results between the two sites, we decided to check inter-assay variability to determine whether there was a technical issue in the assay.

We examined inter-assay variations by comparison of coefficient of variation (%CV) of the two sites as described in the methods section. The inter-assay %CV of the Perth site was 7.85 ± 0.42, while the %CV of the Melbourne site was 18.15 ± 1.09 (Fig. 5-4). As the samples from Perth and Melbourne sites were assayed separately, which produced an obvious technical error, following data analysis from Perth or Melbourne sites were performed separately in order to draw meaningful conclusions. Females and males were also analysed separately owing to the significant difference in serum mBDNF levels.

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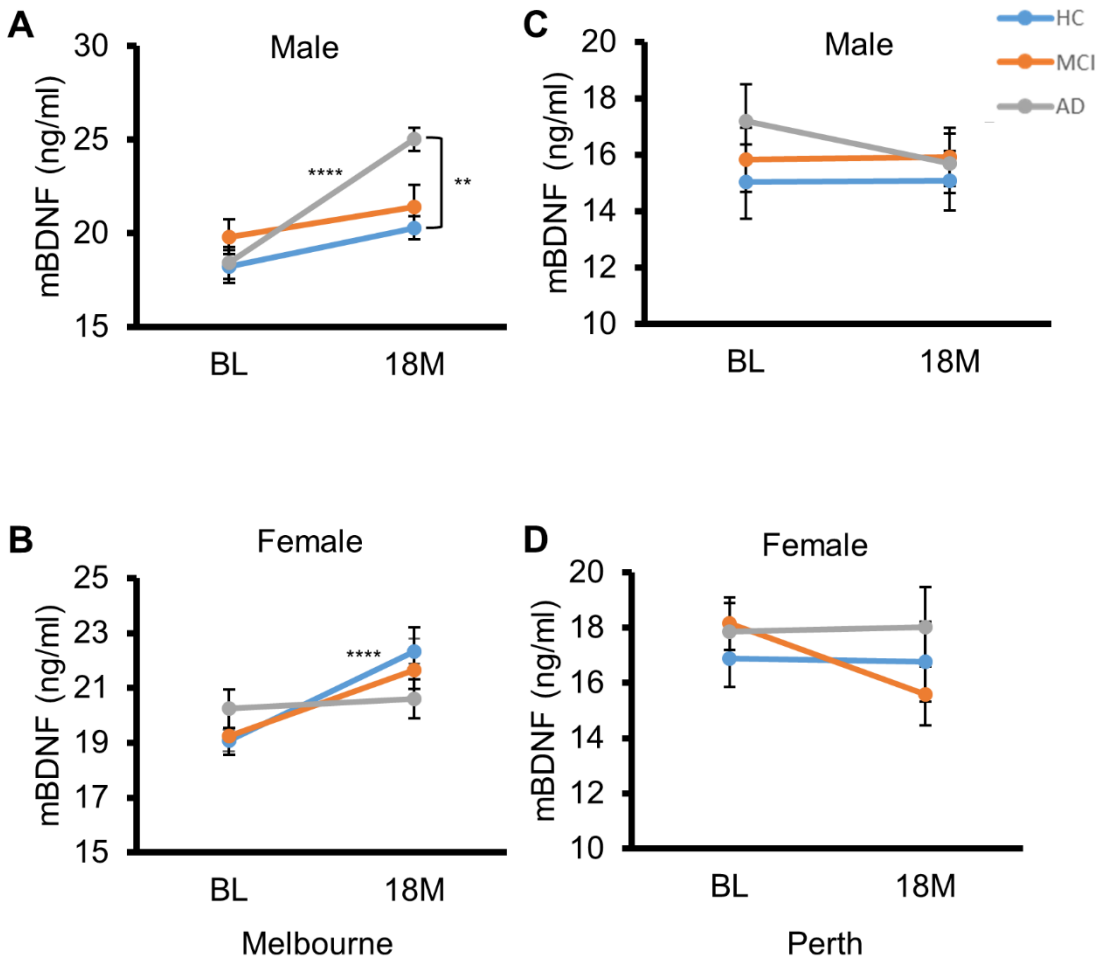


Figure 5-2 Group comparison of mBDNF level in Melbourne and Perth site at BL and 18M.

(A) Male from Melbourne site. HC (BL, n=181; 18M, n=164), MCI (BL, n=32; 18M, n=21), AD (BL, n=54; 18M, n=51). (B) Female from Melbourne site. HC (BL, n= 241; 18M, n=225), MCI (BL, n=45; 18M, n=26), AD (BL, n=88; 18M, n=78). (C) Male from Perth site. HC (BL, n=137; 18M, n=120), MCI (BL, n=22; 18M, n=17), AD (BL, n=25; 18M, n=27). (D) Female from Perth site. HC (BL, n=194; 18M, n=178), MCI (BL, n=27; 18M, n=15), AD (BL, n=35; 18M, n=36). All line graphs are presented as mean ± SEM. ** p <0.01, **** p< 0.0001; One-way ANOVA with Tukey's post-hoc test. BL, baseline; 18M, 18 month follow-ups. HC, healthy control; MCI, mild cognitive impairment; AD, Alzheimer's disease.

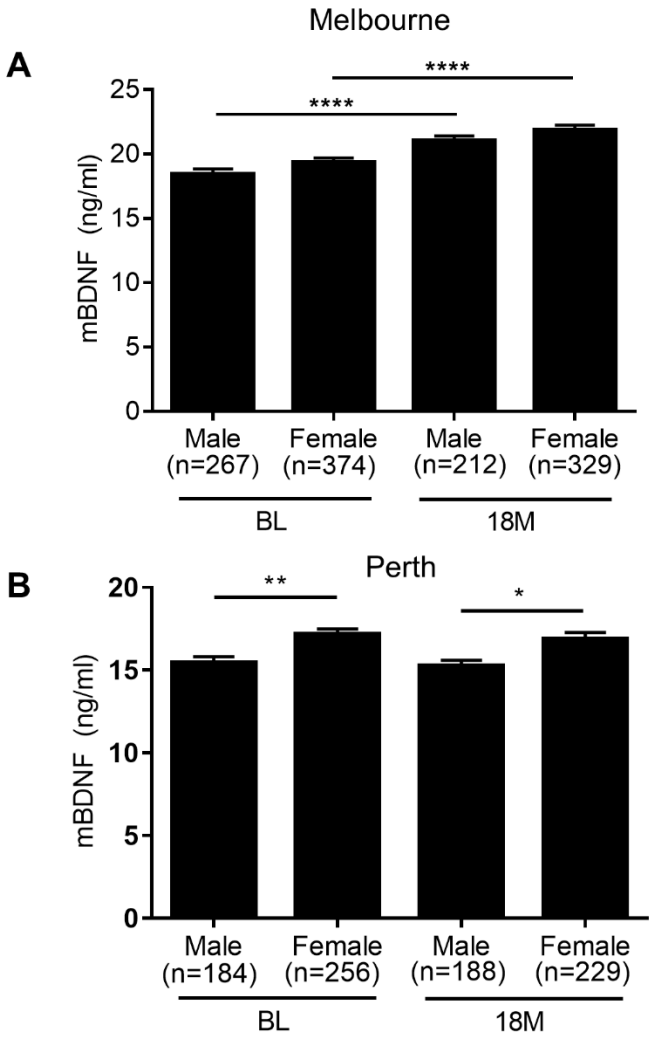


Figure 5-3 Re-analysis of mBDNF serum levels in male and female of entire cohort. Serum mBDNF levels of male and female in the entire cohort at BL and 18M from were compared. (A) Melbourne site. (B) Perth site. All bar graphs are presented as mean ± SEM. * p <0.05, ** p <0.01, **** p< 0.0001; One-way ANOVA with Tukey's post-hoc test. BL, baseline; 18M, 18 month follow-ups.

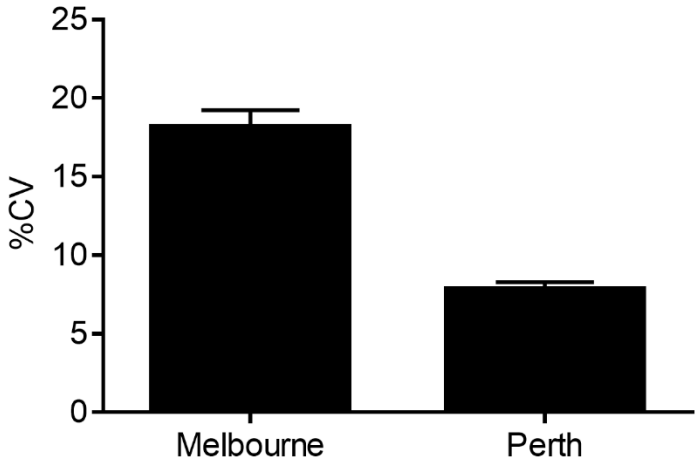


Figure 5-4 Inter-assay variation comparison of the two sample collection sites.
Randomly chosen 96 and 186 serum samples from Melbourne and Perth sites, respectively, were subjected to the inter-assay variation test in mBDNF ELISA. %CV=(SD/mean) x 100. Bar graphs are presenting the mean value of %CV. mean ± SEM

Next, mBDNF serum levels were compared in 36 cases whose clinical classification was changed over the 18 month period (HC → MCI, 14 cases; HC → AD, 3 cases; MCI → AD, 19 cases) to examine whether mBDNF levels change significantly when clinical categories change. As shown in Table 5-2, no cases showed significant mBDNF level difference between BL and 18M. Although, in Perth male samples, mBDNF serum levels decreased in the subjects whose clinical category was converted from HC to MCI (n=5, p=0.05235), these changes failed to reach statistical significance.

As cognitive decline is a feature of AD, a correlation between mBDNF serum level and MMSE was analysed. The subjects whose mBDNF level and MMSE were measured at both BL and 18M were analysed. Negative correlations between the mBDNF level and MMSE scores were observed in the entire Perth cohort ($p < 0.05$) and in Perth male samples ($p < 0.05$) (Fig. 5-5). To examine whether mBDNF level change over the 18 month period is associated with the MMSE score, two groups which showed increase or decrease of serum mBDNF level over the 18 month period were analysed separately. In the group in which the mBDNF levels were increased over the 18 month period, the Perth entire (female + male, $p < 0.0001$), Perth female ($p < 0.05$) and Perth male ($p < 0.0001$) groups showed negative correlations between the mBDNF level and MMSE scores (Table 5-3), while in the group in which the mBDNF was decreased over the 18 month period, the Perth entire (female + male) group showed a positive correlation (Table 5-3).

