MECHANISMS OF BDNF DOWN-REGULATION BY THE PATHOLOGICAL CORRELATES OF ALZHEIMER'S DISEASE

MECHANISMS OF BDNF DOWN-REGULATION BY THE PATHOLOGICAL CORRELATES OF ALZHEIMER'S DISEASE

By

ELYSE ROSA, B.Sc.

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AUTHOR: Elyse Rosa, B.Sc. (McMaster University)

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ABSTRACT

Alzheimer's disease is a progressive neurodegenerative disorder that is the leading cause of dementia among the elderly. Alzheimer's disease presents as global cognitive decline with associated memory loss and altered personality. The neuropathological hallmarks of Alzheimer's disease include extracellular beta-amyloid-containing plaques and intracellular neurofibrillary tangles formed by hyper-phosphorylated tau protein. There is increasing evidence that although the primary insult in Alzheimer's disease may be over-expression of amyloid-β, it may ultimately lead to cognitive dysfunction and neurodegeneration by inducing alterations in tau. While the precise toxic mechanism of these accumulated proteins is not well understood, our hypothesis is that both amyloid-β and tau exert their neurotoxicity via down-regulation of brain-derived neurotrophic factor (BDNF). BDNF is crucial for synaptic function, neuronal survival and learning and memory and is decreased in Alzheimer's disease, tauopathies and in several relevant animal and cellular models of Alzheimer's disease.

Using differentiated, human neuroblastoma cells, we found that treatment with oligomeric Aβ down-regulates basal levels of BDNF as a consequence of Aβ-induced CREB transcriptional down-regulation. Similarly, these cells, when made to over-express wild-type tau, also exhibit reduced BDNF expression. They specifically lose the major CREB-regulated BDNF transcript, transcript IV. Using transgenic mice, we showed that neither tau mutations nor neurofibrillary tangles are required for BDNF reduction, but that wild-type tau over-expression is

sufficient to down-regulate BDNF. Lastly, we crossed APP23 mice, which over-express Aβ and exhibit reduced BDNF, with Tau knockout (TauKO) mice. BDNF levels were partially rescued in the APP23xTauKO animals, indicating that tau is an intermediate in Aβ-induced BDNF down-regulation.

These results demonstrate that both soluble Aβ and tau down-regulate BDNF, which likely contributes to learning and memory deficits. Furthermore, the partial rescue of BDNF levels by tau knockout suggests that tau contributes to Aβ-induced BDNF down-regulation. Thus, loss of BDNF may mediate tau neurotoxicity down-stream of Aβ, which has profound implications for therapeutic intervention in Alzheimer's disease and tauopathies, suggesting that current treatments used to alleviate AD symptoms by targeting Aβ pathology alone may not be sufficient, and that combined treatments targeting tau may be required.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES:

LIST OF ABBREVIATIONS:

AChE	Acetylcholinesterase
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
Aβ	Amyloid-beta
BDNF	Brain-derived neurotrophic factor
CaMKII	Ca(2+)/calmodulin-dependent protein kinase II
CBD	Corticobasal degeneration
CBP	CREB binding protein
CREB	Cyclic AMP response element binding protein
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
FTDP-17	Frontotemporal dementia and parkinsonism linked to chromosome 17
GLM	General linear model
GSK3β	Glycogen synthase kinase-3β
HD	Huntington's disease
LDH	Lactate dehydrogenase
LTP	Long-term potentiation
MAPK2	Mitogen-activated protein kinase 2
MCI	Mild-cognitive impairment

MT	Microtubule
NFT	Neurofibrillary tangle
NGF	Nerve growth factor
NSC	Neural stem cell
PBS	Phosphate buffered saline
PD	Parkinson's disease
PiD	Pick's disease
PKA	Protein kinase A
proBDNF	Pro-brain derived neurotrophic factor
proNGF	Pro-nerve growth factor
PS	Presenilin
PSP	Progressive supranuclear palsy
PVDF	Polyvinylidene fluoride
$p75^{NTR}$	Pan-neurotrophin receptor
qRT-PCR	Quantitative Real-time Reverse Transcription-Polymerase Chain Reaction
RA	Retinoic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
TauKO	Tau knockout
TBS	Tris-buffered saline
TrkB	Tropomyosin receptor kinase

CHAPTER 1: INTRODUCTION

1.1 Alzheimer's Disease

1.1.1 Clinical presentation:

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder that is the most common form of dementia among the elderly. AD presents as global cognitive decline with associated memory loss and altered personality. Perhaps the most clinically apparent symptom of AD is the decline of declarative memory, including both episodic and semantic memories (Aronoff et al., 2006). Episodic memory loss is not only an early event, but in mild cognitive impairment (MCI), a preclinical stage of AD, it is associated with an increased risk for conversion to AD (Aggarwal et al., 2005; Devanand et al., 2007). MCI is considered a preclinical stage of AD, since as many as 8% of persons with MCI progress to AD per year (Mitchell and Shiri-Feshki, 2009). Transgenic mouse models of AD are also significantly impaired compared to control animals on tests of episodic-like memory (Davis et al., 2013). In addition, animal models of AD are impaired in long-term potentiation (LTP), a model for synaptic plasticity and a major cellular constituent underlying learning and memory processes. Like episodic memory in humans, the LTP deficit in rodent models of AD occurs early, prior to plaque formation, and is accompanied by deficits in spatial learning and memory (Jacobsen et al., 2006; Liu et al., 2008).

1.1.2 Pathological features

The neuropathological hallmarks of AD include extracellular amyloid-beta (Aβ)-containing plaques and intracellular neurofibrillary tangles (NFTs) formed by hyper-phosphorylated tau protein, resulting in synaptic loss and neurodegeneration (Coleman and Flood, 1987; Coleman and Yao, 2003; Hyman et al., 2012; Hyman and Trojanowski, 1997; McKhann et al., 1984; Scheff and Price, 2003). Neurofibrillary tangles and amyloid-β plaques are both required for the pathological diagnosis of AD (Hyman et al., 2012). AD primarily affects several regions of the brain that are known for their role in learning and memory, namely the basal forebrain, hippocampus, cortex and entorhinal cortex (Coyle J., 1983; Gomez-Isla et al., 1996; Hyman, 1984).

A significant decline in synaptic connections in AD is followed by the pronounced loss of neurons in the basal forebrain, entorhinal cortex, hippocampus and cortex. AD has been characterized as a disconnection of the hippocampus, as projections from the basal forebrain, entorhinal cortex, and cortex to the hippocampus are lost in AD (Hyman, 1984). Among these brain areas, the entorhinal cortex is one of the earliest areas vulnerable to synaptic degeneration and because of its connection with the hippocampus and cortex, entorhinal cortex damage in AD significantly impacts memory deficits (Van Hoesen et al., 1991). In addition, studies have indicated that the memory deficits seen in AD are also directly related to the degree of basal forebrain cholinergic atrophy (Coyle J., 1983). The basal forebrain is the major cholinergic output of the central nervous system, and these neurons as well as their projections to hippocampal and cortical regions are critical for learning, memory and attention (Baxter and Chiba, 1999). The loss of basal

forebrain innervation to these areas is associated with aging and age-related memory loss (Terry and Katzman, 2001; Ypsilanti et al., 2008). In fact, the major drugs for AD on the market today are cholinergic enhancers.

Several theories exist to explain the molecular mechanisms that could lead to this devastating disease. One such theory (the amyloid cascade hypothesis) suggests that it is the accumulation of toxic Aβ that is the upstream driving force behind subsequent AD neuropathology, and that other pathological correlates are a result of an imbalance between Aβ production and clearance (Hardy and Selkoe, 2002; Iqbal and Grundke-Iqbal, 2008). Nevertheless, amyloid plaques do not correlate well with cognitive decline; neurofibrillary tangles correlate with dementia better than amyloid plaques (Guillozet A.L., 2003). However, it is the loss of functional synapses in AD that correlates most strongly with loss of cognitive abilities (Terry et al., 1991).

In addition to neurodegeneration, there may be a loss of functional neurogenesis associated with AD. In many different AD transgenic mouse models, significantly less proliferation of new neurons is present in the hippocampal formation compared to control animals (Mu and Gage, 2011). Without the generation of new neurons, the synaptic degeneration resulting from AD is not compensated and learning and memory are impaired. The case is not so clear in human hippocampus however, where immunostaining with different markers of immature neurons suggests that there is either increased (Jin et al., 2004), unchanged (Boekhoorn et al., 2006), or decreased (Crews et al., 2010) neurogenesis in AD. More research is needed to determine whether reduced hippocampal neurogenesis contributes significantly to memory loss in AD. In addition, a

close examination of the molecular mechanisms required for learning and memory and how they are disrupted in AD is essential.

1.2 Molecular pathway for learning and memory:

A molecular pathway essential for learning and memory that is disrupted in AD involves the transcription factor cAMP response element binding protein (CREB) (Barco et al., 2003). Aβ interferes with hippocampal LTP via signaling pathways including the Ca(2+)-dependent protein phosphatase calcineurin, Ca(2+)/calmodulin-dependent protein kinase II (CaMKII), and CREB (Yamin, 2009). CREB transcription is decreased in AD and *in vitro* following treatment with Aβ (Pugazhenthi et al., 2011). Further active, phosphorylated CREB is reduced in the AD brain (Yamamoto-Sasaki et al., 1999) and is also decreased in neurons following treatment with Aβ *in vitro* (Garzon and Fahnestock, 2007; Tong et al., 2001). CREB phosphorylation recruits transcriptional coactivators, which are required for the transcription of genes involved in learning and memory. Aβ-induced suppression of transcription induced by the CREB coactivator CRCT1 has been demonstrated in transgenic AD mice, and this is mediated by blockade of L-type voltage-gated calcium channels, reduced calcium influx and disruption of PP2B/calcineurin-dependent CRTC1 dephosphorylation (Espana et al., 2010). Restoration of CREB activity in AD transgenic mice by inducing expression of another coactivator, CREB binding protein (CBP), ameliorates learning and memory deficits (Caccamo et al., 2010). Among the genes induced by CREB and its coactivators that are required for learning and memory, the most important for AD may be brain-derived neurotrophic factor (BDNF).

1.3 Brain-derived neurotrophic factor (BDNF)

Brain-derived neurotrophic factor (BDNF) was initially purified in 1982 (Barde et al., 1982), making it the second neurotrophin to be discovered following the purification and description of nerve growth factor (NGF) (Levi-Montalcini and Hamburger, 1951). In the seminal research paper, BDNF was purified from pig brain and found to support the survival and fibre outgrowth from embryonic chick sensory neurons in culture (Barde et al., 1982). Since its initial discovery, BDNF is now considered to be the most synaptogenic protein known and has been shown to support the survival of neurons and their connections that are vulnerable in aging and diseases of the aging brain, such as AD (Alderson et al., 1990; Knusel et al., 1991). Further, BDNF plays a critical role in neuronal survival, differentiation and learning and memory, making it an extremely important molecule to investigate when considering toxic mechanisms in AD.

1.3.1 BDNF structure and transcripts

In order to investigate BDNF regulation in AD, the complicated structure of BDNF must be understood. The human BDNF gene is 70kb long and consists of 11 exons and 9 functional promoters, which by alternative splicing result in at least 17 different BDNF transcripts (Pruunsild et al., 2007) (Figure 1). This variation in transcript expression allows for tissue-specific regulation of BDNF in response to a variety of developmental and environmental cues (Pruunsild et al., 2007; Timmusk et al., 1993). BDNF transcripts II, III, IV, V and VII are exclusively localized to the brain, while other

BDNF transcripts are expressed in neuronal as well as non-neuronal tissues (Pruunsild et al., 2007). Within the brain, BDNF transcript IV accounts for approximately half of the total BDNF mRNA found in the cortex (Garzon and Fahnestock, 2007). Further, BDNF transcript IV is regulated at least in part by CREB (Pruunsild et al., 2007; Shieh et al., 1998; Timmusk et al., 1995).

BDNF protein is synthesized as a 36kDa precursor, proBDNF, which can be cleaved by enzymes including plasmin, furin and matrix metalloproteases to produce 14kDa mature BDNF (Lee et al., 2001; Lu et al., 2005; Mowla et al., 2001). ProBDNF is expressed widely in the human brain, where it is only partially cleaved to its mature form. Both BDNF and proBDNF are found in relatively high amounts in the hippocampus, cortex, and basal forebrain (Michalski and Fahnestock, 2003), areas that are particularly vulnerable in AD.

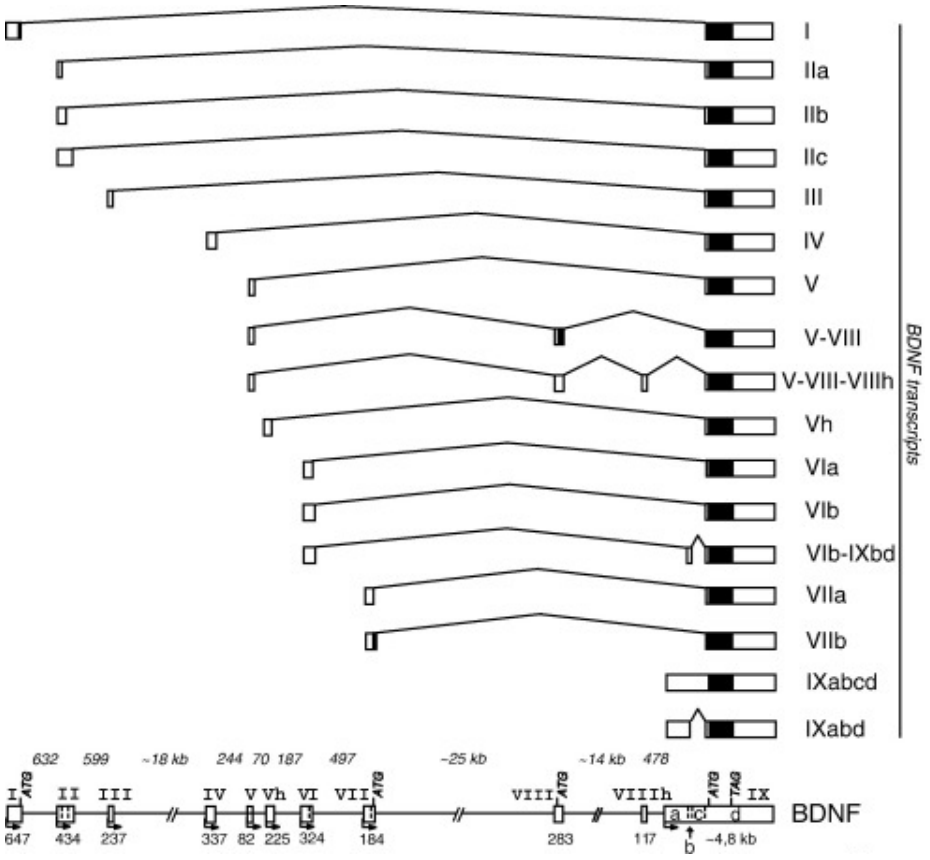


Figure 1: Human BDNF structure. BDNF has a complicated structure whereby alternative splicing results in at least 17 different BDNF transcripts. Filled boxes here represent translated regions of the exons, and unfilled boxes represent untranslated regions. BDNF transcripts are named according to the upstream exons present (Figure from Pruunsild et al., 2007).

1.3.2 BDNF function: Neuronal survival, neurogenesis & dendritic modulation

BDNF supports the survival of neurons and their connections that are vulnerable in AD, such as the hippocampus, entorhinal cortex, neocortex and basal forebrain (Alderson et al., 1990; Ghosh et al., 1994; Hyman, 1984; Knusel et al., 1991; Lindholm et al., 1996; Lowenstein and Arsenault, 1996). Early studies demonstrated a more than 2-fold increase in survival of septal cholinergic neurons following BDNF treatment as measured by acetylcholinesterase (AChE) histochemical staining (Alderson et al., 1990). Further, blocking BDNF signaling either using antibodies or BDNF gene knockdown decreases survival of cortical (Ghosh et al., 1994), hippocampal (Lindholm et al., 1996) and dorsal root ganglion neurons (Korte et al., 1995). Although these results demonstrate that BDNF is a survival-promoting factor for critical neuronal populations during development, conditional knockdown of BDNF in the adult suggests that BDNF is not required for postmitotic neuronal survival, but rather for dendritic maturation and growth of specific neuronal populations such as medium spiny neurons of the striatum (Rauskolb et al., 2010). These results suggest that decreases in BDNF may trigger altered spine morphology, reduced dendritic complexity, synaptic dysfunction and degeneration but not outright neuronal loss.

BDNF also promotes neurogenesis. Direct infusion of BDNF into rat hippocampus increases the number of developing neurons compared to saline injections in both the ipsilateral and contralateral hemispheres, suggesting a widespread effect (Scharfman et al., 2005). This hippocampal neurogenesis is directly related to subsequent cognitive functions, as new neurons are integrated into neural circuitry and play an

important role in specific learning and memory tasks such as learning in the Morris water maze, a spatial memory task (Zhao et al., 2008). Studies investigating the exact molecular mechanisms underlying BDNF-mediated increases in neurogenesis are still underway; however, the activation of CREB is associated with hippocampal neurogenesis, as phosphorylated CREB is consistently found in newborn hippocampal neurons (Jagasia et al., 2009; Nakagawa et al., 2002a; Nakagawa et al., 2002b). Further, BDNF is known to activate CREB by signaling through the TrkB receptor via the PI3K/Akt, Ras/ERK and PLCγ pathways (Blanquet and Lamour, 1997; Finkbeiner et al., 1997; Gonzalez and Montminy, 1989; Minichiello et al., 2002; Pizzorusso et al., 2000). Together, these findings support the possibility that BDNF promotes neurogenesis via mechanisms that rely on its ability to activate the neurogenesis-promoting transcription factor CREB.