As the scores of MMSE > 24 indicate no cognitive impairment (Pezzotti, Scalmana et al. 2008), the subgroups of MMSE scores < 25 were also analysed to establish if there is a correlation between the mBDNF level and MMSE in the subjects who have impaired cognitive function. The Perth male subgroup (MMSE < 25) showed a negative correlation between MMSE score and mBDNF serum level ($p < 0.01$).

Neither groups of increased or decreased mBDNF levels over the period of 18 months of the Melbourne site showed a correlation between mBDNF level and MMSE score (Table 5-3).

Next, we examined whether the change of MMSE score decrease is associated with mBDNF serum level change over the 18 month period. The MMSE scores of the subjects whose MMSE score was decreased over the 18 month period were analysed in relation to the difference of the mBDNF serum levels over the 18 month period. No correlations were observed in either the Melbourne + Perth, Perth or Melbourne groups (Fig. 5-6).

		Clinical classification change over 18 months	Serum mBDNF (ng/ml)		p value
Melbourne	Male	HC→MCI (n=3)	HC-BL	17.68 ± 5.194	0.5428
			MCI-18M	21.70 ± 3.113	
		HC→AD (n=2)	HC-BL	14.55 ± 1.716	0.974
			AD-18M	14.49 ± 0.5660	
		MCI→AD (n=6)	MCI-BL	21.53 ± 2.047	0.187
			AD-18M	18.50 ± 0.6366	
	Female	HC→MCI (n=5)	HC-BL	16.98 ± 1.159	0.7025
			MCI-18M	18.60 ± 3.921	
		HC→AD (n=0)	HC-BL	nd	nd
			AD-18M	nd	
		MCI→AD (n=8)	MCI-BL	20.85 ± 2.059	0.7469
			AD-18M	21.81 ± 2.075	
Perth	Male	HC→MCI (n=5)	HC-BL	21.28 ± 0.9835	0.0535
			MCI-18M	18.89 ± 0.3924	
		HC→AD (n=1)	HC-BL	17.75	nd
			AD-18M	15.96	
		MCI→AD (n=5)	MCI-BL	11.54 ± 1.114	0.87
			AD-18M	11.99 ± 2.431	
	Female	HC→MCI (n=1)	HC-BL	21.76	nd
			MCI-18M	17.09	
		HC→AD (n=0)	HC-BL	nd	nd
			AD-18M	nd	
		MCI→AD (n=6)	MCI-BL	18.93 ± 0.9684	0.9425
			AD-18M	18.76 ± 2.050	

Table 5-2 mBDNF serum level comparisons of BL and 18M of the subjects whose clinical category changed over the 18 month period.

mBDNF serum levels of BL and 18M were compared in those cases when the clinical classification has changed over the 18 month period. Values are mean ± SEM. Student's t-test, two-tailed. BL, baseline; 18M, 18 month follow-ups. HC, healthy control; MCI, mild cognitive impairment; AD, Alzheimer's disease; nd, not determined; n, case number.

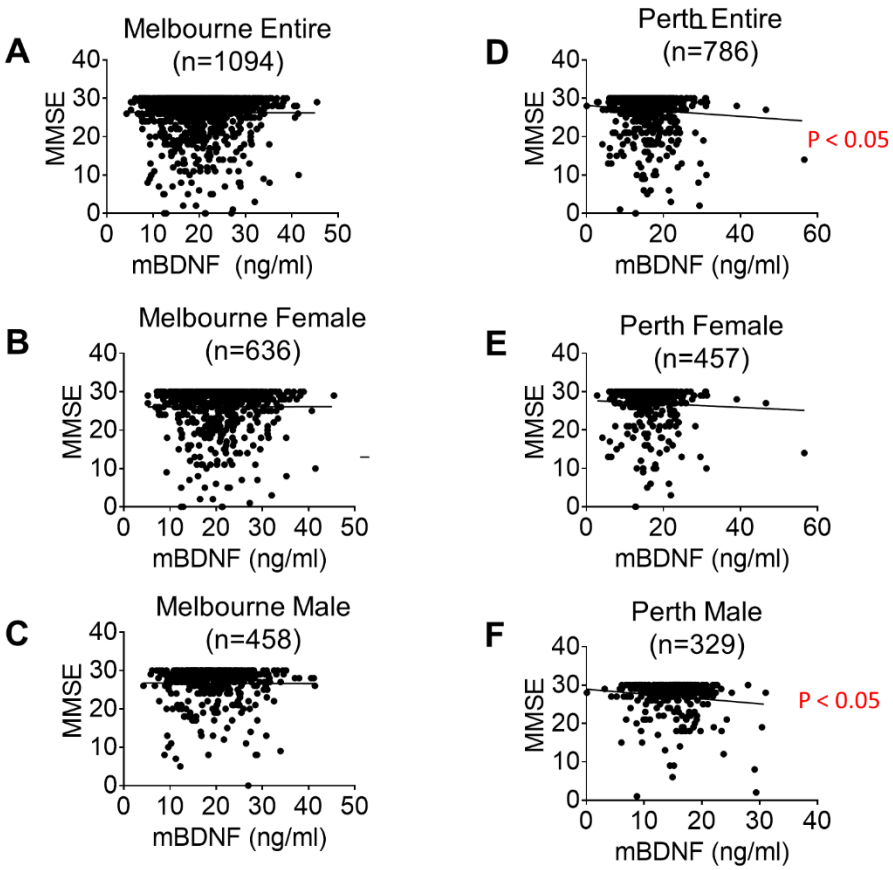


Figure 5-5 Correlation between mBDNF serum level and MMSE score
(A) Entire cohort of the Melbourne site (r=-0.006, p=0.85), (B) Melbourne female samples (r=-0.002, p=0.95), (C) Melbourne male samples (r=-0.004, p=0.92), (D) entire cohort of the Perth site (r=-0.076, p=0.033), (E) Perth female samples (r=-0.05, p=0.28), (F) Perth male samples (r=-0.126, p=0.022). MMSE, Mini-Mental State Examination. The curve was fitted with linear regression using GraphPad Prism 6.0.

			Melbourne	Perth
Serum mBDNF level increased over 18 months	Female + Male	n	684	383
		r	-0.028	-0.17
		p-value	0.36	**< 0.0001**
	Female	n	386	214
		r	-0.04	-0.12
		p-value	0.31	**< 0.05**
	Male	n	298	169
		r	-0.001	-0.31
		p-value	0.98	**< 0.0001**
	Female MMSE < 25	n	70	41
		r	0.2	-0.15
		p-value	0.11	0.13
	Male MMSE < 25	n	54	24
		r	0.01	-0.53
		p-value	0.93	**< 0.01**
Serum mBDNF level decreased over 18 months	Female + Male	n	406	404
		r	0.02	0.12
		p-value	0.6	**< 0.05**
	Female	n	247	244
		r	0.05	0.16
		p-value	0.44	0.06
	Male	n	159	160
		r	-0.01	0.07
		p-value	0.82	0.36
	Female MMSE < 25	n	68	29
		r	0.16	0.45
		p-value	0.23	0.08
	Male MMSE < 25	n	25	28
		r	0.06	0.01
		p-value	0.72	0.96

Table 5-3 Correlation between serum mBDNF and MMSE score.
The correlation between mBDNF and MMSE score was analysed in the males and females of groups that mBDNF level decreased or increased over the 18 month period. $p < 0.05$ were written in bold. Linear regression.

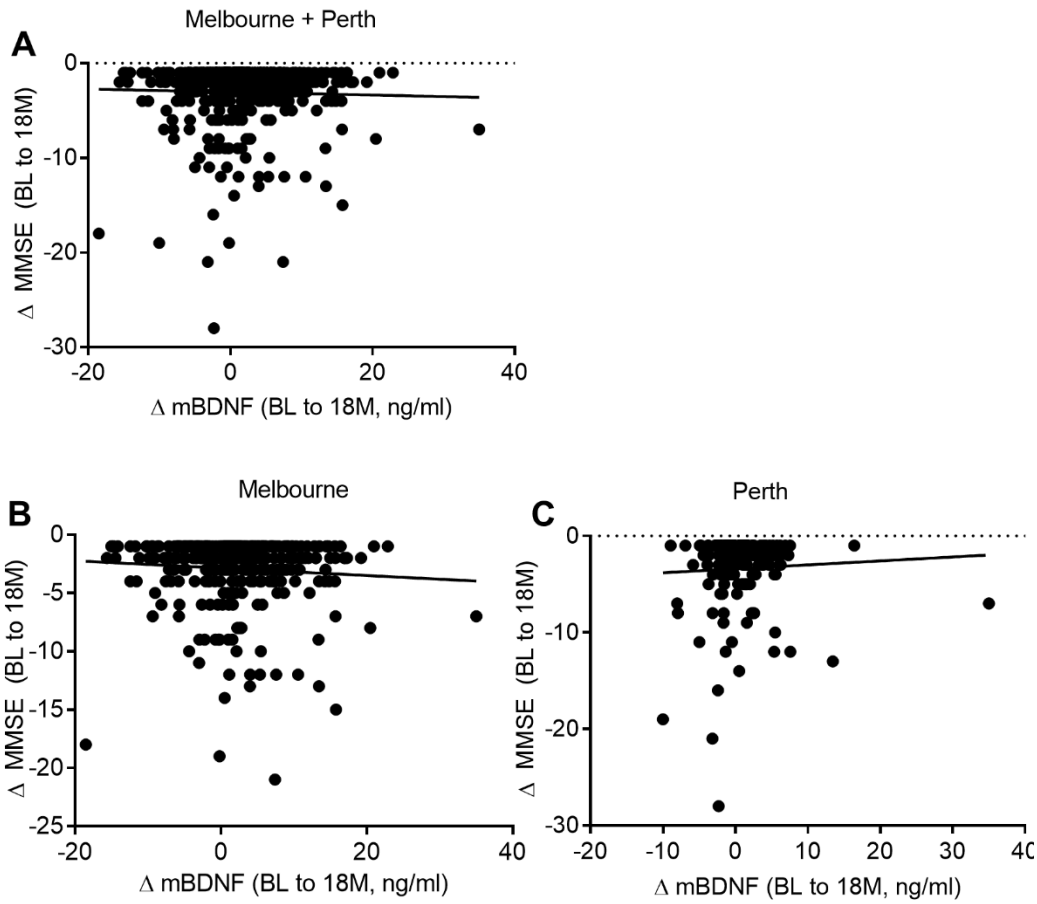


Figure 5-6 Correlation between changes of mBDNF and MMSE of those patients that showed decreased MMSE score over the18 month period were analysed
(A) Melbourne and Perth sites combined (r=-0.009, p=0.81). (B) Melbourne site only (r=-0.071, p=0.18). (C) Perth site only (r=0.045, p=0.56). Δ MMSE= MMSE (18M) – MMSE (BL); Δ mBDNF = mBDNF (18M) – mBDNF (BL). MMSE, Mini-Mental State Examination. The curve was fitted with linear regression using GraphPad Prism 6.0.

As amyloid burden is a hallmark of AD (Masters and Selkoe 2012) and a recent study reported a negative correlation between plasma BDNF and PiB SUVR (Hwang, Lazaris et al. 2015), we examined an association between mBDNF serum level and amyloid burden. To perform this analysis, positron emission tomography (PET) with $[11]^{C}$-Pittsburgh compound B (PiB) (PiB-PET) subset were stratified according to the increase or decrease of mBDNF serum level over the 18 month period. In the Perth sasmples, a negative correlation was observed in AD ($p < 0.05$) at BL in female subgroup in the group of increased mBDNF serum level over 18 months (Table 5-4). I next examined whether increased amyloid burden is associated with mBDNF serum level change over the 18 month period. The subjects whose PET SUVR increased over 18 months were analysed. However, no correlations were observed with mBDNF level change in either the Melbourne + Perth, Perth or Melbourne groups (Fig. 5-7).

				BL			18M	
			HC	MCI	AD	HC	MCI	AD
Serum mBDNF level increased over 18 months	Melbourne	Female	n	26	6	5	23	4	5
			r	0.01	0.04	0	0.004	-0.03	0.002
			p-value	0.68	0.07	1	0.78	0.21	0.97
		Male	n	24	10	6	22	7	9
			r	-0.003	-0.01	-0.01	0.003	-0.05	-0.01
			p-value	0.87	0.93	0.84	0.83	0.4	0.79
	Perth	Female	n	13	2	3	11	0	2
			r	-0.03	0.54	-0.09	-0.01	nd	-0.08
			p-value	0.16	nd	**<0.05**	0.54	nd	nd
		Male	n	22	5	1	19	3	2
			r	-0.03	-0.11	nd	-0.01	0.58	-0.08
			p-value	0.22	0.06	nd	0.78	0.57	nd
Serum mBDNF level decreased over 18 months	Melbourne	Female	n	22	5	9	17	4	7
			r	0.01	0.09	0.01	0.02	-0.15	-0.02
			p-value	0.67	0.37	0.52	0.36	0.5279	0.36
		Male	n	17	6	2	19	0	3
			r	-0.02	0.11	-0.01	0.01	nd	0.11
			p-value	0.39	0.18	nd	0.52	nd	0.62
	Perth	Female	n	22	5	0	19	3	0
			r	0.01	-0.1	nd	0.0001	0.005	nd
			p-value	0.7188	0.11	nd	0.99	0.93	nd
		Male	n	16	2	5	16	2	2
			r	-0.01	-0.03	0.01	0.04	-394.6	-0.22
			p-value	0.86	nd	0.85	0.28	nd	nd

Table 5-4 Correlation between serum mBDNF and PET SUVR.
The correlation between mBDNF serum level and PET SUVR was analysed in the patients that showed decreased or increased mBDNF levels over 18 months. BL, baseline; 18M, 18 month follow-ups. HC, healthy control; MCI, mild cognitive impairment; AD, Alzheimer's disease; nd, not determined; n, case number. $P < 0.05$ were written in bold. Linear regression.

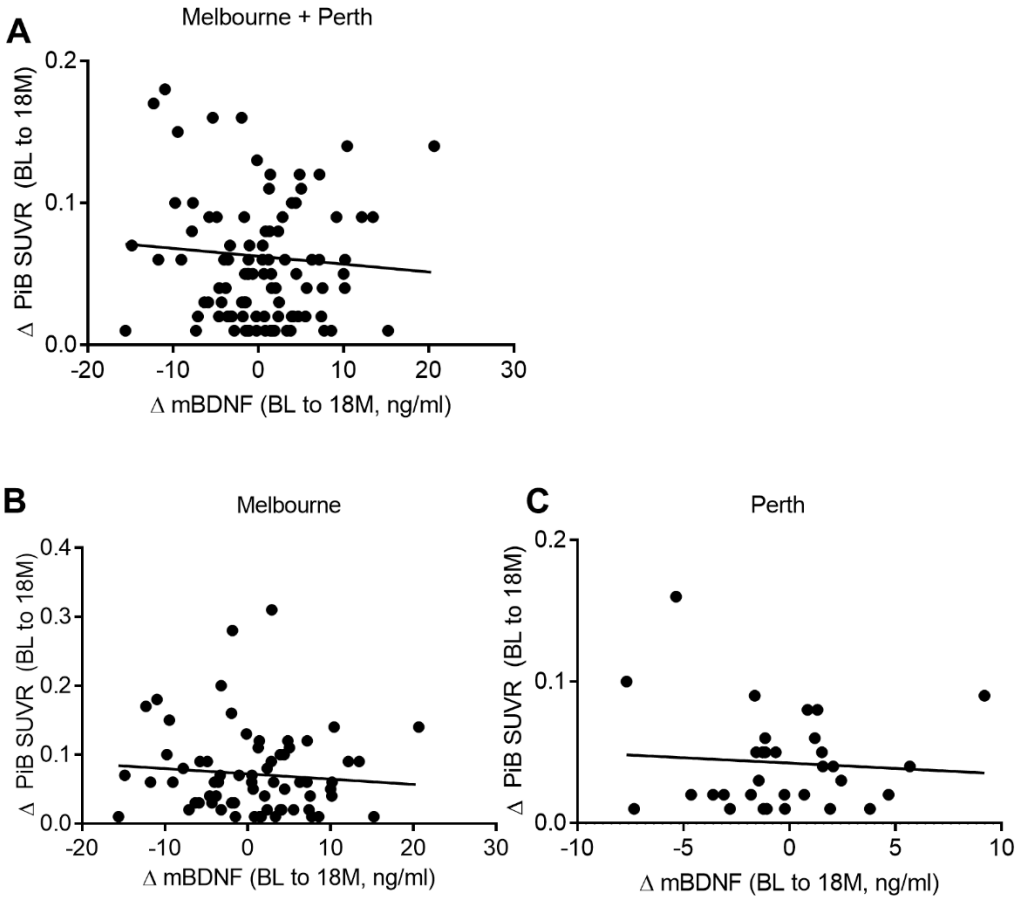


Figure 5-7 Correlation between change of mBDNF and change of PiB SUVR of the group which showed increased PiB SUVR over the 18 month period were analysed. (A) Melbourne and Perth sites combined (n=102, r=-0.063, p=0.53). (B) Melbourne site only (n=69, r=-0.091, p=0.46). (C) Perth site only (n=33, r=-0.077, p=0.67). Δ PiB SUVR= PiB SUVR(18M) – PiB SUVR (BL); Δ mBDNF = mBDNF (18M) – mBDNF (BL). The curve was fitted with linear regression using GraphPad Prism 6.0.

5.5 Discussion

As mentioned in Chapter 1.3.2, a number of variables could have caused the discrepant results in regards to mBDNF serum levels. We used a large well-characterised cohort, the AIBL, to minimise possible variation problems. To our knowledge, this study was the first study analysing serum mBDNF specifically in patients with MCI or AD. We also examined whether serum mBDNF is associated with cognitive decline and amyloid burden in a longitudinal study.

In this study, there were significant discrepancies in mBDNF serum levels between the two sample collection sites. Especially, the significantly increased serum mBDNF levels of HC at 18M compared to BL looked abnormal in the Melbourne samples. As well as that, the two sites samples generated inconsistent results in the most of analyses. %CV is used to validate inter-assay variability and < 15% are generally acceptable for ELISA assays (Thomsson, Strom-Holst et al. 2014). The Perth samples have produced acceptable %CV (7.85 ± 0.42) in the mBDNF ELISA assay; however, the Melbourne samples produced unacceptable %CV (18.15 ± 1.09). Taken together, it is highly likely there was a technical error in the assay of mBDNF with the Melbourne site samples. The high inter-assay variation may be owing to the variability in incubation time with reagents including coating antibody, blocking, capture antibodies, and TMB substrates. Another possibility could be the plate numbers which were tested on the same day. When the Perth sites samples were analysed 4 plates were tested a day, while 6 plates were tested a day when the Melbourne site samples were used. As all the ELISA procedures were done manually, the first plate and sixth plate might have different incubation time or some plates/wells might be washed insufficiently causing high background OD during the development step. Although mBDNF standard would not affect the inter-plate variation, keeping consistent standard molecule for a large cohort analysis is important. In this study, there was about one-year time difference between the two site

samples in running ELISAs and a new batch of mBDNF standard molecule was prepared just before measuring the Melbourne site samples. That might have caused the average mBDNF level discrepancy between the Melbourne and Perth sites however it does not explain the high inter-assay variation. One possibility that should be considered is that the baseline and its matching 18M samples were loaded on the same plate in case of the Perth site assays. However, this sample loading method was not applied to the Melbourne site samples. Using pooled serum as internal control on each plate will help to monitor a possible inter-plate assay variation. It should be also noted that serum mBDNF level can be varied by many factors including storage time, fasting state, blood collection time, smoking status and drinking habits (Bus, Molendijk et al. 2011). In addition, as platelets are major BDNF storage and secretion source (Fujimura, Altar et al. 2002), serum collection protocol should be consistent in all the sample collection sites. Therefore, reanalysis of Melbourne samples would strengthen this study and it is strongly recommended.

We failed to observe any significant difference in mBDNF serum levels among HC, MCI and AD at each time point in the Perth site samples. These results are consistent with previous reports (O'Bryant, Hobson et al. 2009, Woolley, Strobl et al. 2012). We observed there was a trend of decreasing mBDNF levels over 18 months in male AD group and the slope is much steeper than in HC and MCI groups. There was also a trend of decreasing mBDNF levels over 18 months in female MCI and the slope is steeper than in HC and AD samples. Further observation for 36, 54 and 74 months would give more comprehensive results. If there were correlations between mBDNF level and AD, we should have seen them in those cases where the clinical category changed over 18 months especially in those patients that showed fast pathological progression such as HC→MCI or MCI → AD cases. We failed to observe a significant mBDNF level difference between BL and 18M in the pairs whose clinical category changed over 18 months (Table 5-2) however, the case numbers were very low, so no definitive conclusion can be made at this stage. It is of note that in the

male Perth group, five pairs of subjects showed the clinical category change from HC to MCI in parallel with the decreased levels of mBDNF over the 18 month period, however the difference of serum mBDNF level between BL and 18M did not reach statistical significance, although it was very close (p=0.0535). Therefore, more cases should be analysed to investigate the correlation between serum mBDNF level change and fast progression of the disease. Overall, mBDNF serum levels could potentially serve as a biomarker for the fast MCI and AD progression but further studies are required.