BDNF also regulates dendritic growth, branching and spine maturation by a balance between mature BDNF and its precursor form, proBDNF. Both mature BDNF and proBDNF signal through the TrkB receptor and the pan-neurotrophin receptor $p75^{NTR}$, however mature BDNF has a greater affinity for TrkB while proBDNF preferentially binds to $p75^{NTR}$ (Fayard et al., 2005; Teng et al., 2005) (Figure 2). Signaling through TrkB promotes dendritic growth and spine maturation via activation of the PI3K/Akt/mTOR signaling pathway, a pathway long associated with axonal growth and dendritic complexity (Atwal et al., 2000; Kumar et al., 2005; Kuruvilla et al., 2000; Markus et al., 2002). Therefore, the preferential binding of BDNF to TrkB promotes synaptic development through increased dendritic growth and spine maturation. Conversely, proBDNF inhibits neurite outgrowth through its preferential binding to

$p75^{NTR}$, which negatively alters dendrite and spine morphology in addition to spine density (Koshimizu et al., 2009; Singh et al., 2008; Yamashita et al., 1999; Zagrebelsky et al., 2005). $p75^{NTR}$ reduces dendritic morphology through activation of a Rho-GTPase, RhoA (Sun et al., 2012), by releasing Rho from its inactive Rho-GDI (Yamashita and Tohyama, 2003). These findings suggest that the balance between proBDNF and BDNF plays a critical role in the regulation of spine maturation, dendritic growth and synaptic density, which underlie learning and memory processes.

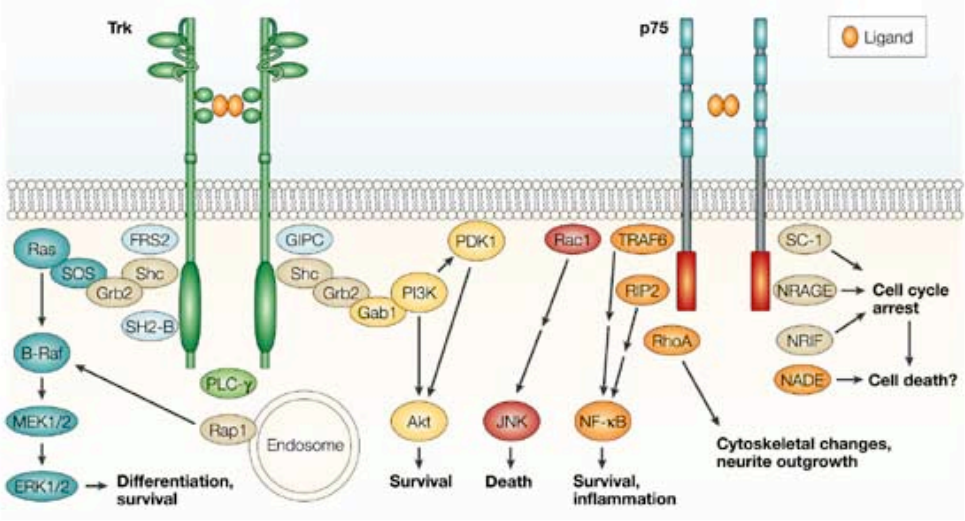


Figure 2: Mature BDNF/proBDNF signaling pathways. Mature BDNF preferentially binds to TrkB, which activates three signaling cascades upstream of CREB to promote differentiation and survival: the PLCγ/CAMKII, PI3K/AKT and Ras/MAPK pathways. Conversely, proBDNF preferentially binds $p75^{NTR}$, which promotes either apoptosis via the JNK pathway, cell survival via the NF-κB pathway or reduces dendritic morphology via activation of RhoA. (Figure from Chao, 2003).

1.3.3 BDNF and learning & memory

Multiple studies have shown that decreases in BDNF such as in heterozygous BDNF knockout mice or BDNF knockdown result in learning and memory deficits (Gorski et al., 2003; Heldt et al., 2007; Linnarsson et al., 1997). A Val66Met polymorphism in the pro-domain of proBDNF, which interferes with BDNF secretion, results in episodic memory impairments and reduced hippocampal volume, key endophenotypes of AD (Egan et al., 2003; Hariri et al., 2003). This finding shows that an alteration in BDNF levels is sufficient to affect hippocampal activity and that BDNF is critically involved in hippocampal learning and memory processes.

The expression of BDNF is also strongly correlated with cognitive status. BDNF mRNA and proBDNF and mature BDNF protein are decreased in the entorhinal, frontal, temporal and parietal cortices as well as the hippocampus of AD subjects compared to controls (Amoureux et al., 1997; Connor et al., 1997; Fahnestock et al., 2002; Ferrer et al., 1999; Hock et al., 2000; Holsinger et al., 2000; Michalski and Fahnestock, 2003; Murray and Lynch, 1998; Narisawa-Saito et al., 1996; Peng et al., 2009; Peng et al., 2005; Phillips et al., 1991). Further, BDNF decreases with increasing age in the cognitively impaired primate brain (Hayashi et al., 1997). In addition, in human post-mortem parietal cortex, lower levels of both mature BDNF and proBDNF protein correlate with decreased cognitive test scores as measured by the Mini Mental State Examination (MMSE) and Global Cognitive Score (GCS) (Peng et al., 2005).

1.4 BDNF down-regulation in Alzheimer's disease & tauopathies

1.4.1 BDNF loss is an early event in the progression of AD

Early in the progression of AD, BDNF levels are diminished. This loss of BDNF occurs prior to plaque and tangle deposition in transgenic mice and coincides with memory deficits (Francis et al., 2012). We now know that amyloid plaques correlate poorly with cognition, and further evidence has suggested that the cause of cognitive decline in AD precedes Aβ pathology (Hanna et al., 2009; Van Dam et al., 2003; Westerman et al., 2002). This has been supported in transgenic mouse models of AD, where deficits in novel object recognition and spontaneous alternation performance in a "Y" maze occurred prior to the deposition of Aβ plaques (Francis et al., 2012; Holcomb et al., 1998). To explain the early deficit in learning in memory, prior to AD pathology, it has been found that BDNF is lost early in the progression of AD, as demonstrated by a significant decline in BDNF in individuals with MCI. While MCI corresponds with the initiation of Aβ and tau aggregation, the distribution of amyloid deposits are thought to be intermediate between that of non-cognitively impaired individuals and those with AD (Markesbery 2010). However, more than a 30% reduction in cortical BDNF expression was found in MCI subjects compared to age- and gender-matched, non-cognitively impaired controls (Peng et al., 2005), an expression nearly as low as in AD subjects. Further, BDNF mRNA levels are significantly decreased and correlated with cognitive status in aged, cognitively impaired canines (Fahnestock et al., 2012), which like humans, develop cognitive impairment and amyloid deposition as they age and are an excellent model of MCI (Cotman and Berchtold, 2002; Cotman and Head, 2008). This result

supports human findings that BDNF loss coincides with memory impairments. Thus, in both animal models and humans, BDNF is lost early, coinciding with memory loss and prior to the appearance of significant plaque deposition typical of AD.

1.4.2 BDNF transcript-specific and protein decreases in AD:

BDNF mRNA expression in the hippocampus is decreased nearly 2-fold in individuals with AD (Phillips et al., 1991), and BDNF mRNA is reduced approximately 3-fold in the AD parietal cortex (Garzon et al., 2002; Yamashita et al., 1999). This decrease in BDNF mRNA is due to a decrease specifically in four BDNF transcripts: I, II, IV & VI (Garzon et al., 2002). Of particular interest is transcript IV, since it is the most prevalent transcript in human cortex (Garzon and Fahnestock, 2007) and is specifically decreased in human and mouse models of AD (Garzon et al., 2002; Peng et al., 2009).

The decreased BDNF mRNA in AD corresponds to a similar decrease in BDNF protein. Mature BDNF protein is significantly reduced in AD hippocampus (Connor et al., 1997; Hock et al., 2000; Narisawa-Saito et al., 1996), frontal cortex (Ferrer et al., 1999), temporal cortex (Connor et al., 1997) and parietal cortex (Hock et al., 2000; Peng et al., 2005) compared to controls, and proBDNF protein is reduced in AD parietal cortex (Michalski and Fahnestock, 2003; Peng et al., 2005).

1.4.3 Increasing BDNF levels can counteract memory impairments:

The strong correlation between lowered BDNF and diminished cognition, learning and memory makes the possibility of rescuing these behavioral symptoms of AD with

BDNF an enticing undertaking. Knocking down BDNF in mice results in loss of synapses, learning and memory deficits and diminished LTP, whereas BDNF administration rescues or increases LTP and synapses and subsequently restores learning and memory (Blurton-Jones et al., 2009; Korte et al., 1995; Nagahara et al., 2009; Patterson et al., 1996). Early studies substantiated findings that BDNF is necessary for LTP, as mice with a deletion in the BDNF gene exhibited significantly weakened LTP expression (Korte et al., 1995). Treating hippocampal BDNF knockout tissue with recombinant BDNF completely restored LTP (Patterson et al., 1996). Similarly, knockdown of the BDNF receptor TrkB can also significantly reduce hippocampal LTP (Minichiello et al., 1999). These early studies led to efforts in recent years to exogenously deliver BDNF as a method of restoring cognitive abilities in animal models of AD. However, expression of truncated TrkB receptors in the ventricular ependyma effectively prevents diffusion of BDNF to target tissues when it is administered by intracerebroventricular infusion (Anderson et al., 1995). Furthermore, BDNF does not readily cross the blood-brain-barrier. Fusions of BDNF with factors that allow it to be transferred across the blood-brain barrier such as an antibody to the transferrin receptor (Zhang and Pardridge, 2001; Zhang and Pardridge, 2006) or to the insulin receptor (Boado et al., 2007) have facilitated entry of intravenously administered BDNF into brain tissue.

The brain, however, is exquisitely sensitive to BDNF levels, which presents another significant barrier to the successful use of this trophic factor *in vivo*. Microinjections of microgram levels of BDNF into the hippocampus increases neuronal

excitability and can even cause spontaneous seizures, whereas chronic infusions of similar amounts will decrease the TrkB receptor and TrkB activation, resulting in loss of responsiveness to BDNF (Scharfman, 1997; Xu et al., 2004). Further, delivery of BDNF can result in impairment of spatial learning rather than improvement if BDNF expression is too high (Pietropaolo et al., 2007). Therefore, more physiological delivery of low doses of BDNF via viral vectors has been extensively investigated.

In the entorhinal cortex-lesioned aged rat, BDNF gene delivery enhanced LTP and partially restored cognitive function (Ando et al., 2002). BDNF gene delivery via lentiviral injection to the entorhinal cortex in a transgenic mouse model of AD not only increased synaptic density (synaptophysin-IR) and synaptic function (p-Erk signaling), but importantly, improved spatial memory performance in the Morris water maze task. BDNF treatment did not affect neuronal number in this model, although it did rescue neurons from cell loss in a perforant path transection model. BDNF administration also exerted similar effects and rescued learning and memory in the aged, cognitively impaired non-human primate (Nagahara et al., 2009). Further, this expression system has been used to maintain delivery of nerve growth factor (NGF) for up to 1 year in primate basal forebrain, eliminating transient expression as a disadvantage of the system. These experiments were carried out after the onset of disease symptoms in the transgenic mice and after cognitive impairment was demonstrated in aged primates, suggesting that BDNF may be an effective therapy for reversal of cognitive deficits as well as prevention. However, this delivery method suffers from poor viral diffusion within the parenchyma. This complication is somewhat ameliorated by the implantation of neural stem cells

(NSC) which can migrate within brain parenchyma. NSC delivery into hippocampus of the triple-transgenic (3xTg-AD) mouse model of AD increased synaptic density and improved hippocampal-dependent learning and memory (Blurton-Jones et al., 2009). BDNF knockdown in these NSCs eliminated the beneficial effects of the cells, demonstrating that BDNF secreted by the NSCs was the active factor (Blurton-Jones et al., 2009).

Another approach, viral delivery of CREB activators (Caccamo et al., 2010; Espana et al., 2010), has successfully reversed synaptic atrophy and learning and memory impairments in transgenic mice. Alternative approaches to gene delivery include methods of increasing endogenous BDNF levels or signaling. For instance, rolipram, a phosphodiesterase inhibitor and CREB activator, can increase BDNF expression (DeMarch et al., 2008) and therefore may have therapeutic effects in AD. Additionally, small molecule BDNF mimetics or TrkB agonists, which promote signalling through the TrkB receptor, can restore synaptic plasticity and cognitive function (Massa et al., 2010). Further, natural products and their derivatives such as certain flavonoids are neuroprotective and can enhance LTP and improve memory by activating CREB, TrkB and its downstream signaling pathways PI3K/Akt and MAPK/Erk (Jang et al., 2010; Maher et al., 2006). Finally, lifestyle adjustments such as exercise, environmental enrichment and dietary restriction have enhanced BDNF expression in both animal models and human studies (Mattson et al., 2004). An enriched environment has been shown to increase BDNF mRNA in rats (Falkenberg et al., 1992), while an exercise routine can regulate transcript-specific BDNF mRNA and protein (Berchtold et al., 2002;

Cotman and Berchtold, 2002; Zajac et al., 2010). Further, the combination of an antioxidant-rich diet and environmental enrichment has been shown to significantly increase BDNF expression which was correlated with an improvement in memory scores in old, cognitively impaired canines (Fahnestock et al., 2012). Although a variety of molecular, pharmacological and lifestyle interventions are under investigation and may pave the way for future clinical trials in subjects with AD, increasing brain BDNF levels safely and effectively is not yet a reality. A greater understanding of the molecular mechanisms in AD that result in reduced BDNF expression is required to more effectively counteract the detrimental down-regulation of BDNF that occurs. As such, the contribution of the main neuropathological components of AD, amyloid-β and tau, must be closely examined in order to understand the down-regulation of BDNF in AD.

1.5 Amyloid-beta

Although Aβ has been recognized as having non-disease associations, such as acting as an anti-microbial agent (Soscia et al., 2010), it is most well studied as the precipitating insult in AD. Early studies suggested that it was the formation of Aβ plaques that was the primary neurotoxic insult in AD. However, plaques do not correlate well with decreased cognition, and more recent evidence suggests that soluble forms of Aβ may be more toxic (Ferreira et al., 2007; Garzon and Fahnestock, 2007; Hardy and Selkoe, 2002; Lacor et al., 2007; Walsh and Selkoe, 2007).

1.5.1 Formation of toxic amyloid-β

Amyloid-β is a peptide formed by the proteolytic processing of the amyloid precursor protein (APP) (Citron et al., 1992; Haass et al., 1992; Hardy and Selkoe, 2002; Shoji et al., 1992). APP is an integral membrane protein with a small intracellular C-terminus and a large extracellular segment, with the Aβ sequence contained mostly in the extracellular segment and partially in the intramembrane sequence (Mattson et al., 2004). APP is initially proteolytically cleaved by α- or β-secretase (also known as beta-site APP cleaving enzyme 1; BACE-1), which releases a soluble fragment of APP (De Strooper and Annaert, 2000; Lahiri and Maloney, 2010). Following primary cleavage by β- or α-secretase, a secondary cleavage of the trans-membrane domain, or C-terminal fragment, of APP by γ-secretase results in either the formation of Aβ peptide or the p3 fragment of APP, respectively (De Strooper and Annaert, 2000; Haass et al., 1992) (Figure 3). Gamma-secretase is a complex of integral membrane proteins that includes presenilin-1 (PS-1) or presenilin-2 (PS-2), which when mutated can dramatically alter the production of Aβ. Gamma-secretase cleavage following cleavage by β-secretase can result in Aβ peptides of various lengths (De Strooper and Annaert, 2000; Haass et al., 1992). $A\beta_{1\text{-}40}$ is most commonly produced following the successive cleavage of β- and γ-secretases, however it is $A\beta_{1\text{-}42}$ that is of particular interest in the progression of AD because of its greater propensity to aggregate (Glabe, 2001; Tabaton et al., 2010). The aggregation of Aβ involves several conformational states from dimers to high-molecular-weight oligomers and protofibrils and finally insoluble Aβ fibrils (Glabe, 2004) and requires a

structural change from an α-helical conformation to a more organized β-sheet configuration characteristic of Aβ aggregation (Xu et al., 2005).

Formation of soluble Aβ is increased by mutations in APP, PS-1 and PS-2, which are hallmarks of familial forms of AD (Citron et al., 1992; Haass et al., 1994; Hutton and Hardy, 1997; Scheuner et al., 1996). Such mutations result either in Aβ over-production or in an increase in the aggregation-prone $A\beta_{42}$ (Burdick et al., 1992; Hardy and Selkoe, 2002; Jarrett et al., 1993; Selkoe, 1994). Increased levels of Aβ increase aggregation and toxicity prior to plaque deposition. Levels of soluble Aβ oligomers in the frontal cortex of individuals with AD are up to 70-fold higher than in control brains (Gong et al., 2003).

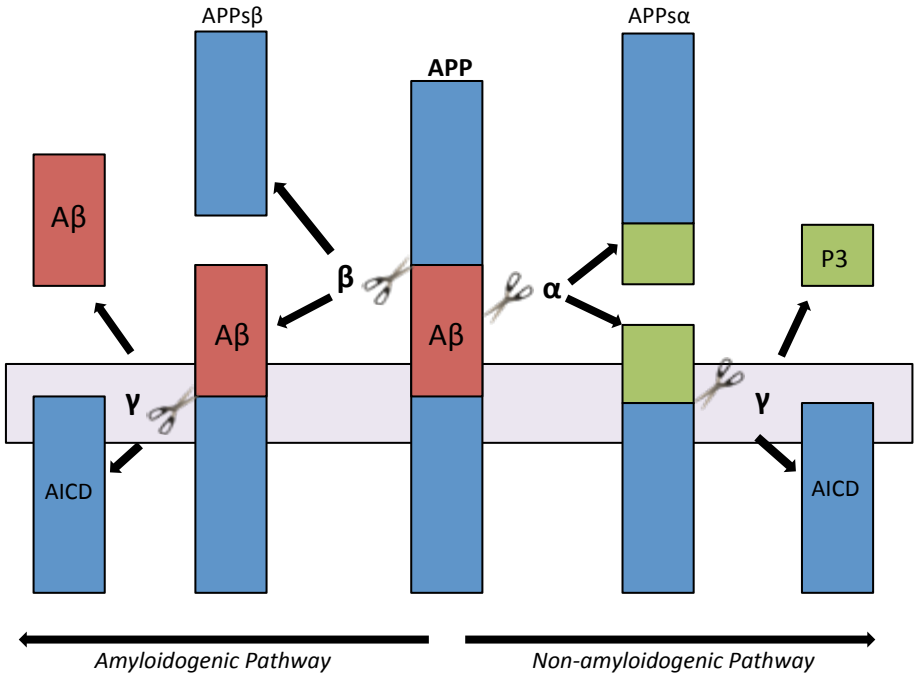


Figure 3: Proteolytic processing of amyloid precursor protein. Initially, amyloid precursor protein (APP) is proteolytically cleaved by either α-secretase or β-secretase followed by a secondary cleavage by γ-secretase to release either soluble amyloid-β or the p3 fragment of APP resulting in dissociation of the APP intracellular domain (AICD). The cleavage of β-secretase followed by γ-secretase is referred to as the "amyloidogenic pathway" as it promotes the accumulation of toxic amyloid-β. Conversely, cleavage by α-secretase followed by γ-secretase is referred to as the "non-amyloidogenic pathway". *Modified figure* (Dislich and Lichtenthaler, 2012).