We found that the entire group and male samples of the Perth site showed significant negative correlations between serum mBDNF levels and MMSE score (Fig. 5-5 D and F). Our result is partially consistent with that of Konukoglu et al. (2012) who showed serum mBDNF is negatively correlated with MMSE score in AD patients with CEI treatment (Konukoglu, Andican et al. 2012), however it is unclear how many participants were treated with CEI in our study. One possible reason for the negative correlation between low MMSE score and high mBDNF levels could be a compensatory mechanism at early stages of neuronal damage to protect neurons. It has been reported that continuous intracerebroventricullar infusion of Aβ leads to increased hippocampal BDNF expression spatiotemporally in rats (Tang, Yamada et al. 2000), which suggests that BDNF can play a role in neuronal protection in AD pathophysiology. We further found that there are negative correlations between MMSE score and mBDNF serum levels in the subjects showing increased mBDNF levels over the period of 18 months in the males, females or male + female. When compared with the subjects whose MMSE scores are less than 25, the females and males still showed negative correlations although the females did not show the significant correlation (p = 0.13). On the other hand, there was a positive correlation between MMSE score and mBDNF serum levels in the subjects whose mBDNF serum levels decreased over the period of 18 months in the female + male group, which is opposite to the subjects whose mBDNF serum levels increased over the period of 18 months. These results

might be owing to the analysis design as the two groups are already separated in regards to the BDNF serum concentrations, so the correlation with MMSE score should be opposite. Overall, we failed to find a correlation between the degree of the cognitive decline and the change of mBDNF serum level over the 18 month period, which suggests that the mBDNF serum level could not be a marker to monitor the severity of cognitive decline in the aged people.

There was a negative correlation between mBDNF and PiB SUVR in female AD ($p < 0.05$) at BL in the Perth group that showed increased mBDNF levels over 18 months, which is consistent with the recent study that BDNF plasma levels showed significantly negative correlation with PiB SUVR in multiple brain areas (Hwang, Lazaris et al. 2015). In our analysis, however, the analysed sample numbers are small (n=3), so more cases at BL and 18M are required to verify the result. We did not observe a significant correlation between the severity of amyloid burden and the change of mBDNF levels over the 18 month period, which suggests that mBDNF may not be related to the amyloid burden progress.

We found that serum mBDNF level is significantly higher in females than in males at both BL and 18M. This finding is in line with previous reports that females have significantly higher plasma BDNF concentration than males (Komulainen, Pedersen et al. 2008, Bus, Molendijk et al. 2011). Therefore, this should be taken into account in clinical studies measuring mBDNF levels in serum.

In the study, a large number of serum samples were analysed and several factors which could have affected the analyses should be discussed apart from the technical issues (specified above). Some studies have demonstrated that brain and serum BDNF levels are increased by the treatment with donepezil (Leyhe, Stransky et al. 2008), which is an acetylcholinesterase inhibitor (AchEI), or by using antidepressant drugs (Schmidt and Duman 2007, Kozisek, Middlemas et al. 2008). At the BL assessment, 134 (64%) out of the

211 AD participants confirmed taking prescribed AD medication; donepezil (74 AD patients), galantamine (43 patients), revastigmine (5 patients), combination of donepezil/memantine (6 patients) and combination of galantamine/memantine (6 patients). Also, the AD group showed significantly higher use of antidepressants (6% (HC), 15% MCI and 25% (AD)) (Ellis, Bush et al. 2009). Therefore, medication status of AD participants in the AIBL cohort is worth considering.

Overall, our findings suggest that mBDNF serum concentration could be associated the cognitive function but not with the cognitive decline over time. Our finding also suggests that mBDNF serum level may not be a reliable biomarker for AD. Further longitudinal analysis is required to evaluate whether mBDNF can be a biomarker for cognitive decline.

Through this study and data analyses, I have learnt how carefully I should perform assays and prepare assay-relating materials including standard materials and internal controls to avoid discrepant inter-assay variation.

Chapter 6: General discussion and future directions

6.1 Summary

BDNF is important in neuronal functions, development, survival, proliferation and long-term potentiation (LTP) in the nervous system (Davies 1994, Huang and Reichardt 2001, Pang and Lu 2004). For the neurotrophic function, BDNF binds TrkB and the BDNF/TrkB complex is then endocytosed and trafficked to the cell body to stimulate downstream signalling pathways. However, how BDNF and its receptor get endocytosed is not clear. HAP1 has been found to be involved in intracellular trafficking and stabilising and recycling membrane receptor including TrkB, we therefore hypothesised that HAP1 is required for BDNF and its receptors endocytosis to regulate TrkB downstream signalling pathway for neuronal proliferation in neurons.

Our study demonstrated that HAP1 interacts with $p75^{NTR}$, TrkB and their interaction is triggered by BDNF stimulation. We also showed that the internalisation of BDNF and TrkB is abolished in cultured cortical neurons of HAP1 KO mice and the WT HAP1 gene introduction can rescue the internalisation deficit. The down-stream signalling of TrkB including Erk, Akt and PLCγ-1 was found to be significantly reduced in cortical neurons of HAP1 KO mice compared to WT. Moreover, *in vitro* and *in vivo* experiments revealed that proliferation of cerebellar granule cell was defected in HAP1 KO mice. Our findings suggest that HAP1 is critically required for BDNF and its receptor internalization and impaired HAP1 function could be linked to the pathology of neurological disorders. As such this study has addressed the role of HAP1 in BDNF and its receptor endocytosis and down-stream signalling pathways in neurons (Fig. 6-1).

AIM1

Outcomes:

- HAP1 interacts with TrkB and $p75^{NTR}$ and BDNF triggers the interaction.
- In HAP1 KO cortical neurons, endocytosis of BDNF and TrkB is abolished.
- In HAP1 KO cortical neurons, TrkB down-stream signalling pathways are reduced.
- Proliferation of cerebellar granule cell is defected in HAP1 KO mice *in vivo* and *in vitro*.

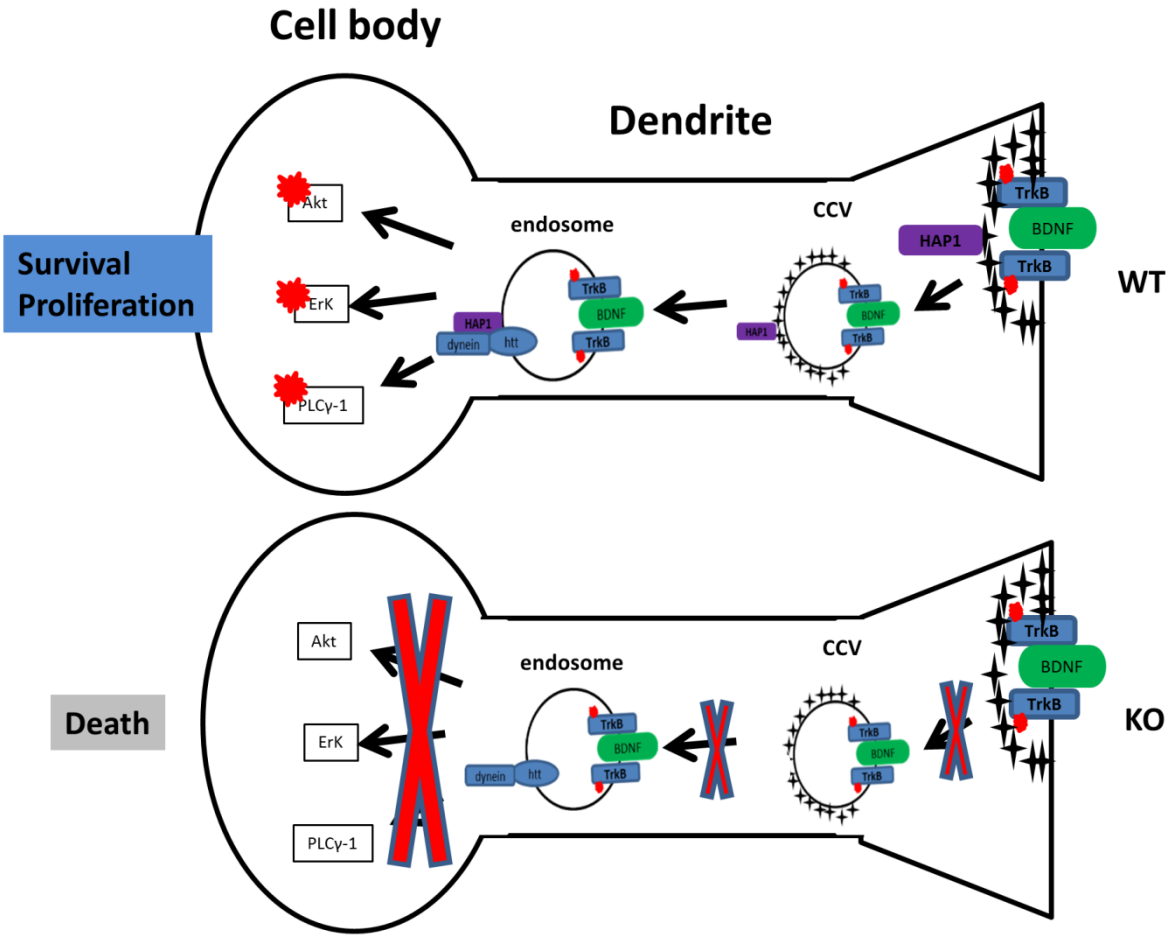


Figure 6-1 Graphical summary of HAP1's role in BDNF and TrkB endocytosis in neurons.

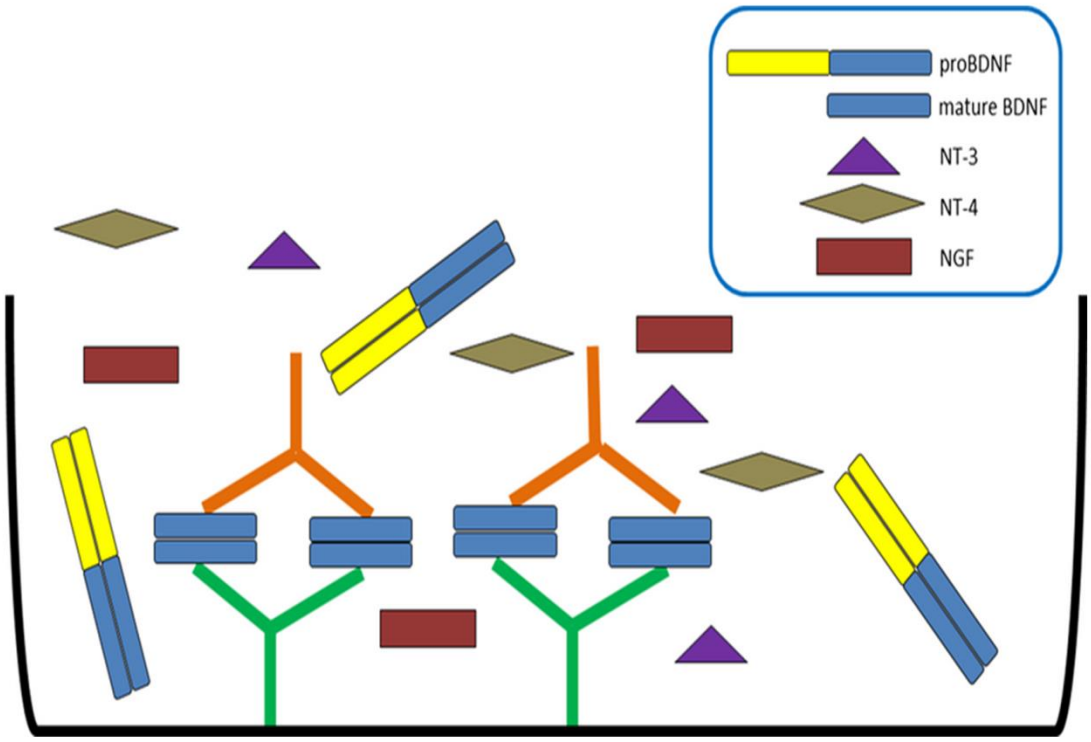
Once BDNF binds TrkB, TrkB is phosphorylated and the BDNF/TrkB complex undergoes clathrin-mediate endocytosis with HAP1's involvement. HAP1 then facilitates retrograde endosomal trafficking to the cell body and Akt, Erk and PLCγ-1 are activated. This signalling endosome leads to neuronal survival and proliferation. In HAP KO mice, BDNF/TrkB complex cannot get endocytosed into neurons so that TrkB down-stream signalling pathways cannot be activated, which leads to neuronal death. CCV, clathrin-coated vesicle

In contrast to mBDNF, proBDNF elicits neuronal cell death and long-term depression (LTD) (Lee, Kermani et al. 2001, Pang and Lu 2004, Woo, Teng et al. 2005, Sun, Lim et al. 2012). The altered BDNF levels have been implicated in neurological disorders (Mogi, Togari et al. 1999, Angelucci, Spalletta et al. 2010, Hashimoto 2010). Commercial BDNF ELISA kits have been used widely to measure BDNF levels in samples, especially, blood samples; however, these kits do not provide a cross-reactivity data to proBDNF or are not specific enough to detect mBDNF. Considering the opposing roles of mBDNF and proBDNF, it is essential to measure mBDNF and proBDNF separately with high specificity. Our second aim therefore was to develop mature BDNF-specific ELISA. In our study, we have developed a new mBDNF-specific sandwich ELISA ((Lim, Zhong et al. 2015), Fig. 6-2 and Chapter 3) that showed no cross-reactivity for other growth factors, NT-3, NT-4 or NGF and higher specificity for mBDNF compared to other commercial BDNF ELISA kits. A few world-widely used commercial BDNF or mBDNF ELISA kits showed 6-18 times higher cross-reactivity to proBDNF compared to our new mBDNF ELISA. We also showed this new ELISA can be used for the mBDNF measurement in various tissues including brain, serum and plasma. Comparison of ELISA and Western blot confirmed our new mBDNF detects mBDNF specifically. We have also showed that the ratio of mBDNF/proBDNF varies in different brain regions and not consistent with the level of mBDNF. Our new mBDNF-specific ELISA would provide a new tool to re-evaluate previous studies presenting BDNF levels in clinical samples and to perform mBDNF and proBDNF measurements-relating research.

AIM2

Outcomes:

- mBDNF-specific ELISA has been developed.
- No cross-reactivity to NT-3, NT-4 and NGF
- Negligible cross-reactivity to proBDNF (1 - 4.5 %)



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Figure 6-2 Graphical summary of mBDNF-specific ELISA development.
New mBDNF-specific ELISA detects mBDNF with high specificity. It does not recognise NT-3, NT-4 and NGF and shows negligible cross-reactivity to proBDNF.

After we developed the new mBDNF-specific sandwich ELISA, we then focused on measurement of proBDNF and mBDNF in samples from patients with neurological disorders. In chapter 4, we examined whether mBDNF, proBDNF and their receptors are altered in major depressive disorder (MDD) and in chapter 5, we examined correlations between serum mBDNF levels and other Alzheimer's disease (AD) biomarkers. The main findings of these studies are presented in Figure 6-3 (AIM 3) and Figure 6-4 (AIM 4) and are discussed below.

Our study described in chapter 4, showed that in serum of MDD patients, mBDNF and TrkB levels are significantly lower while proBDNF, sortilin and $p75^{NTR}$ levels are significantly higher relative to healthy subjects. Similarly, mRNA expression of BDNF and TrkB was significantly lower whereas sortilin and $p75^{NTR}$ were higher in MDD patients than healthy subjects. The levels of mBDNF and TrkB and proBDNF sortilin and $p75^{NTR}$ were negatively and positively correlated with the severity of the MDD, respectively. We also found that serum mBDNF and proBDNF levels are negatively correlated in MDD patients. Our data suggest that balance between mBDNF/TrkB and proBDNF/sotilin/$p75^{NTR}$ is altered in MDD and the disruption of this balance may contribute to pathology of MDD (Fig. 6-3).

AIM 3

Outcomes : In depression,

- proBNDF, p75NTR and sortilin are upregulated.
- mRNA of TrkB and BNDF is reduced
- The ratio of proBDNF/mBDNF altered.
- Severity of depression is correlated with level of mBDNF/TrkB and proBDNF/p75^{NTR}/sortilin.

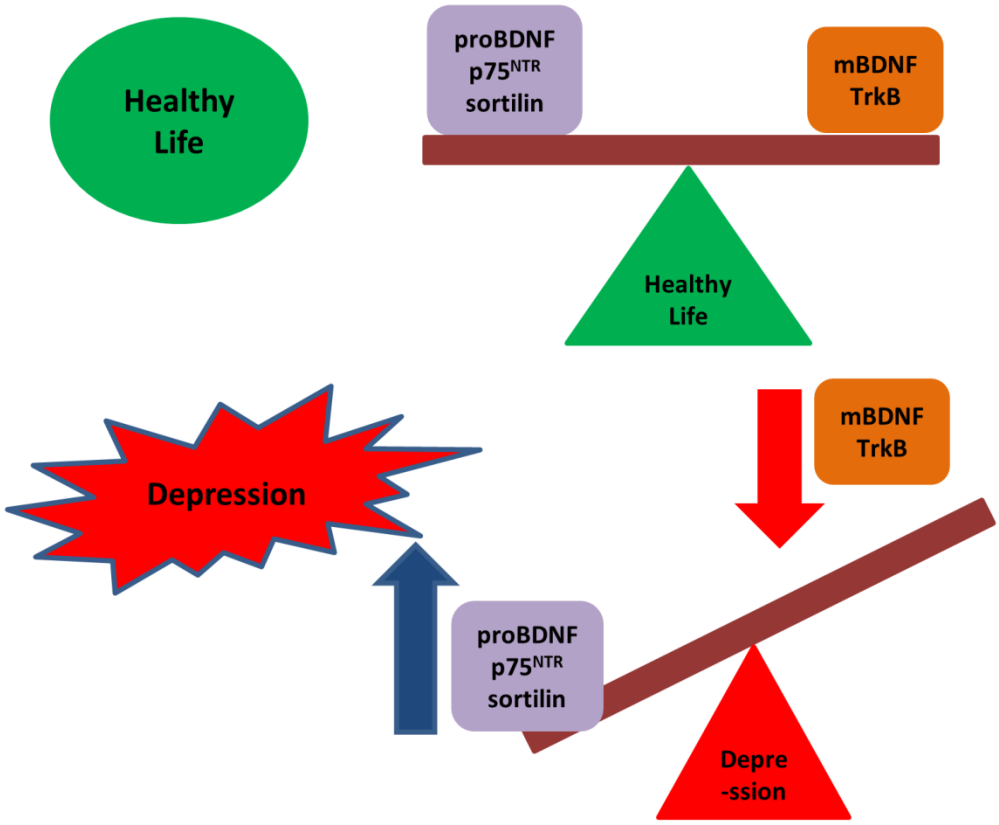


Figure 6-3 Graphical summary of imbalance of mature BDNF/proBDNF and their receptors in depression.
Stressful events and genetic predispositions decrease the expression of BDNF and cause abnormal proBDNF processing, which lead to an increase in proBDNF and its receptors and an imbalance of neurotrophic signals that trigger neurodegeneration and depression. Positive life styles are critical for the maintenance of neurotrophic balance which is essential for healthy life.

Our study described in Chapter 5, showed no significant difference of mBDNF serum concentration among healthy controls (HC), mild cognitive impairment (MCI) and Alzheimer's disease (AD) at either baseline (BL) and 18-month follow-up (18M) or over the period of 18 months. Female showed significantly higher mBDNF serum levels at BL and 18M. We have found a negative correlation between mBDNF serum levels and Mini-Mental State Examination (MMSE) scores in entire cohort and the males of the Perth site. We did not find correlations between MMSE decrease and mBDNF level change over the 18 month period. We did not find meaningful corraltions betwwen serum mBDNF and amyloid burden. The ELISA with Melbourne site samples showed very high inter-assy %CV (18.15 ± 1.09) while that with Perth site samples showed acceptable %CV (7.85 ± 1.09), which caused a technical error in this study. Therefore, the Melbourne site samples need to be re-anlaysed (Fig. 6-4).