1.5.2 Amyloid-beta toxicity

Amyloid-β has many proposed toxic effects that could result in the neuronal dysfunction and neurodegeneration characteristic of AD. One well-studied toxic effect of Aβ accumulation is altered calcium homeostasis. Aβ oligomers have been shown to alter calcium regulation by modulating ion channels, including voltage-gated calcium and potassium channels, nicotinic and NMDA receptors, and by forming its own calcium-conducting pores, thereby increasing levels of cytosolic calcium (Demuro et al., 2005; Ferreiro et al., 2006; Green et al., 2007; Mattson et al., 1993; Resende et al., 2007). Further, Aβ has also been shown to promote increased reactive oxygen species (ROS) production in primary cortical and hippocampal neurons (De Felice, 2007; Sponne et al., 2003), which has been associated with inflammation and neurodegeneration. Amyloid-β may also indirectly induce neurotoxicity through the activation of pro-inflammatory responses (Akiyama et al., 2000; Giovannini et al., 2002; Lukiw and Bazan, 2000), which may have a role in inhibiting hippocampal memory formation (Heneka and O'Banion, 2007; Murray and Lynch, 1998; Tancredi et al., 2000; Tancredi et al., 1992). Further, the APP intracellular domain (AICD) has proposed toxicity resulting from its modulation of intracellular calcium homoestasis (Hamid et al., 2007), and cell death (Nakayama et al., 2008; Passer et al., 2000; Vazquez et al., 2009). However, Aβ has the most direct effect on synaptic degeneration is via its modulation of specific signalling cascades.

1.6 Aβ alters signalling pathways upstream of BDNF:

Aβ toxicity results in neurodegeneration by altering signalling pathways upstream and downstream of CREB. CREB phosphorylation and signalling are reduced by exposure to soluble, oligomeric Aβ *in vitro* (Garzon and Fahnestock, 2007; Tong et al., 2001). Aβ treatment inhibits the Ras/ERK and PI3K/AKT signalling pathways (Tong et al., 2004), which are downstream of BDNF/TrkB but are also essential for the phosphorylation of CREB and subsequent expression of BDNF. It has been shown that inhibiting glycogen synthase-3β (GSK3β), which can be activated by Aβ, is an effective way to decrease inhibitory phosphorylation of CREB and increase BDNF protein (DaRocha-Souto et al., 2012). Additionally, Aβ has been shown to inactivate PKA *in vitro,* and increasing PKA activity reverses decreased CREB activation induced by Aβ treatment (Vitolo et al., 2002). Therefore, Aβ disrupts several kinase cascades, leading to a reduction in activity of CREB and in BDNF expression (Figure 4).

Specifically, soluble oligomeric Aβ, but not the fibrillar Aβ found in plaques, decreases CREB activation and down-regulates BDNF *in vitro*, largely via transcript IV (Garzon and Fahnestock, 2007). In transgenic mouse models of AD, which also exhibit down-regulation of transcript IV, BDNF expression is inversely proportional to the amounts of soluble, high-molecular-weight Aβ oligomers (Peng et al., 2009). Thus, inactivation of CREB by soluble, aggregated Aβ may down-regulate BDNF transcript IV, resulting in the diminished synaptic connections and memory loss characteristic of AD.

A major consequence of Aβ-induced CREB reduction that is mediated by BDNF is diminished LTP. Soluble Aβ oligomers have been shown to effectively inhibit

hippocampal LTP (Lambert et al., 1998; Walsh et al., 2002). Aβ inhibits LTP by specifically interfering with CaMKII and CREB signalling pathways (Yamin, 2009), and Aβ-induced reductions in LTP are rescued by BDNF (Zheng et al., 2010). These effects on LTP suggest that Aβ interferes with learning and memory by similar mechanisms. Therefore, although Aβ has many proposed mechanisms of toxicity, its modulation of kinase pathways affecting transcription of genes involved in learning and memory, particularly BDNF and CREB, is perhaps the most significant for AD.

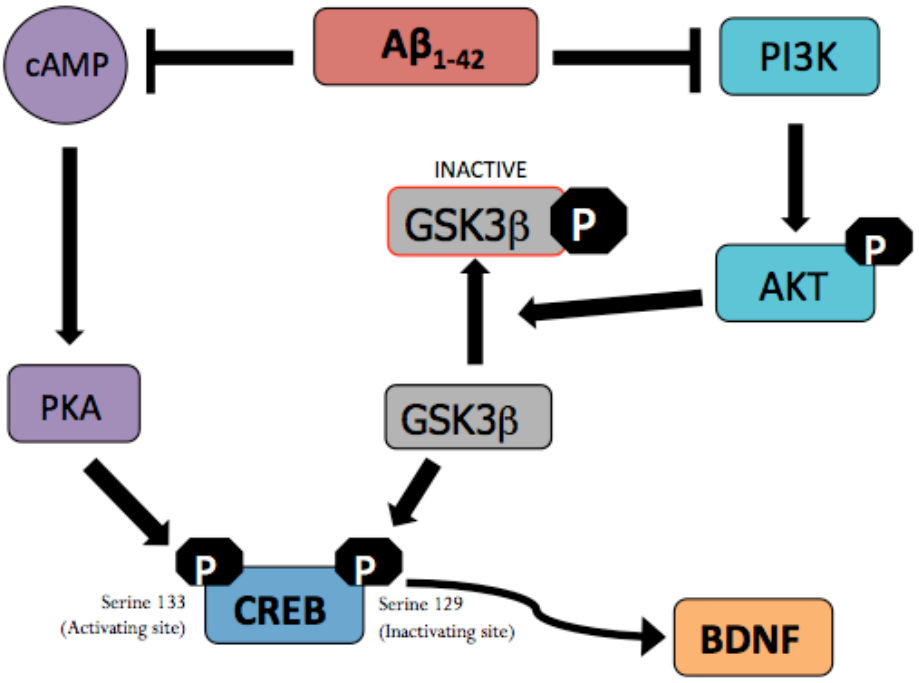


Figure 4: Aβ-induced inactivation of CREB via phosphorylation. Aβ has been shown to inhibit the activation of CREB via phosphorylation both by inhibiting PKA (Vitolo et al., 2002) and activating GSK3β (DaRocha-Souto et al., 2012), possibly via inactivation of the PI3K/AKT pathway (Tong et al., 2004). As such this pathway represents a possible mechanism of Aβ-induced BDNF down-regulation, whereby CREB activity is inhibited via phosphorylation.

1.7 Amyloid cascade hypothesis

The predominant theory to explain neuropathology in Alzheimer's disease is the amyloid cascade hypothesis. This hypothesis essentially states that Aβ is the upstream driving force behind subsequent AD pathology and synaptic degeneration and that all other pathological correlates of AD result from an imbalance of Aβ production and clearance (Hardy and Selkoe, 2002; Iqbal and Grundke-Iqbal, 2008; Selkoe, 1994). As such, tau pathology characteristic of AD is thought to be down-stream of Aβ and therefore possibly a more direct mediator of neurodegeneration (Ittner et al., 2010; Lewis et al., 2001; Masliah et al., 2001; Pennanen and Gotz, 2005). Specifically, it has been shown that Aβ enhances tau hyperphosphorylation and the subsequent formation of neurofibrillary tangles in animal models (Gotz, 2001; Lewis et al., 2001; Masliah et al., 2001; Murray and Lynch, 1998) and in human neuroblastoma cells *in vitro* (Pennanen and Gotz, 2005). This suggests that Aβ may exert its neurotoxic effects via tau hyperphosphorylation and subsequent neurofibrillary tangle formation (Iqbal and Grundke-Iqbal, 2008). Furthermore, Aβ-induced neurodegeneration is prevented in primary neuronal cultures from TauKO mice (Rapoport et al., 2002), and knocking out tau in a transgenic AD mouse model can block Aβ-induced cognitive impairments (Roberson et al., 2007). Whether this rescue of cognitive impairment was mediated by BDNF was not tested. These findings taken together, suggest that tau may be a more direct mediator of toxicity in AD and that further investigation into tau structure, function and pathology are required.

1.8 Tau

Although the primary insult in AD may be Aβ over-expression, ultimately cognitive dysfunction and neurodegeneration are a result of alterations in tau (Gotz, 2001; Iqbal and Grundke-Iqbal, 2008; Lewis et al., 2001; Masliah et al., 2001; Pennanen and Gotz, 2005; Roberson et al., 2007). However, the mechansims that lead from alterations in tau to the physical symptoms of AD are not clear.

1.8.1 Tauopathies

In addition to AD, non-Alzheimer's disease tauopathies, such as frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), Pick's disease (PiD), corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP) are also characterized by intracellular accumulations of tau protein (Crowther and Goedert, 2000; Goedert and Spillantini, 2011; Iwatsubo et al., 1994; Lee et al., 2001; Pollock et al., 1986). While the phenotypic manifestation of specific cell types and brain areas affected vary among tauopathies, the presence of accumulated tau protein in the absence of other pathological hallmarks substantiates the toxicity of tau independent of Aβ. The phenotypic expression of tauopathies vary a great deal because the regional distribution of tau aggregation within the brain is different between these diseases, however they each present with hyperphosphorylation of tau and a change in the ratio of tau isoforms (Lee et al., 2001). This suggests that an increased understanding of tau structure and both physiological and pathological function could lend great support to therapeutically intervening with a number of devastating neurodegenerative diseases.

1.8.2 Tau structure and function

The human tau gene is over 110kb long and is found on the long arm of chromosome 17 at position 17q21. The tau gene contains 16 exons, and in the human adult brain exons 2, 3 and 10 are alternatively spliced (Andreadis et al., 1992). Exons 2 and 3 are always spliced together, while exons 2 and 10 can be found independently. Therefore, splicing of these exons results in six possible tau isoforms (Figure 5). These tau isoforms vary in size by the insertion of 29 or 58 amino acids (corresponding to exon 2 or exon 2+3; also known as 1N or 2N tau) at the N-terminus and by having either three or four repeat-regions (3R or 4R) in the microtubule-binding domain of the C-terminal sequence (Buee et al., 2000). In a healthy adult the ratio of 3R:4R tau isoforms is approximately 1:1 in most regions of the brain (Goedert and Jakes, 1990; Kosik et al., 1989; Lee et al., 2001). Deviations from this ratio could become problematic and is a recognized characteristic of neurodegenerative tauopathies. The complexity of tau is furthered by its propensity to be truncated in disease states. This truncated form of tau is found in AD and is thought to increase tau hyperphosphorylation and aggregation (Hrnkova et al., 2007). Therefore, there seems to be a functional difference between various tau alterations. However, specifically how different forms of tau change its normal functioning and dictate disease progression remains largely unclear.

The most well-known function of tau, as a protein that is most abundant in the axons of neurons (Binder et al., 1985), is its ability to bind to and stabilize microtubules (MTs). However, alterations in tau caused by a number of post-translational modifications can alter tau structure and subsequently its function (Carrell and Gooptu,

1998; Grundke-Iqbal et al., 1986; Kuhla et al., 2007; Min et al., 2010; Mondragon-Rodriguez et al., 2008; Perry et al., 1989; Reynolds et al., 2005). These post-translational modifications can also cause a detrimental missorting of soluble tau to the somatodendritic compartment. Which modifications dictate this intracellular trafficking and which tau isoforms are capable of being mislocalized is poorly understood.

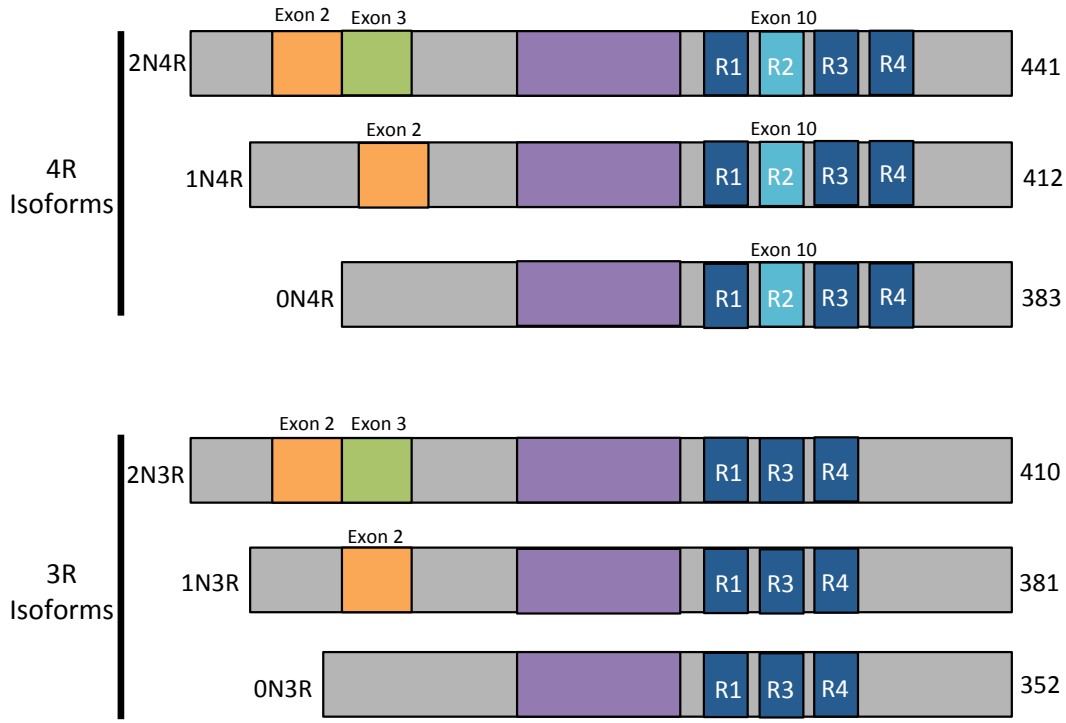


Figure 5: Structural organization of tau isoforms. The six predominant tau isoforms vary by the presence or absence of exon 2 or exon 2+3 in the N terminus and exon 10 in the microtubule-binding domain. The microtubule-binding domain consists of either 3 or 4 repeat domains, depending on the presence or absence of exon 10. The longest isoform contains both exons 2 and 3 in the N terminus and exon 10 and is therefore denoted 2N4R, whereas the shortest isoform lacks exons 2, 3 and 10 and is denoted 0N3R. *Modified figure* (Johnson and Stoothoff, 2004).

1.8.3 Pathological tau modifications

Phosphorylation of tau is thought to be the most relevant and impactful post-translational modification, decreasing its ability to promote microtubule polymerization (Lindwall and Cole, 1984) and to aggregate into toxic species, effectively altering its function (Hernandez and Avila, 2007). There are nearly 80 serine/threonine tau phosphorylation sites identified to date (Kolarova et al., 2012), and phosphorylation is regulated by the coordinate action of kinases and phosphatases. Hyperphosphorylation of tau causes diminished tubulin binding of tau, allowing the free tau proteins to self-polymerize and aggregate (Avila et al., 2008; Iqbal and Grundke-Iqbal, 2008). Most of the phosphorylation sites of tau are located in or near the microtubule (MT)-binding domain (Buee et al., 2000; Kolarova et al., 2012; Sergeant et al., 2008) and have an increased propensity to be phosphorylated in disease (Figure 6). For example, the AT8, AT100 and AT180 phospho-epitopes, located near the MT-binding domain of tau, experience increased phosphorylation in tauopathies. The abnormal phosphorylation of these sites and others could be a result of tau kinase up-regulation and/or tau phosphatase down-regulation (Buee et al., 2000; Trojanowski and Lee, 2005). Further, hyperphosphorylation of tau leading to tubulin disengagement results in somatodendritic mislocalization of tau from the axons (Delacourte and Buee, 2000; Goedert, 2004).

The somatodendritic mislocalization of tau is one of the earliest predictors of neurodegeneration. However, this intracellular trafficking of tau is poorly understood. It is known that the misprocessing and trafficking of tau involves tau that is not associated with MTs (Lee et al., 2012), which suggests that hyperphosphorylation is a critical step in

tau missorting. It is also known that tau can be trafficked by slow axonal transport (Mercken et al., 1995). Further, exposure to exogenous tau can result in dose-dependent changes in tau distribution and phosphorylation (Lee et al., 2012; Thies and Mandelkow, 2007) supporting the idea that tau will be trafficked to MT-poor regions of the cell if the amount of tau present exceeds the MT tau-binding capacity (Lee et al., 2012; Samsonov et al., 2004). Recently, it has also been found that depending on the conformation and size of tau aggregates, tau can be taken up at either the somatodendritic compartment or axonal compartment and transported anterogradely or retrogradely (Wu et al., 2013).

Once soluble tau is transported to the cytoplasm, it can form the neurofibrillary tangles (NFTs) characteristic of neurodegenerative tauopathies. The formation of NFTs from free, unbound tau is thought to be a multi-step process and it is in the form of NFTs that tau has long been thought to result in toxicity and subsequent cell death. The number and progression of NFT-positive neurons correlates relatively well with the cognitive decline seen in AD (Ballatore et al., 2007; Giannakopoulos et al., 2003; Gomez-Isla et al., 1997), and NFTs have been associated with the activation of caspase cascades (Arriagada et al., 1992), as well as with diminished binding of calcium (Bezprozvanny and Mattson, 2008) (Mattson, 2006) and synaptic proteins (Coleman and Yao, 2003). However, nearly 40% of the tau that is mislocalized to the soma remains soluble, and a significant amount of cell death and neuronal dysfunction occurs in the absence of, or precedes, NFT pathology (Arriagada et al., 1992; Giannakopoulos et al., 2003). As such, understanding the toxic mechanisms of soluble tau is of utmost importance.

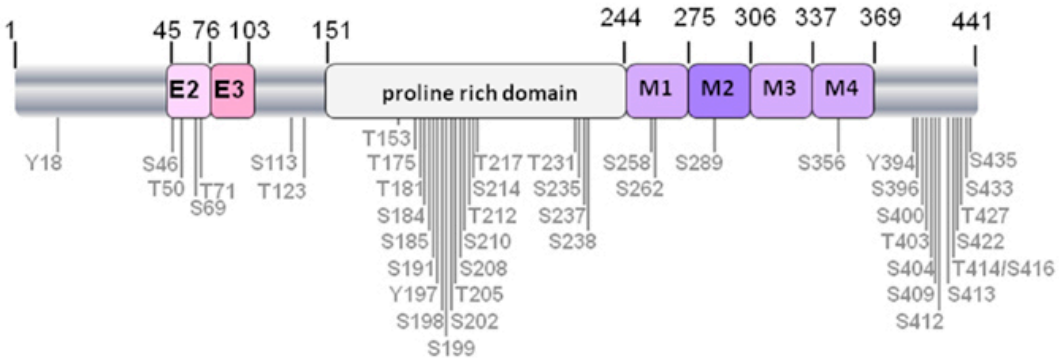


Figure 6: Sites of tau hyperphosphorylation in Alzheimer's disease. Many phosphorylation sites on tau that have been found to be hyperphosphorylated in Alzheimer's disease are located in regions surrounding the microtubule-binding domain (Figure from Noble et al., 2013).

1.8.4 Tau toxicity

Abnormal phosphorylation and mislocalization of tau are accepted as early characteristics of neurodegeneration, which precede NFT formation (Braak et al., 1994; Giannakopoulos et al., 2003) however, the toxic species of tau remains somewhat controversial. Historically, it was believed that NFTs exerted neurotoxic functions. However, more recent evidence suggests that toxicity is also spread by soluble low-molecular-weight oligomers (Kopeikina et al., 2012), which are recognized as NFT precursors (Lasagna-Reeves et al., 2012). Although tau aggregation has been shown to correlate well with neuronal loss and cognitive decline (Santacruz et al., 2005), animal models over-expressing tau without NFT pathology also present with neurodegeneration (Wittmann et al., 2001). Further, in AD before the formation of NFTs, neuronal loss can be detected along with tau oligomers (Gomez-Isla et al., 1996) suggesting that at the early stages of disease, soluble tau species are neurotoxic. Additionally, it has been shown that significant synaptic loss can occur prior to NFT formation (Yoshiyama et al., 2007). Moreover, in a repressible tauopathy mouse model, despite the persistent buildup of NFTs, cognitive function and neuronal survival can be re-established following suppression of transgenic tau (Santacruz et al., 2005). This indicates that NFTs are not sufficient to cause cognitive impairment in this model. However, the mechanism of tau toxicity is still not well understood.

Several hypotheses exist to explain the relationship between tau and neurodegeneration, such as a loss-of-function resulting from tau detachment from MTs and consequent MT instability (Alonso et al., 1996), as well as a gain-of-toxic function

resulting from aggregated tau physically interfering with intracellular transport (Wolfe, 2012). Further, tau has been shown to inhibit the ubiquitin-proteasome system vital for cellular homeostasis (Bandyopadhyay et al., 2007; Keck et al., 2003), and to activate caspases (Rohn et al., 2002), suggesting it may directly signal cellular apoptosis. However, among the most feasible hypotheses for tau-induced neurotoxicity is its ability to decrease trophic support for affected neurons.

1.9 BDNF is down-regulated in human tauopathy subjects

While little investigation has been done focusing on tau regulation of neurotrophic factors, our lab previously showed that there is decreased BDNF mRNA and protein in the parietal cortex of human tauopathy (PiD and CBD) subjects (Belrose et al., 2014). Specifically, in these tauopathies, we reported down-regulation of BDNF transcript IV. These results suggest that tau alone may decrease BDNF expression. However, there have been no *in vitro* investigations of tau-induced BDNF down-regulation, nor have transgenic animal models been utilized to avoid possible confounds of post-mortem human tissue. Further, it is still unclear whether soluble tau, not aggregated as NFTs, is capable of down-regulating BDNF and whether soluble tau mediates Aβ-induced BDNF down-regulation.

CHAPTER 2: SPECIFIC AIMS, HYPOTHESES & RATIONALE

The overall objective of this work is to understand the mechanisms of BDNF down-regulation in Alzheimer's disease. More specifically, the purpose of this work is to investigate the down-regulation of BDNF by the main pathological correlates of AD: amyloid beta and tau.

2.1 Objective #1: To determine the mechanism of Aβ-induced BDNF down-regulation

It has previously been shown that soluble, oligomeric Aβ down-regulates BDNF *in vitro*, largely via transcript IV (Garzon and Fahnestock, 2007). Further, in transgenic mouse models of AD, BDNF expression is inversely proportional to the amounts of soluble, high-molecular-weight Aβ oligomers (Peng et al., 2009). However, the mechanism of Aβ-induced BDNF down-regulation has not been determined.

Following cell stimulation, Aβ has been shown to inhibit both the Ras/ERK and PI3K/AKT signalling pathways (Tong et al., 2004), and to activate GSK3β (DaRocha-Souto et al., 2012), resulting in the inactivation of CREB (DaRocha-Souto et al., 2012; Tong et al., 2004). Given that CREB transcription plays a major role in regulating BDNF expression (Pruunsild et al., 2007; Shieh et al., 1998; Timmusk et al., 1995), the main objective outlined here is to determine if CREB mediates Aβ-induced BDNF down-regulation.

2.1.1 ***Specific Aim #1:*** *To determine if Aβ-induced basal BDNF down-regulation is mediated by inactivation of CREB via phosphorylation*

Aβ has been shown to both decrease the activating phosphorylation of CREB (pCREB133) and increase the inactive form of CREB phosphorylated at Ser-129 (pCREB129) following cell stimulation. However, the effect of this CREB regulation on BDNF expression has not been investigated in the absence of cell simulation. It is hypothesized here that Aβ treatment will down-regulate basal BDNF levels by either increasing the deactivating phosphorylation of CREB (pCREB129) via GSK3β activation and/or by decreasing the activating phosphorylation of CREB (pCREB133) via PKA inactivation.

2.1.2 ***Specific Aim #2:*** *To determine if Aβ-induced basal BDNF down-regulation is mediated by sequestration of CREB outside the nucleus*

Another mechanism of transcription factor inactivation is their sequestration outside of the nucleus. This has been shown to be mechanistically relevant in AD, where the transcription factor SFPQ is sequestered into the cytoplasm (Ke et al., 2012) as well as in Huntington's disease, where mutant huntingtin sequesters CREB-binding protein (CBP) outside the nucleus, preventing it from entering the nucleus to enhance CREB binding (Choi et al., 2012). It is hypothesized here that Aβ treatment will down-regulate BDNF by sequestering CREB outside the nucleus, rendering it inactive.

2.1.3 ***Specific Aim #3:*** *To determine if Aβ-induced basal BDNF down-regulation is mediated by transcriptional down-regulation of CREB*

CREB transcription has been shown to be decreased in AD and *in vitro* following treatment with Aβ (Pugazhenthi et al., 2011). Therefore, it is hypothesized here that Aβ treatment will down-regulate basal BDNF by decreasing CREB mRNA.

2.2 Objective #2: To determine if tau is capable of down-regulating BDNF

There is increasing evidence that although the primary insult in AD may be increased Aβ; it may ultimately lead to cognitive dysfunction and neurodegeneration by inducing alterations in tau (Iqbal and Grundke-Iqbal, 2008; Lewis et al., 2001). How tau might lead to memory loss and neurodegeneration is unclear. Recent findings showing that BDNF mRNA is down-regulated in the cortex of human non-AD tauopathy subjects (FTDP-17, PiD and CBD) (Belrose et al., 2014) suggest that tau might decrease BDNF expression. However, there have been no *in vitro* investigations of tau-induced BDNF down-regulation, nor have transgenic models been utilized to avoid possible confounds of post-mortem human tissue. Thus, the main objective outlined here is to investigate tau-induced BDNF down-regulation.

2.2.1 ***Specific Aim #1****: To determine if over-expression of wild-type tau is capable of down-regulating BDNF*

While, amyloid-β has been shown to decrease BDNF expression (DaRocha-Souto et al., 2012; Garzon and Fahnestock, 2007; Rosa and Fahnestock, 2015; Tong et al., 2004) tau is thought to be down-stream of Aβ and therefore possibly a more direct mediator of neurodegeneration (Ittner et al., 2010; Lewis et al., 2001; Masliah et al., 2001; Pennanen and Gotz, 2005). It is hypothesized here that over-expression of wild-type tau alone in transgenic mice and tau-transfected cells will down-regulate BDNF.

2.2.2 ***Specific Aim #2****: To determine if NFTs are required for BDNF down-regulation*

In AD, before the formation of NFTs, neuronal loss can be detected along with the presence of tau oligomers (Gomez-Isla et al., 1996) suggesting that soluble aggregated tau species are neurotoxic. In addition, soluble tau over-expressing animal models exhibit neurodegeneration despite their lack of NFT-like pathology (Andorfer et al., 2005; Wittmann et al., 2001), supporting the notion that NFTs are not the primary cause of neurodegeneration. Therefore, it is hypothesized here that NFTs are not required for BDNF down-regulation, and rather that soluble tau is responsible for down-regulating BDNF.

*2.2.3 **Specific Aim #3**: To determine if BDNF Transcript IV is down-regulated by tau*

BDNF transcript IV is down-regulated in human AD cortical tissue (Garzon et al., 2002), in mouse models of AD (Peng et al., 2009) and in SH-SY5Y cells treated with Aβ (Garzon and Fahnestock, 2007). BDNF transcript IV is also down-regulated in the parietal cortex of human tauopathy (PiD and CBD) patients (Belrose et al., 2014). Therefore, it is hypothesized that soluble tau will specifically down-regulate BDNF transcript IV in the tested models.

2.3 Objective #3: To determine if tau mediates Aβ-induced BDNF down-regulation

Aβ is thought to be the upstream driving force behind subsequent AD pathology and synaptic degeneration (Hardy and Selkoe, 2002; Iqbal and Grundke-Iqbal, 2008; Selkoe, 1994). As such, tau pathology characteristic of AD is thought to be down-stream of Aβ and therefore a more direct mediator of neurodegeneration (Ittner et al., 2010; Lewis et al., 2001; Masliah et al., 2001; Pennanen and Gotz, 2005). The main objective of this work is to determine if tau mediates Aβ-induced BDNF down-regulation.

*2.3.1 **Specific Aim #1:** To determine if Aβ over-expressing APP23 mice down-regulate BDNF*

Aβ has been shown to down-regulate BDNF in both cell culture (DaRocha-Souto et al., 2012; Garzon and Fahnestock, 2007; Rosa and Fahnestock, 2015) and in transgenic

mice (Peng et al., 2009). However, this has yet to be determined for the APP23 mice. It is hypothesized that the over-expression of Aβ characteristic of APP23 mice will result in significantly diminished BDNF expression.

2.3.2 ***Specific Aim #2:*** *To determine if TauKO rescues BDNF levels in APP23 mice.*

It has been suggested that Aβ may exert its neurotoxic effects via tau hyperphosphorylation and subsequent neurofibrillary tangle formation (Iqbal and Grundke-Iqbal, 2008). Furthermore, Aβ-induced neurodegeneration is prevented in primary neuronal cultures from TauKO mice (Rapoport et al., 2002), and knocking out tau in a transgenic AD mouse model can block Aβ-induced cognitive impairments (Roberson et al., 2007). Therefore, it is hypothesized here that TauKO animals as well as those crossed with Aβ over-expressing animals (APP23xTauKO) will not display the down-regulated BDNF expression seen in Aβ over-expressing animals (APP23) alone, suggesting that tau mediates Aβ-induced BDNF down-regulation.

CHAPTER 3: METHODOLOGY

3.1 OBJECTIVE 1:

3.1.1 SH-SY5Y cell culture & treatment

Human neuroblastoma SH-SY5Y cells are an immortalized cell line that exhibits neuronal morphology, expresses BDNF and its receptor TrkB and is dependent on BDNF for survival (Encinas et al., 2000; Feng et al., 2001; Kaplan et al., 1993). This cell line was chosen instead of primary neuronal cultures for this study, as it allows for more complex manipulations, such as stable transfections, which traditionally have very low efficiency in primary neuronal cultures. SH-SY5Y cells (ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (GIBCO BRL, Carlsbad, CA), 1% L-glutamine (GIBCO) and 1% penicillin/streptomycin (GIBCO). Cells were incubated at 37ºC and 5% CO_2 in a 75cm^2 flask and split at a ratio of 2:3 with growth medium every 3-4 days. Cells were differentiated (Figure 7) and treated with 5μM oligomeric Aβ as previously described (Garzon and Fahnestock, 2007) and outlined in Table 1.

Lyophilized $A\beta_{42}$ peptide (rPeptide, Athens, GA) was dissolved to 1mM in 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma-Aldrich) and dried to create biofilms as described (Garzon and Fahnestock, 2007). Aβ oligomers, which have previously been shown to specifically down-regulate BDNF (Garzon & Fahnestock 2007), were prepared 24 hours prior to treatment by dissolving the biofilm in 100% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to obtain a 2mM solution and sonicating at 37°C for 10

minutes. This solution was diluted 1:10 in Ham's F-12 (phenol red free; BioSource, Camarillo, CA), vortexed for 30 seconds and incubated for 24 hours at 4^{o}C. The Aβ solution was then diluted 1:40 in DMEM containing 1% FBS, 1% N2 Supplement, 1% L-glutamine and 1% penicillin/streptomycin (treatment medium), giving a final concentration of 5μM Aβ and 0.25% DMSO. This treatment concentration of Aβ was determined previously to both disrupt cellular signalling (Tong et al., 2001, 2004) and down-regulate BDNF mRNA (Garzon and Fahnestock 2007) without affecting cell viability. Vehicle-treated cells were exposed to 0.25% DMSO in treatment medium. There was no difference in BDNF expression between DMSO-treated cells and non-vehicle (medium only) treated controls (*data not shown,* p=1.00).

CT 99021 is an ATP-competitive inhibitor of GSK3β and is considered the most potent GSK3β inhibitor available (Bain et al., 2007). CT 99021 was used here to decrease GSK3β activity, in order to determine if increased levels of inactivated pCREB129 mediate Aβ-induced BDNF down-regulation. Conversely, to determine if decreased levels of activated pCREB133 mediate Aβ-induced BDNF down-regulation, the PKA activator forskolin was used. Forskolin is a naturally occurring diterpene compound which effectively activates adenylyl cyclase to subsequently increase levels of cAMP and increase the activity of PKA (Seamon & Daly 1981). Both CT 99021 (Sigma-Aldrich) and forskolin (Sigma-Aldrich) were solubilised in 100% DMSO to obtain 4mM and 50mM solutions, respectively. Stock solutions of both CT 99021 and forskolin were diluted further into treatment medium, giving a final concentration of 2μM CT 99021 (Bain et al., 2007) with 0.05% DMSO and 30μM forskolin with 0.06% DMSO. The

treatment concentration of forskolin was determined through my own experimental manipulations. I tested a variety of forskolin treatment concentrations and durations: 50μM for 15 minutes, 30μM for 30 minutes, and 10μM for 24 hours. Treatments with either 50μM forskolin for 15 minutes and 10μM forskolin for 24 hours were not sufficient to increase PKA activity (*data not shown*), while PKA activity was significantly increased (as assessed by increased levels of pCREB133) by a 30-minute treatment with 30μM forskolin, when compared to vehicle-treated control cells (Figure 14). Additionally, a 30-minute pre-treatment with CT 99021 has been shown to be sufficient to prevent Aβ-induced inhibition of LTP (Jo et al., 2011). As a result of this previous CT 99021 literature, and my own experimentation with forskolin, cells were treated with either CT 99021 or forskolin for 30 minutes prior to 48 hour Aβ treatment. 30μM forskolin was also administered 24 hours after Aβ treatment and remained on the cells to determine if forskolin is capable of rescuing BDNF expression following down-regulation by Aβ. Following treatment, GSK3β inactivation or PKA activation (via Western blot) and BDNF mRNA levels (via qRT-PCR) were compared to cells treated with $A\beta_{42}$ alone and to vehicle-treated groups.

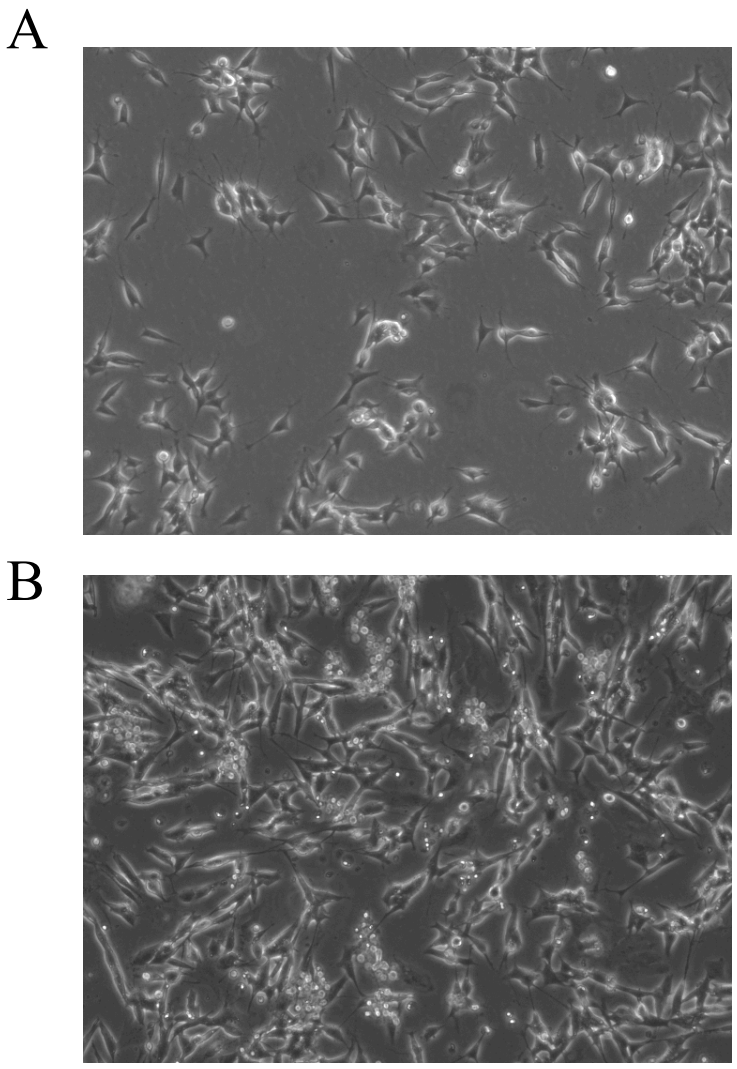


Figure 7: SH-SY5Y cell culture. A) Undifferentiated SH-SY5Y cells at passage 11; **B)** SH-SY5Y cells after 10 days of differentiation with retinoic acid have a more neuronal appearance, with increased elongation and neurite outgrowth compared to undifferentiated SH-SY5Y cells.