AIM4

Outcomes:

- Negative correlation found between serum mBDNF levels and MMSE.
- Female showed significantly higher mBDNF levels.
- No significant differences of mBDNF serum level were found among HC, MCI and AD.
- No correlations were found between serum mBDNF and amyloid burden.

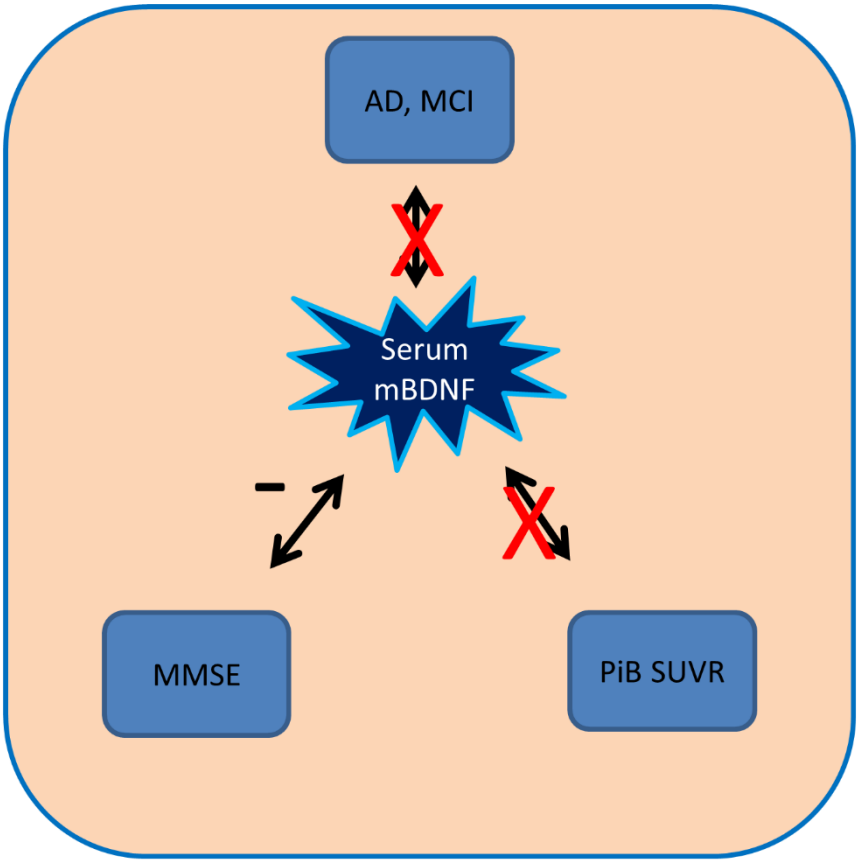


Figure 6-4 Graphical summary of correlations of serum mBDNF with AD, MCI, MMSE and PiB SUVR.
MMSE, Mini-Mental State Examination; HC, healthy control; MCI, mild cognitive impairment; AD, Alzheimer's disease; PiB SUVR, $[11]^{C}$-Pittsburgh compound B standardized uptake value ratio

In summary, we have showed that HAP1 is critical for BDNF and its receptor endocytosis and down-stream signalling pathways which lead neuronal proliferation and the hap1 gene deficiency leads impaired endocytosis of BDNF and its receptor and further down-stream singalling pathways, resulting defected neuronal proliferation. We also have developed mBDNF-specific ELISA and it will allow to measure and analyse mBDNF level specifically

in various neuroscience studies. We also have demonstrated that the decreased mBDNF/TrkB levels and increased proBDNF/p75^{NTR}/Sortilin are associated with severity of depression and mBDNF serum levels are correlated with cognitive decline in the elderly group. In conclusion, my PhD project have presented that impaired HAP1's role and imbalance between mBDNF/proBDNF and their receptors could cause neurological disorders and be a biomarker for diseases (Fig. 6-5).

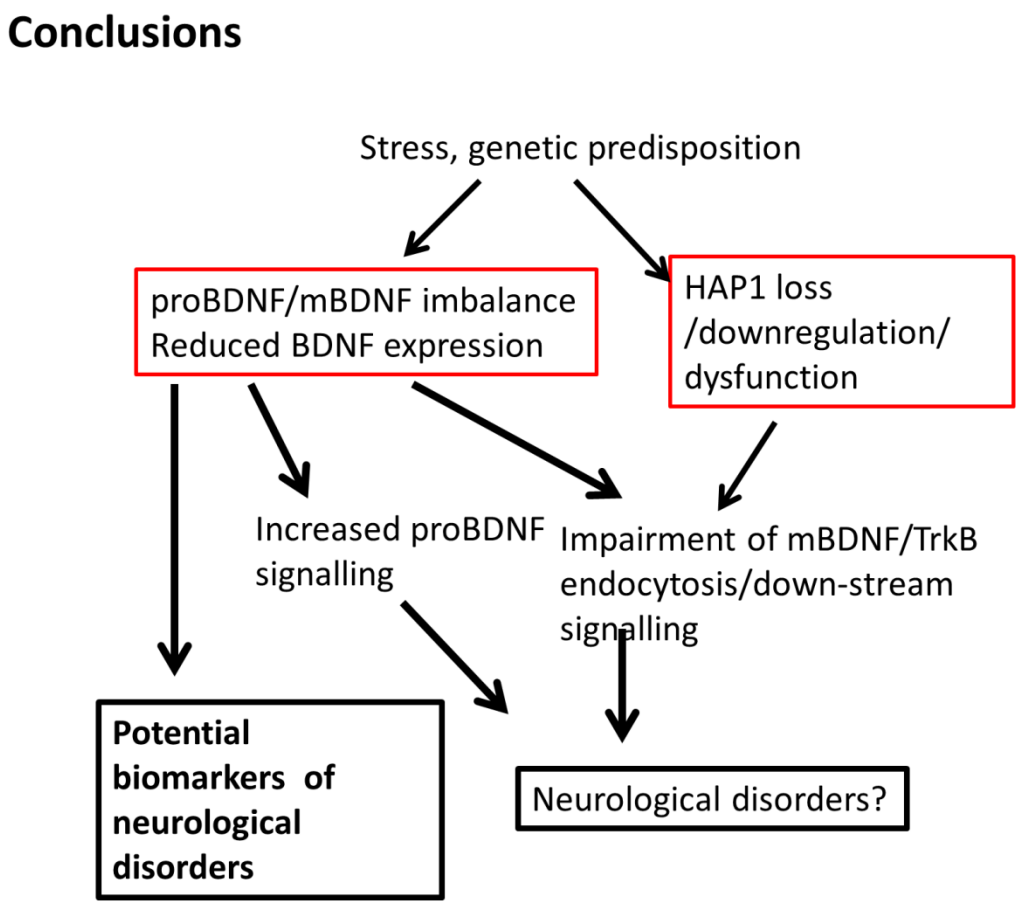


Figure 6-5 Summary of conclusions of this project.

6.2 Future Directions

6.2.1 The role of HAP1 in clathrin-mediated endocytosis of TrkB and $p75^{NTR}$; possible interaction between HAP1 and clathrin light chain

In our study, we have shown that HAP1 is an essential molecule in neurons to facilitate endocytosis of BDNF and TrkB and the following down-stream signalling pathways. However, how HAP1 regulates the initial internalisation of the BDNF/TrkB complex is unclear. The endocytosis of TrkB and $p75^{NTR}$ is clathrin dependent (Saxena, Howe et al. 2004, Deinhardt, Reversi et al. 2007, Zheng, Shen et al. 2008). Huntingtin interacting protein 1 (HIP1) was identified by yeast two-hybrid screening after HAP1 discovery (Kalchman, Koide et al. 1997, Wanker, Rovira et al. 1997) and binds clathrin and α-adaptin which are involved in receptor-mediated endocytosis (Waelter, Scherzinger et al. 2001). HAP1 was found to be associated with clathrin heavy chain, α-adaptin A and α-adaptin C by HIP1-affinity purification and subcellular fractionation (Waelter, Scherzinger et al. 2001). Together with our current findings, we propose that HAP1 regulates clathrin-mediated endocytosis (CME) of TrkB and $p75^{NTR}$, and possibly, the general cellular endocytosis.

To prove our hypothesis, we would use a CME-required molecule such as transferrin (Le Roy and Wrana 2005) or clathrin-independent molecules such as cholera toxin B or dextran (Cheng, Singh et al. 2006). These molecules which are conjugated with fluorescence will be added to the culture of neurons and fibroblasts from WT and HAP1 KO mice. Images will be then taken to analyse the fluorescent intensity of internalised molecules. If HAP1 is required for CME, the internalisation of transferrin but not cholera toxin B and dextran will be reduced or abolished in HAP1 KO cells. If the internalisation is altered, a rescue experiment will be performed by transfection of HAP1 plasmid into HAP1 KO cells to see whether the internalisation of these molecules is recovered after re-expression of HAP1. The experiment will show whether HAP1 is required for CME in neurons and/or fibroblasts.

Addition to that, HIP1 modulates CME by regulating clathrin light chain assembly (Legendre-Guillemin, Metzler et al. 2005). Furthermore, our proteomics analysis of HAP1 antibody affinity column using rat brain homogenates showed that clathrin light chain interacts with HAP1 (non-published data). As the DLL motif of HIP1 interacts with clathrin light chain (Legendre-Guillemin, Metzler et al. 2005) and HAP1 also contains a single DLL motif at amino acid 208 - 210, we would mutate the DLL motif of HAP1 to examine both the interaction between HAP1 and CLC and endocytosis of BDNF, transferrin. The result will reveal whether DLL motif of HAP1 is essential for CME.

6.2.2 The role of HAP1 in AD

Our finding showed that BDNF/TrkB signalling is impaired in HAP1 KO cortical neurons (Fig. 2-6). In AD, the BDNF/TrkB signalling is dysregulated (Zhang, Kang et al. 2012) and amyloid-beta (Aβ) impairs BDNF retrograde transport (Poon, Blurton-Jones et al. 2011, Poon, Carlos et al. 2013). Our pilot data showed that HAP1 is down-regulated in human AD brain. We demonstrated that HAP1 plays an important role in endocytosis of BDNF and its receptors (Fig. 2-3 and 2-4) and others showed that HAP1 is critical in postnatal neurogenesis through the signalling of BDNF and TrkB (Xiang, Yang et al. 2014). Moreover, we also found that HAP1 regulates APP trafficking to the non-amyloidogenic pathway (Yang, Yang et al. 2012) and appears neuroprotective. Therefore, we propose that HAP1 plays a positive role in protecting the brain from the development of AD by promoting the signalling of BDNF/TrkB and reduces amylolidosis in AD (Fig. 6-6).