3.1.2 RNA extraction

SH-SY5Y conditioned medium was removed and cells were harvested 48 hours after treatment. After removal of cell medium, 1ml of Trizol™ (Invitrogen, Burlington, ON) was used to lyse $3.65x10^5$ cells, which were then stored at -80°C until RNA extraction took place. RNA was extracted using RNeasy™ Mini Spin Columns (Qiagen, Mississauga, ON) according to the manufacturer's protocol. Once RNA was bound to the column, it was treated with RNase-free DNaseI in Buffer RDD (Qiagen) for 15 minutes prior to RNA elution with autoclaved water. Resulting RNA concentration and purity were determined by Multiskan GO and SkanIT software (Thermo Scientific, Nepean, ON) at 260/280 nm (Rosa & Fahnestock, 2015).

3.1.3 Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

BDNF expression was determined by qRT-PCR. This method allows for an accurate quantification of BDNF mRNA and is a superior method over protein quantification because of a lack of specific BDNF protein antibodies commercially available at this time. However, we have previously shown that a down-regulation of BDNF mRNA as quantified by qRT-PCR correlates well with decreased BDNF protein levels as measured by ELISA (Belrose et al., 2014). Here, 1µg of SH-SY5Y RNA was reverse transcribed with Superscript™ III, following the manufacturer's protocol (Invitrogen). Briefly, a total reaction volume of 20ul was composed of 1ug of sample RNA, 200 units of Superscript™III RT reagent, 250ng of random primers (Invitrogen), 0.5mM deoxynucleotide triphosphates (Invitrogen), 1x first strand buffer, 0.05mM

dithiothreitol and 1 unit of RNaseOUT™ (Invitrogen). Negative controls ("No-RT") were included in the reverse transcription reaction that had water substituted for Superscript III. The reverse transcription reaction was carried out in a GeneAmp PCR system 2400 thermal cycler (Applied Biosystems, Streetsville, ON) at 25°C for 5 minutes, 50°C for 50 minutes, and 70°C for 15 minutes.

The real-time PCR reaction mixture was composed of 300 nM each forward and reverse BDNF primers or 300 nM each forward and reverse β-actin primers or 300 nM each forward and reverse CREB primers (Mobix, Hamilton, ON, Canada) (Table 2). In addition to primers, the reaction mixture was composed of 10 µl SYBR Green qPCR Supermix UDG ™ (Invitrogen), 30 nM of reference dye ROX (Stratagene, La Jolla, CA, USA or Invitrogen), and 1 µL of cDNA (from 50 ng RNA), in a total volume of 20 µL (Rosa & Fahnestock, 2015; Rosa et al., 2015; Michalski et al., 2015; Garzon & Fahnestock, 2007). BDNF and CREB PCR standards were generated from purified PCR products using the primers listed, whereas β-actin standard was generated from a plasmid obtained from Invitrogen (Garzon and Fahnestock, 2007). Amplifications of samples, standards and controls (no-RT and no-template controls) were run in triplicate. A MX3000P real-time PCR system (Stratagene) was used to run the following thermal profile: 2 min at 50°C, 2 min at 95°C followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 45 s (Rosa & Fahnestock, 2015; Michalski et al., 2015; Garzon & Fahnestock, 2007). A dissociation curve after 40 PCR cycles confirmed a single product for both targets. Copy numbers using absolute quantification and PCR efficiencies were calculated with MXPro Mx3000P Software (Stratagene). Only experiments in which the

real-time PCR efficiency was between 90%-100% and standard curves yielded a R^2>0.990 were used for analysis. BDNF mRNA expression and CREB mRNA expression were normalized to the housekeeping gene β-actin (Rosa & Fahnestock, 2015).

3.1.4 Cell viability assay

Cell viability was determined by quantifying the release of lactate dehydrogenase (LDH) into conditioned medium. LDH is a stable enzyme released from all cell types upon plasma membrane damage (Koh and Choi, 1987) and is a commonly used marker to assess cytotoxicity because of its sensitivity. The procedure used here was modified in our lab from previous reports (Koh and Choi, 1987; Lobner, 2000). Briefly, 30µl of conditioned medium was combined with 200µl of LDH buffer (1M Tris pH 7.4, 1.4mM Na pyruvate, 3.15mM NADH), and LDH levels from each sample were quantified using a Multiskan GO microplate reader and SkanIT software (Thermo Scientific). Rabbit muscle LDH type II, in ammonium sulfate suspension (800-1200 units/mg protein) (Sigma-Aldrich), was diluted 1:5000 and 1:25000 and used as a positive control, and medium alone was used as a negative control. All samples were read at 340nm every 30 seconds for 5 minutes.

3.1.5 Protein extraction

$3.65x10^5$ SH-SY5Y cells were lysed in 150 µL of lysis buffer [50mM Tris pH 7.4, 150mM NaCl, 5mM EDTA, 1% Triton, 1 complete EDTA-free tablet (Roche, Mississauga, ON) and 1 complete PhosSTOP Phosphatase Inhibitor Cocktail Tablet

(Roche) per 10ml cell lysis buffer] (Michalski et al., 2015; Rosa et al., 2015; Rosa and Fahnestock, 2015). Cell lysates were centrifuged at 14000 x g for 5 minutes, supernatants were collected and protein concentrations determined using the DC™ Protein Assay (Bio-Rad Laboratories, Mississauga, Ontario, Canada) as described by the manufacturer, prior to Western blotting.

3.1.6 Nuclear/Cytoplasmic localization of CREB

SH-SY5Y cells were harvested using NE-PER™ Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) reagents according to manufacturer's protocols. Briefly, cells were lysed in a cytoplasmic extraction buffer and centrifuged. Nuclear extraction buffer was added to the pellet, which was vortexed and centrifuged (14000 x g) to extract the remaining nuclear fraction. Protein concentrations of the samples were then determined using the DC™ Protein Assay (Bio-Rad Laboratories) prior to Western blotting.

3.1.7 Western blotting

12% sodium dodecyl sulfate (SDS)-polyacrylamide gels were used to separate 15-35ug of total protein under reducing conditions prior to transferring to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Membranes were then blocked with a 1:1 solution of phosphate-buffered saline (PBS) pH7.4 and Odyssey Blocking Buffer (BB) (Cedarlane, Burlington, Ontario, Canada) for 1 hour. After blocking, the blots were probed overnight at 4˚C with the following primary antibodies:

human CREB, pCREB-Ser133, pCREB-Ser129, β-catenin or alpha-tubulin (Table 3). After washing with PBS containing 0.5% Tween-20 (PBS-T), blots were incubated with the secondary antibodies IRDye 680-conjugated goat anti-rabbit and IRDye 800CW-conjugated goat anti-mouse (Table 3) for 1 hour at room temperature, washed with PBS-T and scanned using an Odyssey Infrared Imaging System (LI-COR Biosciences). Band intensities were quantified by densitometry with local background subtraction using LI-COR Odyssey Software, version 2.0 (Michalski et al., 2015; Rosa & Fahnestock, 2015; Nicolini et al., 2015).

3.1.8 Statistical analysis

Data collected throughout this work is defined as either interval or ratio data and as such, parametric analysis was carried out using IBM Statistics 22 software (SPSS, Chicago, IL). A two-sample *t*-test, assuming equal variances or a one-way ANOVA with *post-hoc* Tukey's test for pairwise comparisons was done, according to the experimental design. Significance was set at $p<0.05$, using a two-tailed critical value.

3.2 OBJECTIVE 2:

3.2.1 Transgenic mice

Cortical tissue from tau over-expressing mice (hTau and 8c-het mice), were compared to their non-transgenic wild-type control mice (Swiss Webster/B6D2F1 mice). Cortical tissue from these mice was dissected, frozen and generously donated by Dr. Stephen Ginsberg from the Nathan Kline Institute and New York University Langone

Medical Center.

Three to 16 month-old hTau transgenic mice, which over-express human tau on a mouse tau knockout background (Andorfer et al., 2005; Andorfer et al., 2003; Levine et al., 2009) and 8c-het transgenic mice, which over-express human tau on a heterozygous mouse tau background (Duff et al., 2000), were used in this study. hTau mice develop NFTs in a similar pattern and distribution as seen in human tauopathies (Andorfer et al., 2003) and experience age-dependent cognitive and physiological impairments similar to that observed in AD patients (Polydoro et al., 2009). hTau mice exhibit insoluble pre-tangles at approximately 9 months of age (Andorfer et al., 2003), neuronal loss in the piriform cortex between 8 and 17 months of age and NFT pathology beginning at 15 months of age (Andorfer et al., 2005; Andorfer et al., 2003; Levine et al., 2009). In contrast, 8c-het transgenic mice exhibit increased tau phosphorylation and altered tau isoform expression prior to 8 months of age compared to wild-type mice but do not develop insoluble tau aggregates (Duff et al., 2000). Both of these tau over-expressing models have an advantage over other models in that they over-express wild-type tau without any pathological mutations. Historically, transgenic animals harboring tau mutations, such as the P301L tau mutation, which is the pathogenic mutation in FTLD-17 (Lewis et al., 2000), are the most widely used. However, studying animals that over-express wild-type tau such as the 8c-het and hTau mice is highly relevant to understanding the pathophysiology of sporadic AD, by far the most common form of this neurodegenerative disease and a disorder which does not exhibit mutations in tau.

3.2.2 Cell culture

Human neuroblastoma SH-SY5Y cells (ATCC, Manassas, VA, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (Gibco BRL, Carlsbad, CA, USA), 1% L-glutamine (Gibco) and 1% penicillin/streptomycin (Gibco), as described previously in *Section 3.1.1*.

3.2.3 Construction of hTau40-V5/DEST plasmid

The human 4-repeat tau isoform, hTau40, was expressed in a pENTR entry vector that was generously donated by Dr. Lars Ittner from University of New South Wales (Sydney, Australia). This plasmid was then transformed into DH5α *Escherichia coli* (Invitrogen) prior to purification using the QIAprep Spin Miniprep Kit (Qiagen, Mississauga ON). Following purification, the hTau-pENTR plasmid underwent a Gateway® L-R cloning reaction using L-R Clonase® (Invitrogen) with pcDNA3.2-V5/DEST vector (Invitrogen) to insert the hTau gene into the destination vector. This destination vector includes a V5 epitope tag, which is a 14 amino acid sequence derived from simian parainfluenza virus type 5 (Southern et al., 1991) and is a commonly used epitope tag because of its small size and availability of high-affinity antibodies. Following the cloning reaction, the plasmid was again transformed into DH5α *Escherichia coli* (Invitrogen) prior to purification. The purified hTau40-pcDNA3.2/V5-DEST plasmid (Figure 8) was then sequenced using T7 promoter (5'-TAATACGACTCACTATAGGG-3') and V5 reverse primers (5'-

ACCGAGGAGAGGGTTAGGGAT-3') to ensure successful insertion of hTau40 into the pcDNA plasmid (MOBIX, Hamilton, ON) prior to mammalian transfection.

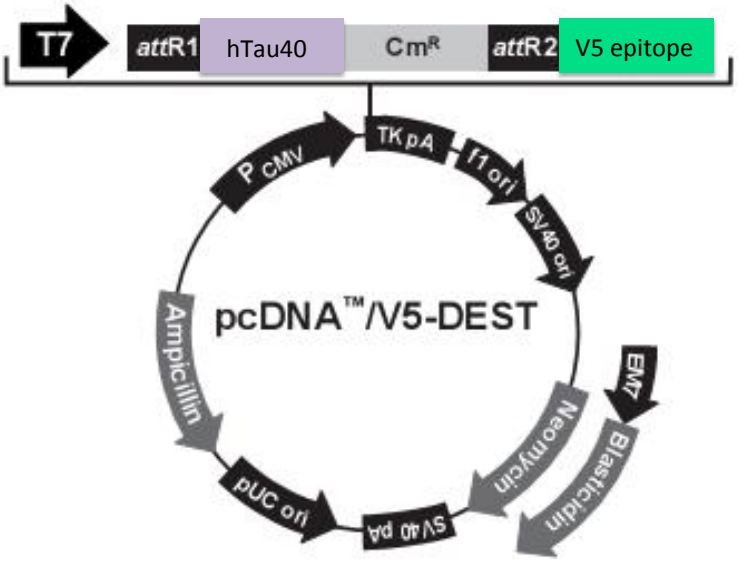


Figure 8: hTau40-pcDNA3.2/V5-DEST plasmid map. Following Gateway® L-R cloning, a pcDNA3.2/V5-DEST plasmid containing the hTau40 sequence with V5 epitope tag for quantification was generated.

3.2.4 Cell transfection

2µg of human 4-repeat wild-type tau plasmid, hTau40/V5-DEST, was transfected into $1.95x10^6$ SH-SY5Y cells using Lipofectamine 3000®, with slight modifications to the maunfacturer's instructions (Invitrogen). In brief, transfectants were grown on $100mm^2$ $cell^+$ dishes (Sarstedt, Montreal, QC, Canada) prior to selection with 300µg/ml G418 (BioShop, Burlington, ON, Canada) for 30 days (Pennanen and Gotz, 2005). To induce neuronal differentiation, cells were treated with 10µM retinoic acid (Sigma-Aldrich, Oakville, ON) for 10 days, as described in *Section 3.1.1* (Garzon and Fahnestock, 2007; Rosa and Fahnestock, 2015). V5 expression was visualized via immunocytochemistry and quantified via Western blotting to ensure hTau expression following differentiation *(see Section 3.2.5),* and total BDNF mRNA and BDNF transcript IV mRNA levels were quantified via qRT-PCR and compared to non-transfected control cells (*see Section 3.2.6)*.

3.2.5 Immunocytochemistry for detection of V5-tagged plasmid

Following 30 days of selection with G418 and 10 days of retinoic acid-induced differentation, hTau40-V5-pcDNA transfected SH-SY5Y cells were fixed and stained to visualize the V5-tagged protein present and confirm that cells stabilized the transfection and that it was maintained following differentiation. In brief, media was removed from wells containing $3.65x10^5$ SH-SY5Y cells, and wells were rinsed twice with PBS. Cells were then fixed by exposure to 100% methanol for 5 minutes followed by 5 washes with PBS. Next, cells were blocked with the addition of PBS + 10% FBS for 20 minutes prior

to the addition of a V5-FITC conjugated antibody (Life Technologies, Burlington, Ontario, Canada) at a dilution of 1:500, which was incubated for 1 hour in the dark. The antibody was then removed and wells washed again with PBS prior to mounting coverslips with ProLong® Gold Antifade Mountant with DAPI (Life Technologies) prior to imaging on a Zeiss Axiovert A1 microscope using filter set 09 (excitation/emission of 450 nm/515 nm) and filter set 49 (excitation/emission of 365 nm/450 nm) (Carl Zeiss International, Toronto, Ontario, Canada).

3.2.6 Quantitative real-time reverse transcription-polymerase chain reaction

Frozen cortical tissue (13-27mg) from all transgenic mice and SH-SY5Y cells were sonicated (Sonic Dismembrator Model 100, Fisher Scientific) in a 1:20 w/v ratio in Trizol® (Invitrogen, Burlington, Ontario). Sonicates were centrifuged for 3 minutes at 9000 x g at 4^{o}C, and RNA was extracted as described in *Section 3.1.2* (Rosa et al., 2015a). Resulting RNA concentration and purity were determined by Multiskan GO and SkanIT software (Thermo Scientific, Nepean, ON, Canada) at 260/280 nm, where 260/280 ratio values were consistently within a reliable range of purity between 1.8-2.0. 1µg of RNA from each sample was used for reverse transcription with Superscript^{TM} III, following the manufacturer's protocol (Invitrogen). Real-time PCR was carried out as described in *Section 3.1.3* (Rosa and Fahnestock, 2015), using 300 nM each forward and reverse BDNF primers, BDNF Transcript IV primers or β-actin primers (Mobix, Hamilton, ON, Canada) (Table 2). Amplifications of samples, standards and controls (no-RT and no-template controls) were run in triplicate as described previously (Rosa et al.,

2015; Rosa and Fahnestock, 2015).

3.2.7 Protein extraction and western blotting

$3.65x10^5$ hTau-transfected SH-SY5Y cells were lysed in 150 µL of lysis buffer [50mM Tris pH 7.4, 150mM NaCl, 5mM EDTA, 1% Triton-X, 1 complete EDTA-free tablet (Roche, Mississauga, ON, Canada) and 1 complete PhosSTOP Phosphatase Inhibitor Cocktail Tablet (Roche) per 10ml cell lysis buffer], as described previously in *Section 3.1.5* (Rosa and Fahnestock, 2015) prior to Western blotting. Frozen cortical tissue from tau over-expressing transgenic mice (8c-het and hTau and non-transgenic controls) was sonicated (Sonic Dismembrator Model 100, Fisher Scientific) in a 1:15 w/v ratio in lysis buffer without the presence of Triton-X detergent, in order to isolate the TBS-soluble protein fraction (Michalski et al., 2015). Tau present in the TBS-soluble fraction may be considered the most soluble, while an additional fraction of tau protein may be extracted using buffer containing detergent, known as the detergent-soluble tau fraction (Eckermann et al., 2007; Greenberg and Davies, 1990; Michalski et al., 2015; Planel et al., 2009; Sahara et al., 2002). Lastly, insoluble tau can be extracted by adding an acid such as formic acid to the lysis buffer (Eckermann et al., 2007; Ishihara et al., 1999; Julien et al., 2012). For the purposes of this study, to confirm genotyping of the transgenic animals used, only the TBS-soluble fraction was examined.

Twelve percent sodium dodecyl sulfate (SDS) polyacrylamide gels were used to separate 25-30µg of total protein under reducing conditions before transferring to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA) as described

previously in *Section 3.1.7* (Rosa & Fahnestock 2015). After blocking, the blots containing transgenic mouse cortex homogenates were probed overnight at 4^{o}C with total tau antibody 39E10 (Table 3), while blots containing human tau-transfected SH-SY5Y cells were probed with Anti-V5 antibody (Table 3). After washing with PBS containing 0.5% Tween-20 (PBS-T), blots were incubated with the secondary antibody IRDye 800CW-conjugated goat anti-mouse for 1 hour at room temperature, washed with PBS-T, and scanned using an Odyssey Infrared Imaging System (LI-COR Biosciences). Band intensities were quantified by densitometry with local background subtraction using LI-COR Odyssey Software, version 2.0.

3.2.8 Statistical analysis

Statistical analyses were carried out using IBM Statistics 22 software (SPSS, Chicago, IL, USA) as described in *Section 3.1.8*. A Pearson correlation was also used according to the experimental design. PCR product synthesis for the 8c-het and hTau mouse data was modeled as a function of mouse genotype using a general linear model (GLM; McCulloch et al. 2011). Significance was judged at the level (α = 0.05), two-sided.

3.3 OBJECTIVE 3:

3.3.1 Transgenic mice

Cortical tissue from Aβ over-expressing APP23 mice and Tau knockout (TauKO)

animals were compared to their non-transgenic wild-type control mice (C57BL6 mice). Dr. Lars Ittner from the Dementia Research Unit at the University of New South Wales generously donated cortical tissue from these animals.

APP23 mice exhibit a 7-fold over-expression of mutated APP harboring the Swedish mutation, resulting in significantly increased soluble Aβ and plaques depositing at about 6 months of age (Sturchler-Pierrat et al., 1997) with cognitive impairments beginning as early as 3 months of age (Van Dam et al., 2003). TauKO (*Mapt* -/-) mice (Tucker et al., 2001) lack tau but do not have any detectable phenotype. Mice used in this study were 24 months of age.

3.3.2 Quantitative real-time reverse transcription-polymerase chain reaction

Frozen cortical tissue (20-50mg) from non-transgenic control mice and APP23, TauKO and APP23xTauKO transgenic mice were sonicated (Sonic Dismembrator Model 100, Fisher Scientific) in a 1:20 w/v ratio in Trizol® (Invitrogen, Burlington, Ontario) and purified as described in *Section 3.2.4* (Rosa et al., 2015a), where 260/280 ratio values were consistently within a reliable range of purity between 1.8-2.0. 1µg of RNA from each sample was used for reverse transcription with Superscript™ III, following the manufacturer's protocol (Invitrogen). Real-time PCR was carried out as described in *Section 3.1.3* (Rosa & Fahnestock 2015), using 300 nM each forward and reverse BDNF primers or β-actin primers (Mobix, Hamilton, ON, Canada) (Table 2). Amplifications of samples, standards and controls (no-RT and no-template controls) were run in triplicate as described previously (Rosa et al., 2015; Rosa and Fahnestock, 2015).

3.3.3 Statistical analysis

Statistical analyses were carried out using IBM Statistics 22 software (SPSS, Chicago, IL, USA) as described in *Section 3.1.8*. A one-way ANOVA with *post-hoc* Tukey's test for pairwise comparisons was performed. Significance was judged at the level ($\alpha = 0.05$), two-sided.

Day	Treatment
1	Seed SH-SY5Y cells at $1.625x10^5$ cell/ml in growth medium
2	Replace all growth medium with differentiation medium
3	
4	Replace ½ differentiation medium
5	
6	Replace ½ differentiation medium
7	
8	Replace ½ differentiation medium
9	
10	**Treatment**: Remove ½ differentiation medium and replace with treatment medium (+/- Aβ and/or CT 99021 or Forskolin)
11	
12	Terminate experiment, harvest

Table 1: SH-SY5Y differentiation protocol

Gene	Species	Forward Primer	Reverse Primer
BDNF	Human	5'-AAACATCCGAGGACAAGGTG-3'	5'-AGAAGA GGAGGCTCCAAAGG-3'
β-actin	Human	5'-AGCCATGTACGTAGCCATCC-3'	5'- CTCTCAGCTGTGGTGGTGAA-3'
CREB	Human	5'-CTGCCTCTGGAGACGTACAA-3'	5'-CAAGCACTGCCACTCTGTTT-3'
BDNF	Mouse	5'-GCGGCAGATAAAAAGACTGC-3'	5'-CTTATGAATCGCCAGCCAAT-3'
BDNF TransIV	Mouse	5'- GAGTATTACCTCCGCCATGC-3'	5'-ATTCACGCTCTCCAGAGTCC-3'
β-actin	Mouse	5'-AGCCATGTACGTAGCCATCC-3'	5'-CTCTCAGCTGTGGTGGTGAA-3'

Table 2: Real-time PCR primers

	Antibody	Dilution	Company
Primary	CREB	1:200	Cell Signaling Technology, Danvers MA, USA
	pCREB-133	1:200	Cell Signaling Technology
	pCREB-129	1:200	Santa Cruz Biotechnology, Dallas, TX, USA
	β-catenin	1:500	Cell Signaling Biotechnology
	α-tubulin	1:8000	Sigma-Aldrich
	V5	1:5000	Life Technologies
	Tau (39E10)	1:500	Covance
Secondary	IRDye 680	1:8000	LI-COR Biosciences, Lincoln, NE, USA
	IRDye 800 CW	1:8000	LI-COR Biosciences, Lincoln, NE, USA

Table 3: Western blotting antibodies

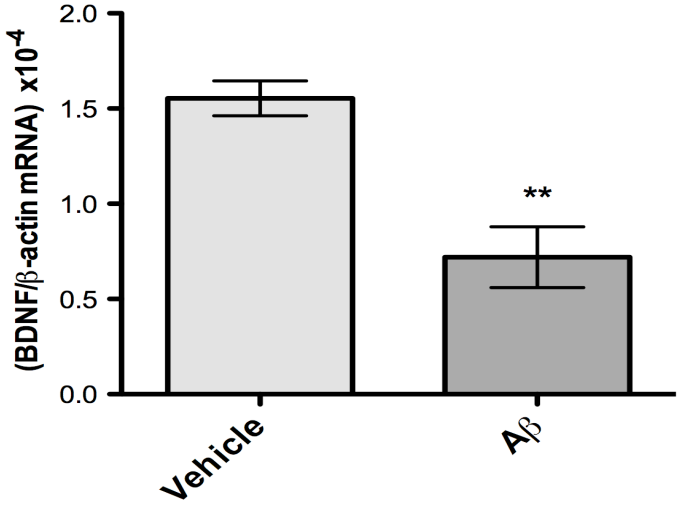
CHAPTER 4: RESULTS

4.1 OBJECTIVE 1

4.1.1 Oligomeric $A\beta_{1-42}$ down-regulates basal BDNF expression without affecting cell viability

BDNF mRNA was significantly down-regulated in differentiated SH-SY5Y cells following treatment with 5μM oligomeric Aβ (Figure 9A; $p<0.001$), without affecting cell viability (no difference in LDH released; Figure 9B; $p=0.65$), as we previously reported (Garzon and Fahnestock, 2007). This concentration of Aβ has also been shown to decrease cellular signaling without affecting cell viability following cell stimulation with KCl and NMDA (Tong et al., 2001). However, it is important to note that our experimental conditions do not include cell stimulation.

A


(BDNF/β-actin mRNA) x10-4
2.0
1.5
1.0
0.5
0.0
**
Vehicle
Aβ

B

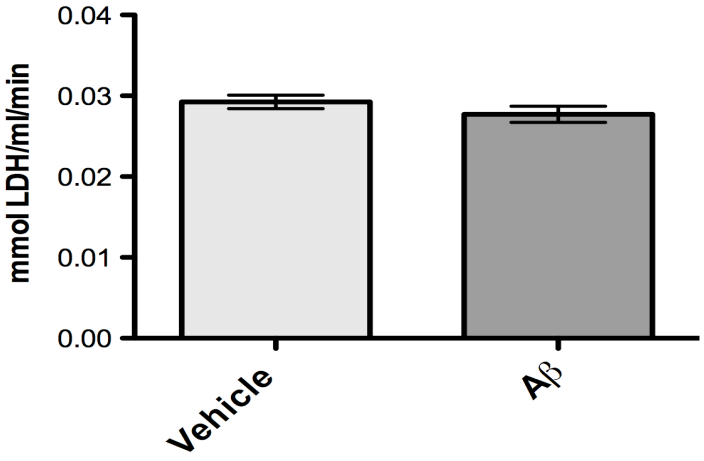

mmol LDH/ml/min
0.04
0.03
0.02
0.01
0.00
Vehicle
Aβ

Figure 9: Aβ significantly down-regulates BDNF mRNA without affecting cell viability. Following treatment of differentiated SH-SY5Y cells with 5μM oligomeric Aβ, **A)** BDNF mRNA was significantly reduced (Student's *t*-test; **p<0.001). Error bars represent S.E.M. n=6/group. This experiment was carried out 7 times with similar group sizes and results. **B)** There was no difference in amount of LDH released between control cells (treated with 0.25% DMSO in treatment medium) and cells treated with 5μM oligomeric Aβ also exposed to 0.25% DMSO (Student's *t*-test; p=0.65). Error bars represent S.E.M. n=11-12/group. This experiment was carried out 3 times with similar group sizes and results.

4.1.2 Aβ treatment of differentiated SH-SY5Y cells decreases CREB transcription but does not alter CREB phosphorylation or nuclear localization

CREB mRNA was significantly down-regulated in differentiated SH-SY5Y cells treated with 5μM Aβ compared to control cells (Figure 10; p=0.009). To determine whether decreased BDNF transcription following Aβ treatment, in the absence of cell stimulation, could also be due to altered CREB phosphorylation, the activating phosphorylation (pCREB133) and inactivating phosphorylation (pCREB129) of CREB were quantified by Western blotting in Aβ-treated cells compared to control cells. Phosphorylated CREB at Ser-133 (normalized to total CREB) in cells treated with 5μM Aβ was not significantly different than in control cells (Figure 11A; p=0.65). Similarly, phosphorylated CREB at Ser-129 (normalized to total CREB) in cells treated with 5μM Aβ was not significantly different than in control cells (Figure 11B; p=0.43).

To assess whether Aβ treatment of differentiated SH-SY5Y cells inactivates CREB by sequestering it outside the nucleus, phosphorylated and total CREB levels were quantified from both the nuclear and cytoplasmic fractions following Aβ treatment and compared to controls. Phosphorylated CREB at Ser-133 was not significantly different in either the cytoplasmic (Figure 12A; p=0.55) or the nuclear (Figure 12B; p=0.39) fractions following Aβ treatment compared to controls. However, total CREB protein was significantly decreased in both the cytoplasmic (Figure 12C; p=0.009) and the nuclear (Figure 12D; p=0.008) fractions following Aβ treatment compared to controls.

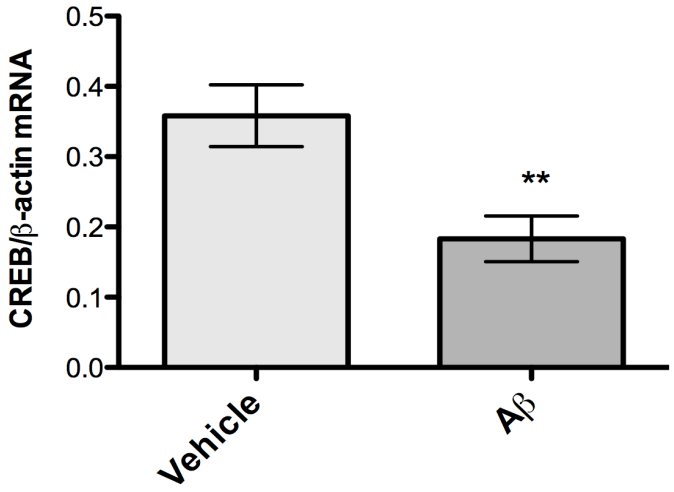
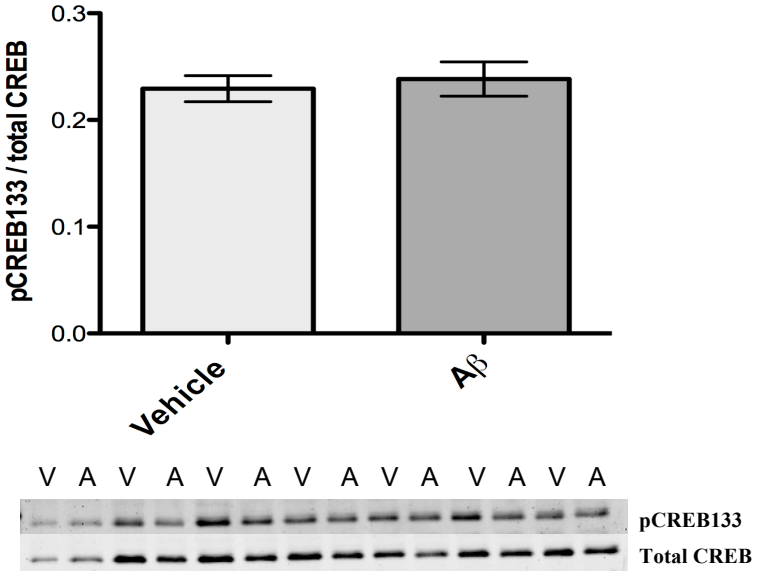


Figure 10: Aβ significantly down-regulates CREB mRNA. Following treatment of differentiated SH-SY5Y cells with 5μM oligomeric Aβ, CREB mRNA was significantly reduced (Students *t-test*; **p=0.009). CREB mRNA expression was normalized to β-actin mRNA for each sample. Error bars represent S.E.M. n=6/group. This experiment was carried out 3 times with similar group sizes and results.

A


0.3
0.2
0.1
0.0
pCREB133 / total CREB
Vehicle
Aβ
V A V A V A V A V A V A V A
pCREB133
Total CREB

B

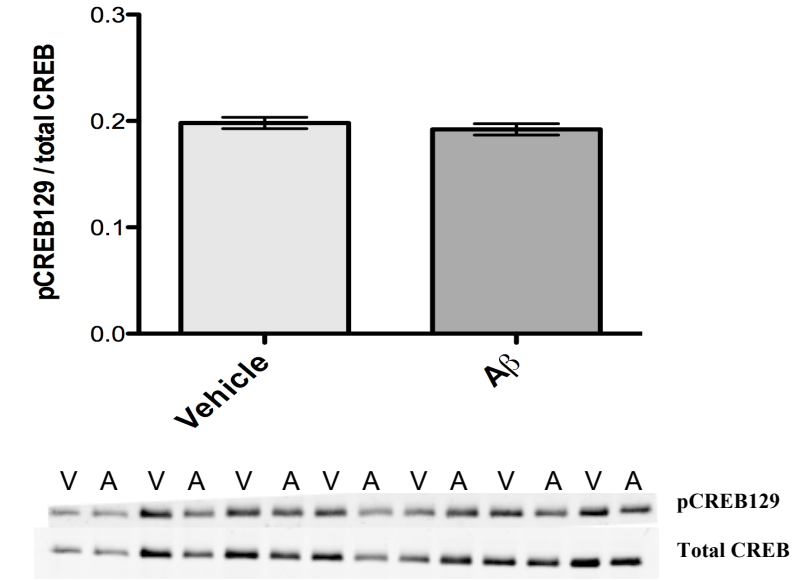

0.3
0.2
0.1
0.0
pCREB129 / total CREB
Vehicle
Aβ
V A V A V A V A V A V A V A
pCREB129
Total CREB

Figure 11: Aβ treatment does not affect phosphorylation of CREB. Western blot analysis of vehicle (V) and Aβ (A) treated SH-SY5Y cells revealed that, following treatment with 5μM oligomeric Aβ, there was no difference in **A)** pCREB133 levels (Students *t*-test; p=0.65) or **B)** pCREB129 levels (Student's *t*-test; p=0.43) compared to untreated controls. Graphs represent integrated intensity of each target as defined by densitometric counts/mm^2. Error bars represent S.E.M. n=18/group. These experiments were carried out 3 times with similar group sizes and results.