The proposed role of HAP1 in AD pathogenesis and possible interaction between HAP1 and clathrin light chain

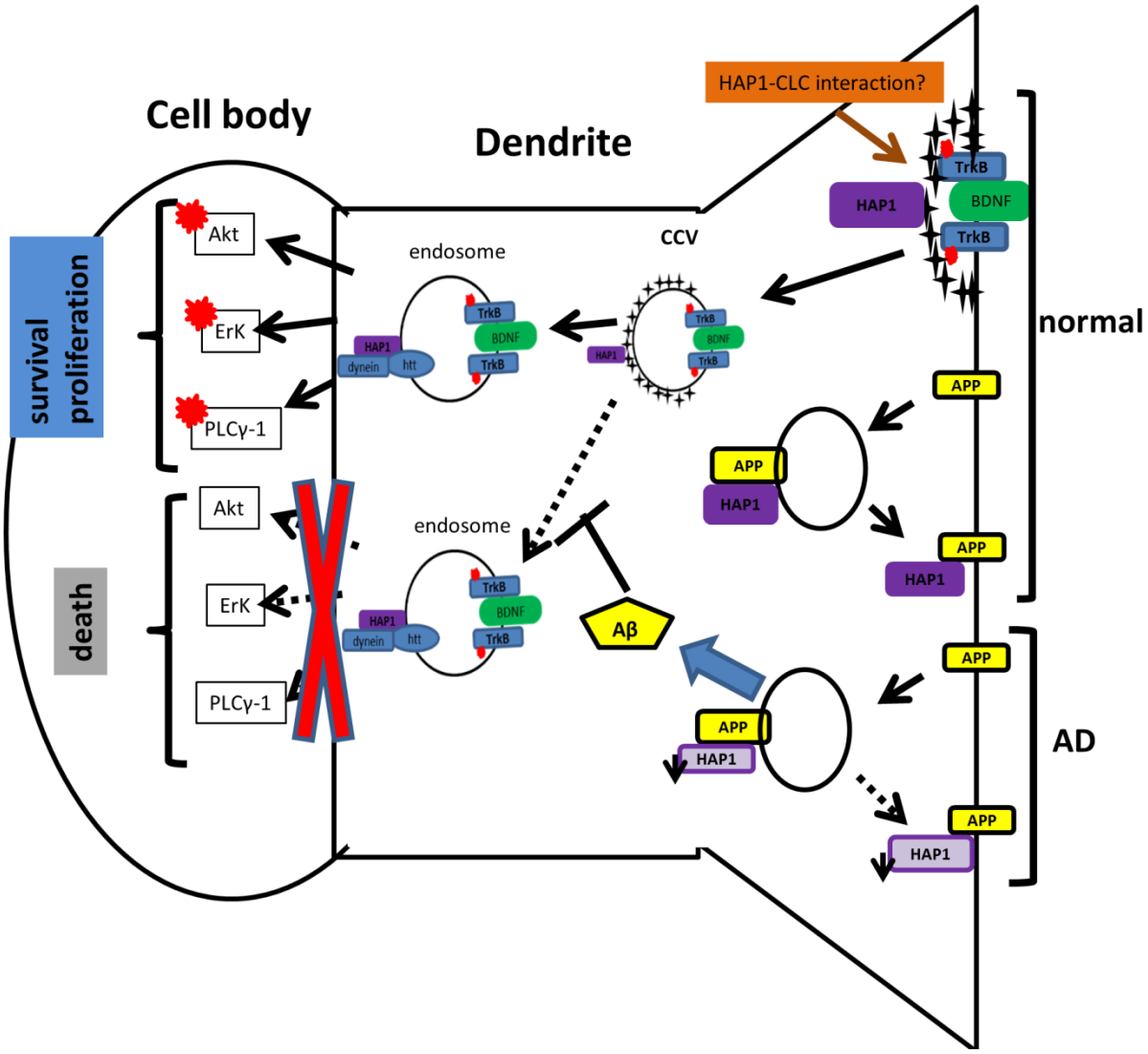


Figure 6-6 Graphical summary presenting hypothesis of correlation of HAP1 with AD pathogenesis and interaction between HAP1 and clathrin light chain (CLC).
In normal status, HAP1 facilitates BDNF/TrkB signalling and recycling of APP to the plasma membrane to prevent Aβ production intracellularly. In AD, HAP1 is downregulated and it causes impaired APP recycling to the plasma membrane. Consequently, more Aβs are produced and Aβ impairs retrograde trafficking of BDNF/TrkB-containing endosome, which causes neuronal death in brain. Also, HAP1 may interact with CLC through 'DLL' motif to mediate clathrin-mediated endocytosis.

To prove our hypothesis, we would examine the HAP1 expression by Western blot in AD brain of human with extended number of AD cases in order to obtain solid statistical evidences. We would also culture WT hippocampal neurons in the presence of various concentration of Aβ peptide or scramble peptide for three days and mRNA and protein level of HAP1 would be determined. These experiments would show whether HAP1 expression is down-regulated in AD brains and whether Aβ causes suppression of HAP1 gene expression. It would be interesting to examine the effects of HAP1 on Aβ-induced impairment of BDNF/TrkB axonal trafficking. Hippocampal neurons and viral particles which carry BDNF-GFP, TrkB-GFP or HAP1-mCherry would be used for the experiment. Hippocampal neurons would be cultured in a fluidic chamber so that axons are separated from cell bodies and straighten in the presence of Aβ or scrambled Aβ. BDNF-GFP and TrkB-GFP vesicles with or without co-transduction of HAP1-mCherry in axons would be monitored with live-imaging microscopy. This experiment would reveal whether axonal transport of BDNF and TrkB is impaired by Aβ and whether HAP1 overexpression can ameliorate such defect. It would be also beneficial to examine whether the HAP1 overexpression reduces AD pathology. To investigate this possibility AD transgenic mice will be crossed with HAP1-overexpressing transgenic mice to generate AD: WT and AD:HAP1. One group of mice will be culled at 6 months old to investigate AD pathology using pathological and biochemical assays. At 12 months old, when AD:WT mice show cognitive decline, the mice will be subjected for behavioural tests including Morris Water Maze, open field and elevated plus tests. After the tests the mice brains will be subjected to pathological and biochemical analyses. This experiment will show whether overexpression of HAP1 could improve the cognitive functions, reduce amyloid plaques and increase TrkB and BDNF levels.

6.2.3 Correlations among mBDNF/proBDNF and their receptors in neurological disorders

We have developed mBDNF-specific ELISA and proBDNF ELISA (Chapter III). Furthermore, the mBDNF-specific ELISA was used to measure mBDNF levels in serum of MDD cohort (Chapter 4) and AD cohort (Chapter 5). As mentioned in Chapter 1, BDNF is related to neurological disorders and proBDNF and mature BDNF have opposing functions. Therefore, our new mBDNF-specific ELISA with proBDNF ELISA can be applied to examine the levels of mBDNF and proBDNF and the ratio of mBDNF/proBDNF to investigate any correlations with status or diagnosis of diseases.

One potential application of mBDNF-specific ELISA could be to re-evaluate reports showing determined BDNF levels by ELISA. We have demonstrated that the most widely used commercial ELISA kits have 30 – 90 % cross-reactivity for proBDNF while our new ELISA has only 4.99 % cross-reactivity (Figure 3, Chapter 3). For example, Teche et al. (2013) reviewed 28 previous BDNF level measurements reports in MDD (Teche, Nuernberg et al. 2013); the data published in these 28 studies most likelyhave presented the combined levels mBDNF and proBDNF. Therefore, we propose that further studies should be performed to investigate if mBDNF and proBDNF levels are altered in MDD using the same and/or other MDD patient samples We expect that the (Shimada, Makizako et al. 2014)results may be different from the previously published work.

Major depressive disorder (MDD) is the most common mental illness. The underlying pathogenesis of depression is not fully understood (Wong and Licinio 2001, Wong, Dong et al. 2012). Inconsistent or even conflicting reports in the literature raise the need for a re-evaluation of the original neurotrophic hypothesis of depression (Shirayama, Chen et al. 2002, Sen, Duman et al. 2008), and justify demands for further investigations to refine it (Groves 2007). For example some anti-depressants increase the overall BDNF gene

expression (Branchi, D'Andrea et al. 2006) but may decrease the processing of proBDNF to mature BDNF, which results in accumulation of proBDNF in the brain and blood (Zhou, Xiong et al. 2013) . Even though the processing of proBDNF is not affected, simply increasing BDNF gene expression may have no effect on depression as the neurodegenerative effect of the gene product, proBDNF, may cancel the neurotrophic effect of mature BDNF. Thus understanding the details of BDNF gene regulation, intracellular trafficking, processing, release and signalling pathways is essential for elucidating the functions of neurotrophins in depression and developing drugs effective for major depression.

Our published preliminary data ((Zhou, Xiong et al. 2013), Chapter 4) show that in subjects with major depression, the balance of mBDNF and proBDNF and their respective receptors in lymphocytes is disrupted: m BDNF and TrkB are significantly reduced, which is consistent with previous studies (Dwivedi, Rizavi et al. 2003, Molendijk, Bus et al. 2011, Yoshida, Ishikawa et al. 2012), but in addition, proBDNF and its receptors, $p75^{NTR}$ and sortilin, are upregulated and positively correlated with the severity of depression (Zhou, Xiong et al. 2013). Our data suggest that balance between mBDNFD/TrkB and proBDNF/sotilin/$p75^{NTR}$ is critical and the disruption of this balance could contribute to pathology of MDD. This finding is the first study to suggest that not only is the genetic expression of BDNF in major depression is dysregulated, but the conversion (processing) from proBDNF to mature BDNF is deficient, resulting in an accumulation of proBDNF in the blood and brain. Two receptor systems mediate signal transduction of BDNF. For neurotrophic functions, mBDNF binds and activates a tyrosine kinase TrkB which is autophosphorylated and activates down-streaming MAPK/Erk, PI3K/Akt and PLC-γ1 signal pathways (Huang and Reichardt 2003). ProBDNF, on the other hand, binds predominantly to $p75^{NTR}$ and its co-receptor sortilin (Teng, Teng et al. 2005) and mediates a number of neurodegenerative events such as apoptosis, LTD and neurite collapse (Woo, Teng et al. 2005, Sun, Lim et al. 2012). Therefore, we propose that the conversion from proBDNF to

mature BDNF is dysregulated in MDD and the ratio of proBDNF/mature BDNF and their receptors can be a diagnostic marker for the disease (Fig. 6-7).