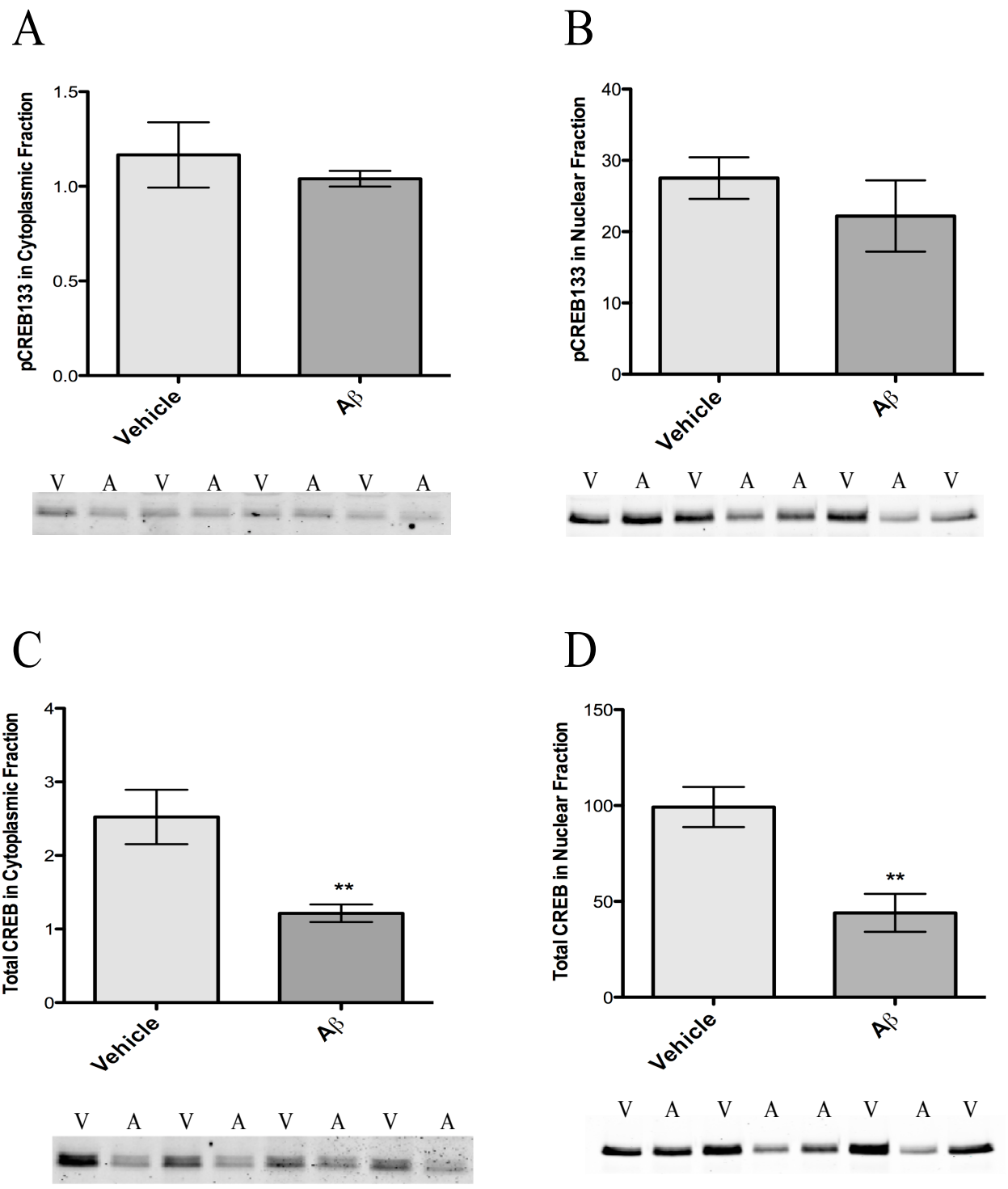

A
pCREB133 in Cytoplasmic Fraction
1.5
1.0
0.5
0.0
Vehicle
Aβ
V A V A V A V A
B
pCREB133 in Nuclear Fraction
40
30
20
10
0
Vehicle
Aβ
V A V A A V A V
C
Total CREB in Cytoplasmic Fraction
4
3
2
1
0
**
Vehicle
Aβ
V A V A V A V A
D
Total CREB in Nuclear Fraction
150
100
50
0
**
Vehicle
Aβ
V A V A A V A V

Figure 12: Aβ does not sequester CREB outside the nucleus; Total CREB protein is decreased by Aβ treatment. Western blot analysis of vehicle (V) and Aβ (A) treated SH-SY5Y cells revealed that, following treatment with 5μM oligomeric Aβ, there was no change in pCREB133 levels in either **A)** cytoplasmic fraction (Student's *t*-test; p=0.55) or **B)** nuclear fraction (Student's *t*-test; p=0.39) of differentiated SH-SY5Y cells compared to untreated controls. Conversely, 5μM oligomeric Aβ treatment resulted in significantly reduced total CREB protein in both the **C**) cytoplasmic fraction (Student's *t*-test; **p=0.009) and **D)** nuclear fraction (Student's *t*-test; **p=0.008). Graphs represent integrated intensity of each target as defined by densitometric counts/mm^2. Error bars represent S.E.M. n=4-5/group.

4.1.3 Activation of PKA, but not inactivation of GSK3β, prevented Aβ-induced down-regulation of BDNF

An inhibitor of GSK3β or activator of PKA was added in conjunction with $A\beta_{42}$ treatment to determine if manipulating either pathway could prevent Aβ-induced BDNF down-regulation. CT 99021 is a potent inhibitor of GSK3β that prevents phosphorylation of GSK3β substrates (Bain et al., 2007). Cells were exposed to either 2μM CT 99021 alone or in combination with $A\beta_{42}$ and compared to both $A\beta_{42}$ alone and vehicle treated groups. CT 99021-treated SH-SY5Y cells exhibited significantly reduced activation of GSK3β, as indicated by significantly increased levels of β-catenin (normalized to alpha-tubulin), which active GSK3β works to decrease, compared to control cells (Figure 13A; $p=0.012$). There was no effect of CT 99021 treatment on BDNF expression in the absence of Aβ (Figure 13B; $p=0.50$). Furthermore, CT 99021 inactivation of GSK3β was not sufficient to prevent Aβ-induced down-regulation of BDNF, as there was no difference between cells treated with Aβ and those treated with Aβ+CT 99021 (Figure 13B; $p=0.87$).

Forskolin, a selective activator of adenylate cyclase (Seamon and Daly, 1981; Vitolo et al., 2002), was used to activate PKA. Following treatment with 30μM forskolin, differentiated SH-SY5Y cells exhibited significantly increased activation of PKA, as indicated by significantly increased pCREB133 levels (normalized to total CREB), compared to control cells (Figure 14A; $p<0.001$). However, there was no effect of forskolin treatment on BDNF expression in the absence of Aβ (Figure 14B; $p=0.86$). Unlike the inactivation of GSK3β, activation of PKA prior to Aβ administration was sufficient to prevent Aβ-induced BDNF down-regulation. Cells treated with $A\beta_{42}$ alone

had significantly lower BDNF mRNA than cells treated with Aβ + forskolin (Figure 14B; p=0.01). However, when forskolin was administered 24 hours after Aβ administration (a time at which BDNF was down-regulated compared to vehicle-treated cells, *data not shown*; p=0.006), forskolin was not able to rescue Aβ-induced BDNF down-regulation. Cells exposed to forskolin after Aβ treatment exhibited BDNF levels that were not significantly different from cells treated with $A\beta_{42}$ alone (Figure 14C; p=0.654).

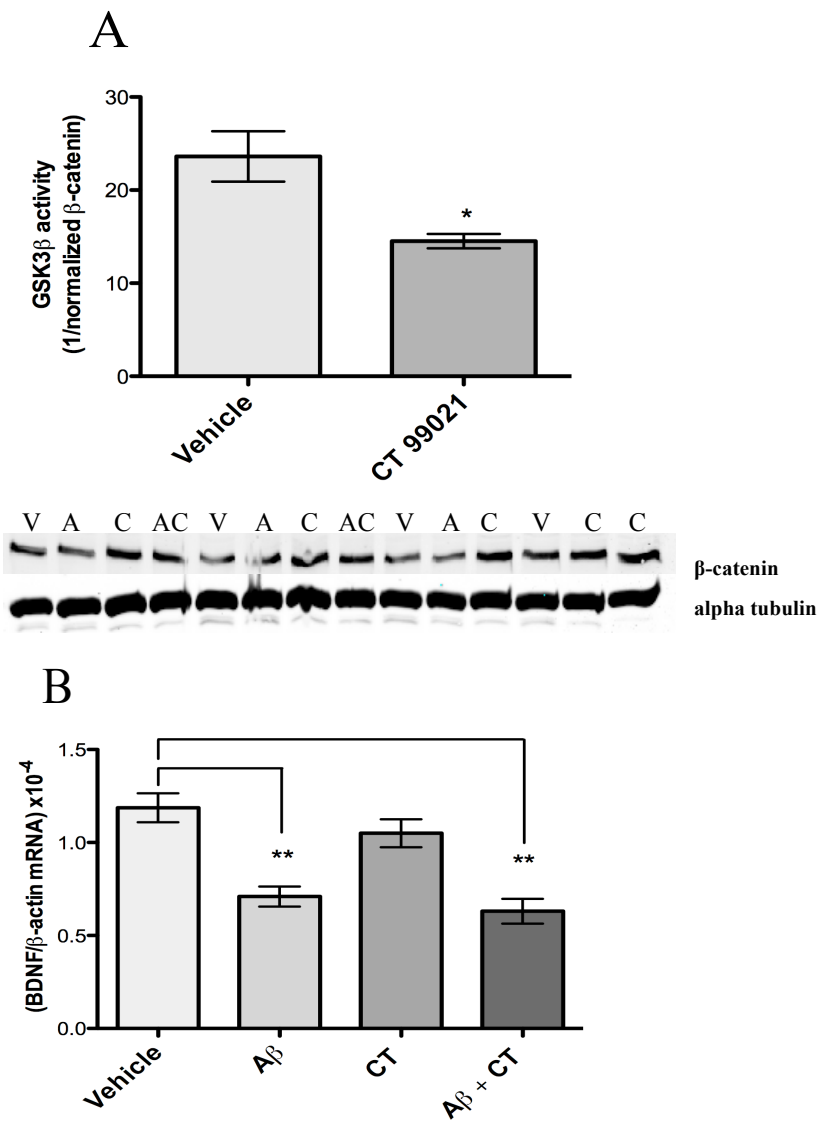
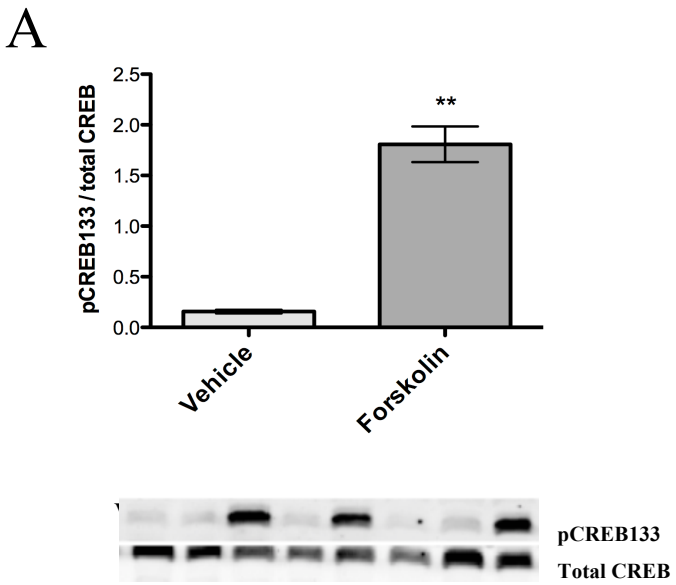
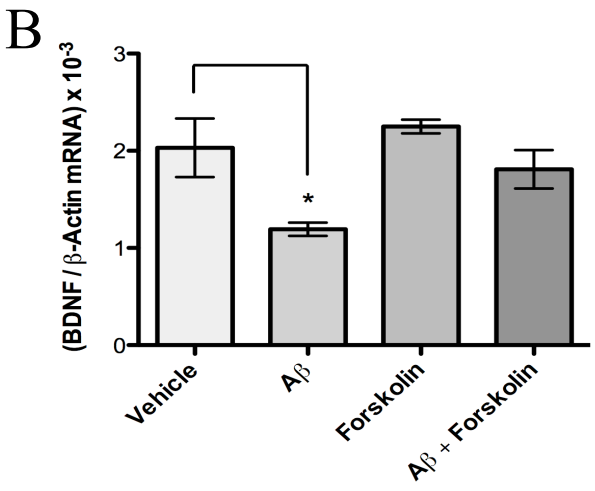


Figure 13: Inhibiting GSK3β is not sufficient to prevent Aβ-induced BDNF down-regulation. Treating differentiated SH-SY5Y cells with 2μM CT 99021 was sufficient to **A)** significantly decrease GSK3β activity as measured by increased levels of β-catenin levels (normalized to alpha-tubulin) (Student's *t*-test; *p=0.012). Western blot analysis was performed on all groups: vehicle (V), Aβ alone (A), CT 99021 (C) and Aβ+CT (AC). CT 99021 was unable to rescue Aβ-induced BDNF down-regulation: **B)** Aβ alone and Aβ+CT significantly down-regulated BDNF compared to control cells (one-way ANOVA and *post-hoc* Tukey's test **p<0.001). Error bars represent S.E.M. n=5-6/group.


A
pCREB133 / total CREB
2.5
2.0
1.5
1.0
0.5
0.0
**
Vehicle
Forskolin
pCREB133
Total CREB


B
(BDNF / β-Actin mRNA) x 10-3
3
2
1
0
*
Vehicle
Aβ
Forskolin
Aβ + Forskolin

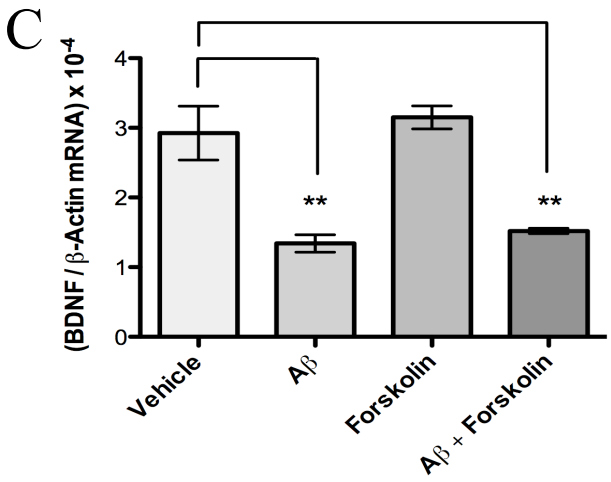

C
(BDNF / β-Actin mRNA) x 10-4
4
3
2
1
0
**
**
Vehicle
Aβ
Forskolin
Aβ + Forskolin

Figure 14: Activating PKA is sufficient to prevent but not rescue Aβ-induced BDNF down-regulation. A) Treating differentiated SH-SY5Y cells with 30μM forskolin significantly increased PKA activity as measured by phosphorylated CREB Ser-133 levels (normalized to total CREB) (Students *t*-test; **p<0.001). Western blot analysis was performed on all groups: vehicle (V), Aβ alone (A), forskolin (F) and Aβ+forskolin (AF). **B)** Aβ alone significantly down-regulated BDNF compared to control cells (one-way ANOVA and *post-hoc* Tukey's test *p=0.02), but when forskolin was administered prior to Aβ (Aβ+forskolin), this treatment resulted in levels of BDNF mRNA that were not significantly different from control cells (one-way ANOVA and *post-hoc* Tukey's test, p=0.86). Error bars represent S.E.M. n=5-6/group. **C)** Administration of forskolin 24 hr following Aβ did not rescue BDNF levels. Both Aβ alone (one-way ANOVA and *post-hoc* Tukey's test **p<0.001) and Aβ+forskolin (one-way ANOVA and *post-hoc* Tukey's test **p=0.006) groups had significantly reduced BDNF mRNA normalized to β-actin mRNA. Error bars represent S.E.M. n=6/group.

4.2 OBJECTIVE 2:

4.2.1 Over-expression of wild-type tau in transgenic mice down-regulates BDNF

hTau and 8c-het transgenic mice both over-express human, wild-type tau, but 8c-het mice are on a heterozygous mouse tau background whereas hTau mice are on a homozygous mouse tau knockout (KO) background (Andorfer et al., 2003; Duff et al., 2000). The expression of tau in 8c-het and hTau mice and lack of tau expression in tau KO mice was confirmed via Western blotting (Figure 15A). In both of these transgenic tau over-expressing mouse models, BDNF mRNA was significantly down-regulated [hTau mice vs. wild-type mice (GLM and *post hoc* Tukey's test, $p=0.006$) and 8c-het mice vs. wild-type mice (GLM and *post hoc* Tukey's test, $p=0.017$); Figure 15]. BDNF mRNA levels, however, did not differ between TauKO mice and non-transgenic controls (GLM and *post hoc* Tukey's test, $p=0.537$, Figure 15B).

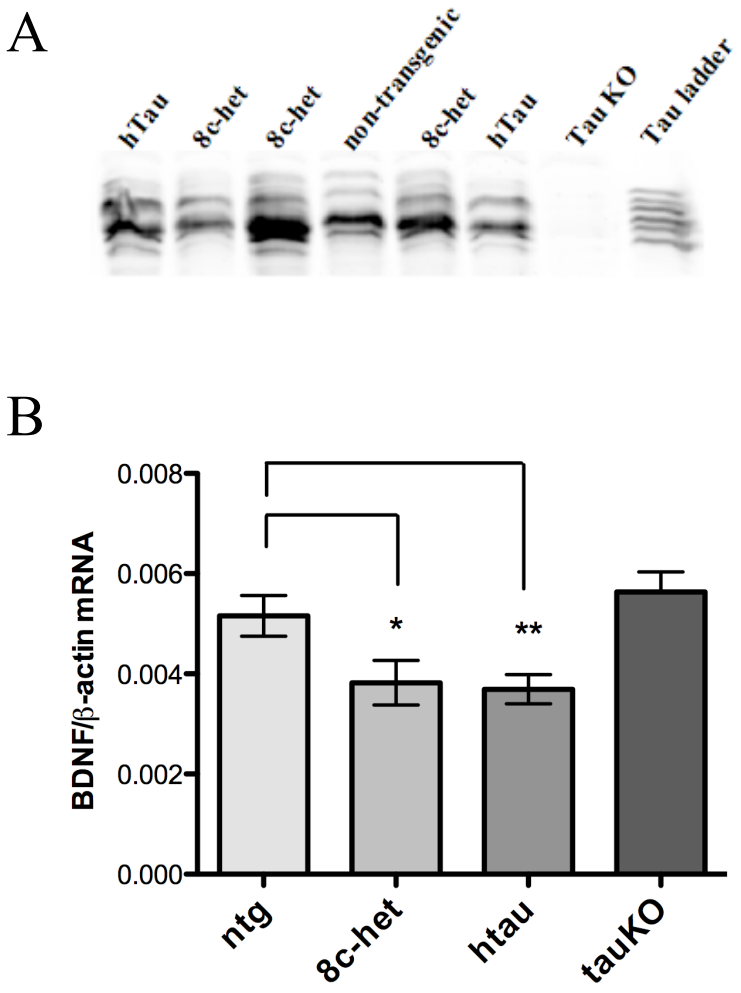
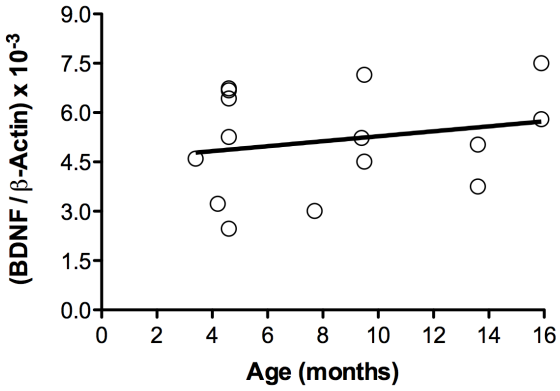


Figure 15: Expression of human, wild-type tau in transgenic mice down-regulates BDNF expression. A) Both 8c-het and hTau mice express TBS-soluble tau protein as measured via Western blot, while TauKO mice are completely devoid of tau protein expression. **B)** Both 8c-het (*p=0.017) and hTau (**p=0.006) transgenic mice show significantly down-regulated BDNF mRNA compared to non-transgenic controls. There was no significant difference in BDNF expression between TauKO mice and non-transgenic controls (p=0.537). Generalized Linear Model (GLM) and *post hoc* Tukey's test. Error bars represent S.E.M. n=11-15/group.