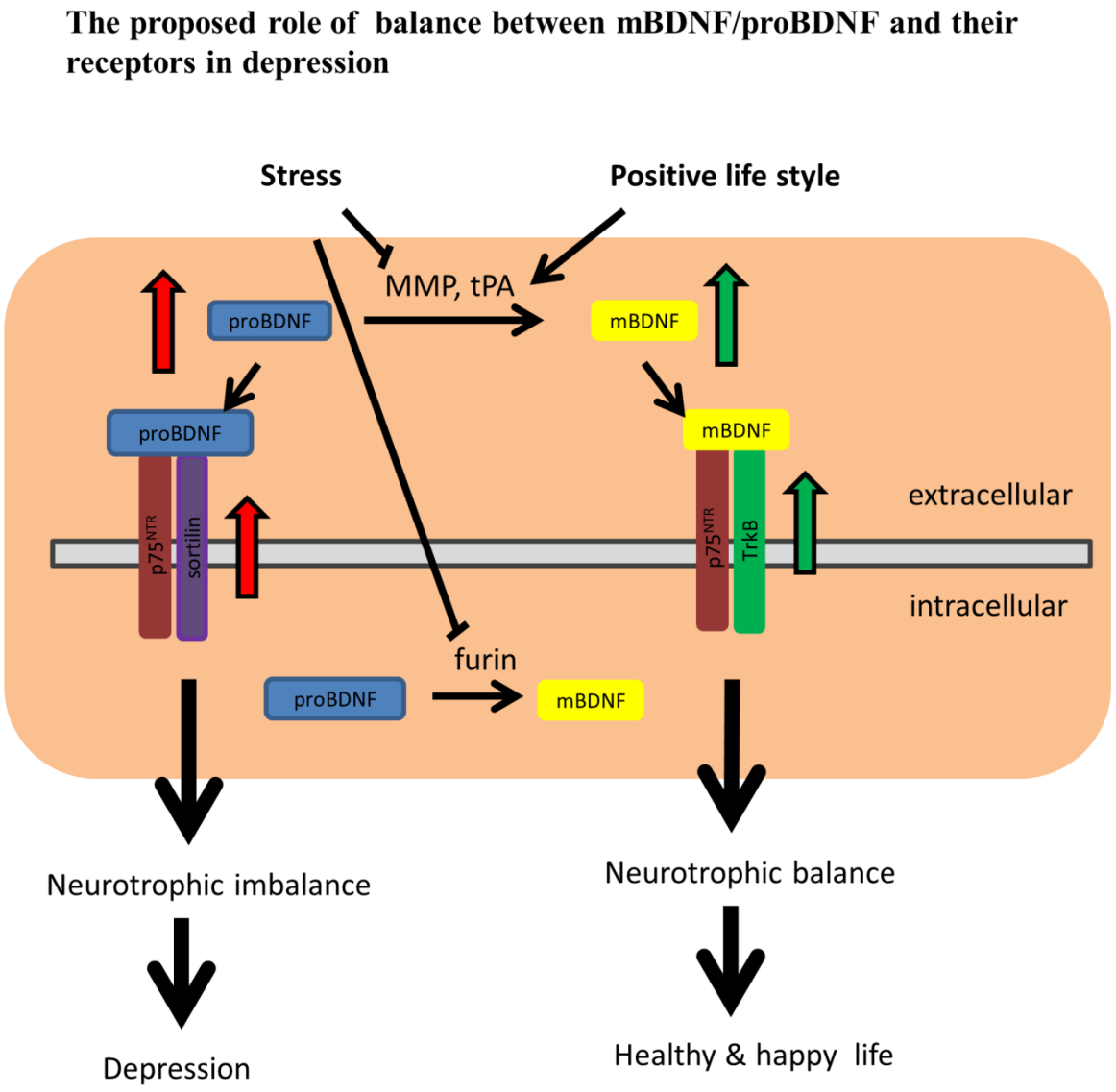


Figure 6-7 Graphical summary of modified BDNF hypothesis for depression.
Stressful events and genetic predispositions decrease the expression of BDNF and cause abnormal proBDNF processing, which lead to an increase in proBDNF and its receptors and an imbalance of neurotrophic signals that trigger neurodegeneration and depression. Positive life styles are critical for the maintenance of neurotrophic balance which is essential for healthy and happy life.

We would analyse protein and/or mRNA levels of mBDNF, proBDNF, Sortilin, TrkB and $p75^{NTR}$ in lymphocytes and serum within a large MDD cohort. We would also conduct same

analyses with post-mortem brain samples. These studies would strengthen our preliminary findings and allow us to infer whether the levels of mBDNF and proBDNF and their receptors and the correlations amongst them in the periphery reflect the change in the brain of MDD. In a separate experiment, we will give chronic unpredictable mild stress to WT mice to cause a depression. When mice show depression –like behaviours such as an increase immobility in the forced swimming test and tail suspension test and a decreased sucrose consumption in the sucrose preference test (Ruan, Wang et al. 2014, Yang, Bai et al. 2014, Yang, Zhang et al. 2014), we will cull the mice and analyse the levels of mBDNF and proBDNF and their receptors and the correlations in serum and brains. This experiment will show whether balance between mBDNF/TrkB and proBDNF/Sortilin/$p75^{NTR}$ is impaired in depressed mice.

Another potential application of the new mBDNF ELISA could be measurement of mBDNF to monitor medical drug efficacy. For example, antidepressant drug treatment has been found to increase serum BDNF levels (Aydemir, Yalcin et al. 2006, Hashimoto 2010, Teche, Nuernberg et al. 2013). This suggests that the determined mBDNF levels in serum or plasma could be used for new antidepressant agents screening or even predicting the response to an antidepressant treatment for an individual by monitoring the BDNF level changes and the clinical response. Similarly, AchEI, an AD treatment drug, was found to increase serum BDNF level (Leyhe, Stransky et al. 2008) . Therefore, we propose that measurement of mBDNF levels can be used to monitor the efficacy of the drug treatment in AD patients. Our mBDNF measurement within the AIBL cohort is a longitudinal study. We have measured BL and 18M only and the data in Chapter 5 are not conclusive and informative enough. Therefore, it could be worthwhile mesuring mBDNF and analyse the correlations with MMSE or other AD biomarkers at 36 months and 54 months follow-ups. As previous studies which demonstrated BDNF levels measured by ELISA did not used mBDNF-specific ELISA kits, this study would reveal whether mBDNF levels are related to AD pathology and

other AD biomarkers. It would also be worth measuring proBDNF levels and analyse the ratio of mBDNF/proBDNF to establish whether the conversion of proBDNF to mBDNF is altered in AD patients.

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Appendices

PUBLICATIONS

Publications from this thesis

1. **Lim, Y**., Zhong, JH., Zhou, XF (2015). "Development of mature BDNF-specific sandwich ELISA." J Neurochem 134(1): 75-85.

2. Zhou, L., J. Xiong, **Lim, Y**., Ruan, Y., Huang, C., Zhu, Y., Zhong, J., Xiao, Z., Zhou XF (2013). "Upregulation of blood proBDNF and its receptors in major depression." J Affect Disord **150**(3): 776-784. **First equal co—author**

Other publications during PhD

- Xiong J, Zhou LI, **Lim Y**, Yang M, Zhu YH, Li ZW, Fu DL, Zhou XF. Mature brain-derived neurotrophic factor and its receptor TrkB are upregulated in human glioma tissues Oncol Lett. 2015 Jul;10(1):223-227. Epub 2015 May 6.
- Xiong J, Zhou L, **Lim Y**, Yang M, Zhu YH, Li ZW, Zhou FH, Xiao ZC, Zhou XF. Mature BDNF promotes the growth of glioma cells in vitro. Oncol Rep. 013Dec;30(6):2719-24.
- Saadipour K, Yang M, **Lim Y**, Georgiou K, Sun Y, Keating D, Liu J, Wang YR, Gai WP, Zhong JH, Wang YJ, Zhou XF. Amyloid beta$_{1-42}$ (Aβ_{42}) up-regulates the expression of sortilin via the p75(NTR)/RhoA signaling pathway. J Neurochem. 2013 Oct;127(2):152-62.
- Yang M, Virassamy B, Vijayaraj SL, **Lim Y**, Saadipour K, Wang YJ, Han YC, Zhong

JH, Morales CR, Zhou XF. The intracellular domain of sortilin interacts with amyloid precursor protein and regulates its lysosomal and lipid raft trafficking.PLoS One. 2013 May 21;8(5):e63049.

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Conference and Seminar Presentations

- **Lim, Y**, Wu LY, Chen S, Sun Y, Lekha S, Yang M, Bobrovskaya L, Keating D, Li XJ, Zhou XF. HAP1 is required for endocytosis and signalling of BDNF and its receptors in neurons. Australian Society for Medical Research (ASMR), June 2015, Adelaide, Australia **(Oral presentation).**
- **Lim, Y**, Annie Sun, Zhou XF. Development of mature and proBDNF-specific sandwich ELISA system. 5th Australian Neurotrauma Symposium, October 2014, Australia **(Poster presentation).**
- **Lim, Y**, Annie Sun, Zhou XF. Development of mature and proBDNF-specific sandwich ELISA system. Alzheimer's Association International Conference, Jul

2014, Denmark **(Poster presentation).**

- **Lim, Y**, Annie Sun, Zhou XF. Development of mature and proBDNF-specific sandwich ELISA system Australasian Neuroscience Society 34th Annual Meeting, Jan 2014, Australia **(Oral presentation).**
- **Lim, Y**, Annie Sun, Zhou XF. Development of mature BDNF-specific antibody and sandwich ELISA system. Feb 21st 2013, Neuroscience Seminar, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, Australia **(Oral presentation).**
- **Lim, Y**, Annie Sun, Zhou XF. Development of mature BDNF-specific sandwich ELISA system. Australasian Neuroscience Society 32h Annual Meeting, Jan 2012, Australia (**Poster presentation**).

AWARDS

- Postgraduate scholarship by NHMRC, 2011 - 2014
- Flinders University Top-up scholarship by Flinders University, Feb 2011 – Feb 2012
- Neuroscience Top-up scholarship by University of South Australia, Mar 2012 – Jun 2014
- UniSA Top-up scholarship by, Mar 2012 – Jun 2014
- Travel Award, Australasian Neuroscience Society, Jan 2012
- Overseas travel grant by University of South Australia, May 2014