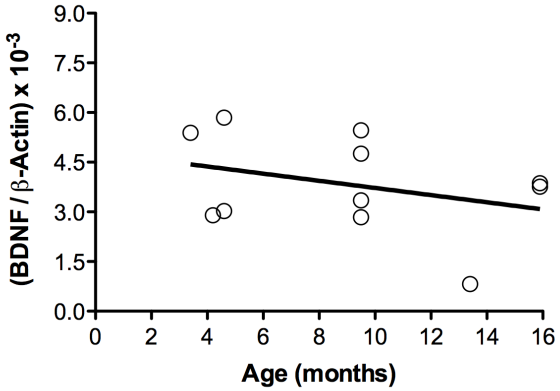
4.2.2 Tau-induced BDNF down-regulation is independent of age

Tau-induced neurotoxicity is thought to precede the formation of NFTs (Gomez-Isla et al., 1996; Yoshiyama et al., 2007). Further, loss of BDNF is an early event in the progression of AD, occurring in mild cognitive impairment (MCI) and in mouse models of amyloid-β and tau over-expression prior to the formation of plaques and NFTs (Francis et al., 2012; Peng et al., 2005). In this study, mice sampled were between 3 and 16 months of age. This age range was intentionally large to determine if BDNF expression preceded the development of NFTs in the hTau animals. Our findings support the early loss of BDNF expression in both 8c-het and hTau mouse models, where BDNF levels are consistently lower than non-transgenic control animals across all ages tested. Furthermore, our results support the toxicity of soluble tau, prior to the formation of NFTs in hTau mice (Figure 16). There was no correlation between age and BDNF expression in non-transgenic control animals (Pearson correlation $r=0.22$, $p=0.438$; Figure 16A), 8c-het mice (Pearson correlation $r=-0.24$, $p=0.345$; Figure 16B) or hTau mice (Pearson correlation $r=-0.12$, $p=0.690$; Figure 16C).

A


(BDNF / β-Actin) x 10-3
9.0
7.5
6.0
4.5
3.0
1.5
0.0
0
2
4
6
8
10
12
14
16
Age (months)

B


(BDNF / β-Actin) x 10-3
9.0
7.5
6.0
4.5
3.0
1.5
0.0
0
2
4
6
8
10
12
14
16
Age (months)

C

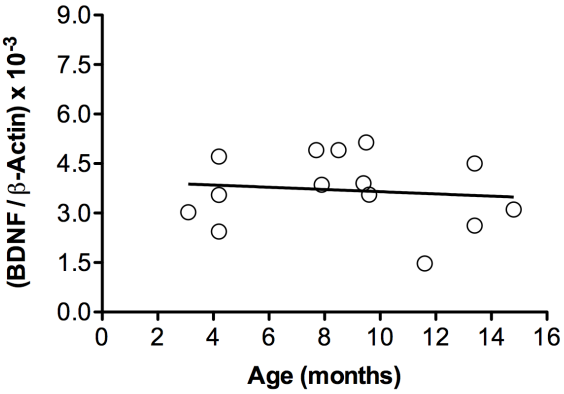

(BDNF / β-Actin) x 10-3
9.0
7.5
6.0
4.5
3.0
1.5
0.0
0
2
4
6
8
10
12
14
16
Age (months)

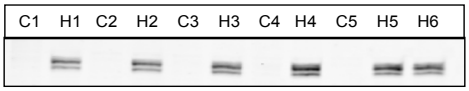
Figure 16: Tau-induced BDNF loss is an early event, prior to the formation of NFTs in hTau mice. Both 8c-het and hTau transgenic mice show significantly down-regulated BDNF expression, compared to control mice, starting at the earliest developmental age tested. BDNF expression (normalized to β-actin) in these tau over-expressing transgenic models, as well as in non-transgenic control mice, does not correlate with age. There was no correlation between BDNF expression and age in **A)** non-transgenic control mice (Pearson correlation r=0.22, p=0.438, **B)** 8c-het mice (Pearson correlation r=-0.24, p=0.345) or **C)** hTau animals (Pearson correlation r=-0.12, p=0.690).

4.2.3 Over-expression of wild-type tau in transfected human cells down-regulates total BDNF and BDNF transcript IV

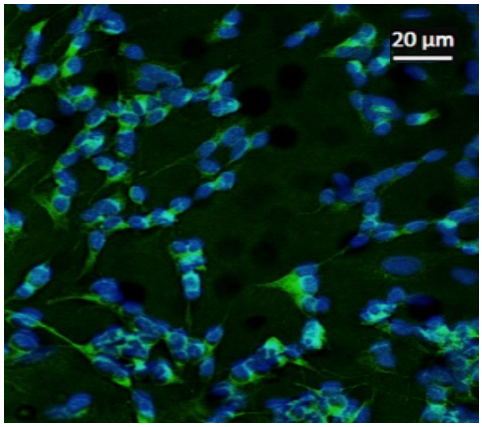
Tau-transfected, differentiated SH-SY5Y cells expressed significant amounts of V5 protein compared to non-transfected, V5-negative controls (Figure 17A), which was indicative of the presence of hTau40-V5-pcDNA3.2 plasmid in the transfected cells following differentiation (Figure 17B).

BDNF mRNA was significantly down-regulated in wild-type human tau hTau40-transfected SH-SY5Y cells compared to non-transfected controls (Student's *t*-test, $p<0.0001$; Figure 17C). Additionally, BDNF Transcript IV mRNA was also significantly decreased in these wild-type tau over-expressing human neuroblastoma cells compared to controls (Student's *t*-test, $p<0.0001$; Figure 17D).

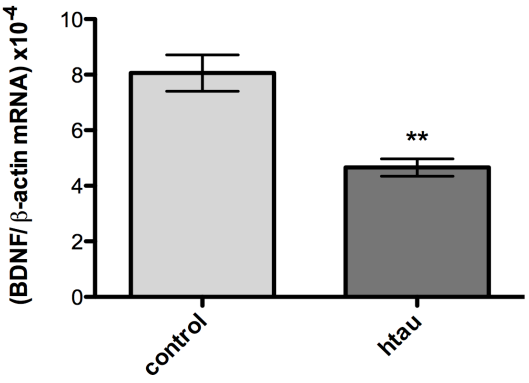
A


C1 H1 C2 H2 C3 H3 C4 H4 C5 H5 H6

B


20 µm

C


(BDNF/ β-actin mRNA) x10-4
10
8
6
4
2
0
**
control
htau

D

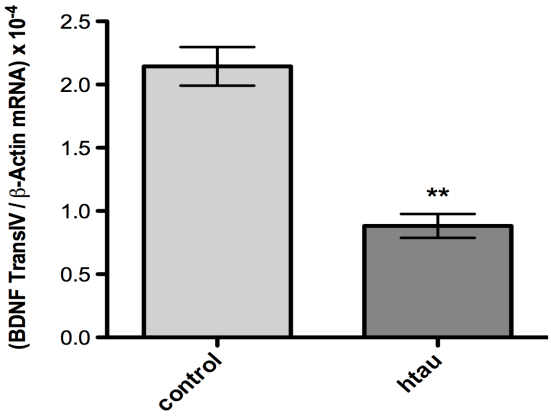

(BDNF TranslV / β-Actin mRNA) x 10-4
2.5
2.0
1.5
1.0
0.5
0.0
**
control
htau

Figure 17: Human neuroblastoma cells over-expressing wild-type human tau down-regulate BDNF and BDNF Transcript IV expression. A) SH-SY5Y cells transfected with hTau40-V5-pcDNA3.2 plasmid (H) express V5-tagged protein that was absent from non-transfected control cells (C) **B)** Fluorescent micrograph depicting V5-tagged human tau proteins (green) and DAPI-stained nuclei (blue) in hTau40-V5-pcDNA3.2 transfected SH-SY5Y cells after 30 days of selection with G418. **C)** BDNF mRNA (normalized to β-actin) compared to controls (Student's *t*-test, $^{**}p<0.0001$) and **C)** BDNF Transcript IV mRNA (normalized to β-actin) compared to controls (Student's *t*-test, $^{**}p<0.0001$). Error bars represent S.E.M. n=9/group.

4.3 OBJECTIVE 3:

4.3.1 Aβ over-expressing APP23 mice significantly down-regulate BDNF

BDNF mRNA was significantly reduced in APP23 mice compared to wild-type animals (one-way ANOVA and *post hoc* Tukey's test, p=0.017; Figure 18). In agreement with previous reports of Aβ-induced BDNF down-regulation *in vitro* and *in vivo* (Garzon and Fahnestock, 2007; Tong et al., 2004), the over-expression of Aβ in APP23 mice was sufficient to significantly decrease BDNF expression.

4.3.2 Tau partially mediates Aβ-induced BDNF down-regulation

While APP23 mice significantly down-regulate BDNF, crossing these APP23 mice with TauKO mice partially rescued BDNF levels, as BDNF expression in APP23xTauKO mice was intermediate between non-transgenic control mice and APP23 mice and was not statistically different from non-transgenic mice (one-way ANOVA and *post hoc* Tukey's test, p=0.128; Figure 18).

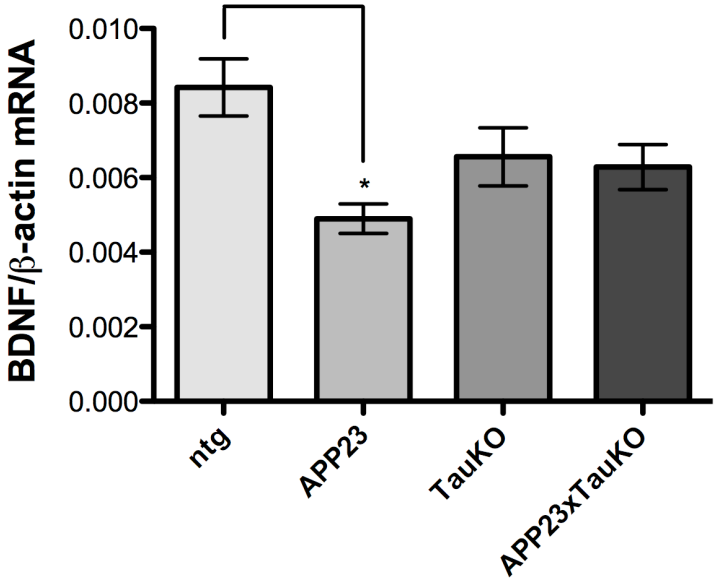


Figure 18: Tau partially mediates Aβ-induced down-regulation of BDNF expression. Over-expression of Aβ in APP23 transgenic mice resulted in a significant reduction of BDNF mRNA compared to non-transgenic animals (one-way ANOVA and *post hoc* Tukey's test *p=0.017). However APP23xTauKO mice expressed levels of BDNF mRNA that were intermediate between wild-type and APP23 mice and were no different from non-transgenic animals (one-way ANOVA and *post hoc* Tukey's test p=0.262). Error bars represent S.E.M. n=5-9/group.

CHAPTER 5: DISCUSSION

5.1 OBJECTIVE 1

In Alzheimer's disease, soluble aggregated amyloid-β is thought to be the primary neurotoxic insult leading to synaptic loss and neurodegeneration. However, the mechanisms that lead from Aβ aggregation to the pathological and physical symptoms of AD are not clear. In this study, we confirmed that oligomeric Aβ significantly down-regulates basal BDNF transcription (Garzon and Fahnestock, 2007) and determined the mechanism. While previous work has examined the regulation of CREB and BDNF following cell stimulation (Vitolo et al., 2002; Tong et al., 2004), this work focuses on unstimulated regulation of BDNF. BDNF is sorted into vesicles of both the constitutive and regulated protein secretion pathways (Brigadski et al., 2005; Heymach et al., 1996; Mowla et al., 2001; Mowla et al., 1999; Thomas and Davies, 2005). Further, the localization of different BDNF transcripts within a neuron is altered following cell stimulation, which is thought to be crucial for conferring spatial and temporal specificity to the different effects of BDNF (Baj et al., 2013; Tongiorgi et al., 2006). As such, understanding the regulation of basal levels of BDNF in the absence of exogenous stimulation is also critical. Further, the dramatic down-regulation of BDNF in AD is not solely activity-dependent. The data presented here demonstrate a new mechanism for Aβ-induced BDNF down-regulation, different from its activity-dependent regulation.

5.1.1 Oligomeric $A\beta_{1\text{-}42}$ down-regulates basal BDNF expression without affecting cell viability

In this study, we used retinoic acid (RA)-differentiated human neuroblastoma SH-SY5Y cells to replicate previous findings that sub-toxic treatment with oligomeric amyloid-β results in significant reduction of basal BDNF expression (Garzon and Fahnestock, 2007). RA-differentiated SH-SY5Y cells were chosen because they exhibit neuronal morphology, express BDNF and its receptor TrkB and are dependent on BDNF for survival (Encinas et al., 2000; Feng et al., 2001; Kaplan et al., 1993). Further, the response of these cells to Aβ treatment mirrors that of human cortical neurons (Lambert et al., 1994). In addition, using this immortalized cell line allows for further manipulations to these cells, such as stable transfections, which traditionally have very low efficiency in primary neuronal cultures. Here, these differentiated SH-SY5Y cells were treated with 5μM Aβ, specifically because this concentration has been shown to disrupt cellular signaling (Tong et al., 2001, 2004) and has been shown previously in our lab to specifically down-regulate BDNF mRNA (Garzon & Fahnestock 2007) without resulting in cell death. Treatment with this sub-toxic dose of Aβ allows us to demonstrate that basal BDNF expression is down-regulated by Aβ specifically and not as a consequence of cell death. The down-regulation of BDNF was quantified via qRT-PCR, which allows for an accurate assessment of the quantity of BDNF mRNA. This study did not include an analysis of BDNF protein via Western blot to correlate with the decreased BDNF expression, given a lack of specific BDNF protein antibodies commercially available at this time. However, we know from previous work in our lab and other reports that both

cortical BDNF mRNA (Holsinger et al., 2000; Garzon et al., 2002; Yamashita et al., 1999) and BDNF protein (Connor et al., 1997; Ferrer et al., 1999) are down-regulated in Alzheimer's disease, suggesting that the reduction in BDNF mRNA correlates with reduced BDNF protein. BDNF expression under sub-toxic Aβ conditions is reduced by half (Figure 9), approximating the amount of BDNF decrease found in cortex of Alzheimer's disease patients (Holsinger et al., 2000). This degree of BDNF down-regulation is consistent with the notion that BDNF reduction still allows neurons to survive, albeit with reduced function, producing synaptic loss and memory dysfunction long before frank cell loss (Fahnestock, 2011).

It has been shown previously that RA-differentiated SH-SY5Y cells express all seven BDNF transcripts tested at similar levels as in human cortical tissue, with BDNF transcript IV accounting for more than half the total BDNF expressed (Garzon and Fahnestock, 2007). BDNF transcript IV is not only the most highly expressed BDNF transcript in RA-induced SH-SY5Y cells and in human cortex, but it is significantly reduced in AD and following Aβ treatment (Garzon et al., 2002; Garzon and Fahnestock, 2007). BDNF transcript IV is regulated at least in part through CREB (Pruunsild et al., 2011; Shieh et al., 1998; Tao et al., 1998). The phosphorylation and subsequent activation of CREB results from the activity of several kinase pathways including PKA, PKC and PI3K/AKT (Walton and Dragunow, 2000).

5.1.2 Aβ treatment of differentiated SH-SY5Y cells decreases CREB transcription but does not alter CREB phosphorylation or nuclear localization

It has been shown that following cell stimulation, Aβ can inactivate PKA *in vitro*, which decreases pCREB133 (Vitolo et al., 2002). Aβ also inhibits the Ras/ERK and PI3K/AKT pathways (Tong et al., 2004), thereby decreasing CREB activation by increasing GSK3β activity and pCREB129 levels. While the inactivation of CREB via phosphorylation may play an important role in the effect of Aβ on stimulated cells *in vitro*, we show here that the levels of phosphorylated CREB (both pCREB133 and pCREB129) are unaffected by Aβ treatment in the absence of cell stimulation. Understanding the regulation of basal levels of BDNF in the absence of stimulation remains critically important, as the dramatic down-regulation of BDNF in AD is not solely activity-dependent. Results here suggest that Aβ-induced basal BDNF down-regulation is not mediated by changes in CREB phosphorylation. Thus, Aβ reduces basal and activity-induced BDNF expression by different mechanisms, which implies that therapeutic interventions targeting BDNF regulation in AD must consider both mechanisms of Aβ-induced BDNF down-regulation in order to be fully effective.

Another mechanism of transcription factor inactivation is their sequestration outside of the nucleus. For example, the transcription factor SFPQ is sequestered in the cytoplasm in AD and in mutated tau-transfected SH-SY5Y cells (Ke et al., 2012). Additionally, mutant huntingtin sequesters CREB binding protein (CBP), preventing it from entering the nucleus to enhance CREB binding (Choi et al., 2012). We investigated whether Aβ could sequester CREB outside the nucleus, but instead we found that the levels of total

CREB in both the nucleus and the cytoplasm of Aβ-treated cells were significantly lower than in control cells. Thus, CREB is not sequestered in the cytoplasm, but rather the amount of total CREB protein in the cell is reduced following treatment with Aβ oligomers. This was verified by our finding that Aβ treatment significantly down-regulates CREB mRNA. Reduced CREB, in turn, significantly decreases BDNF transcription.

CREB is an essential component of molecular pathways required for learning and memory (Barco et al., 2003). Therefore, down-regulation of CREB by Aβ is expected to lead to cognitive deficits in AD. While it was long believed that CREB regulation was mediated only by phosphorylation, there have been several reports of CREB transcriptional regulation (Brecht et al., 1994; Walker et al., 1995; Widnell et al., 1996; Widnell et al., 1994). It has been reported that CREB mRNA expression may be regulated at least in part by activation of the cAMP pathway (Widnell et al., 1996; Widnell et al., 1994). However, this finding was shown to be dependent on the cell type used (Coven et al., 1998; Widnell et al., 1996). Recent findings have shown that CREB transcription is reduced in both AD post-mortem hippocampal tissue and in Aβ-treated rat hippocampal neurons (Pugazhenthi et al., 2011). Our work confirms these data in an Aβ-treated human neuroblastoma cell line. The current report is the first to show that this Aβ-induced CREB mRNA down-regulation is associated with the significant down-regulation of basal levels of BDNF expression. It has been suggested that Aβ may down-regulate CREB transcription via oxidative stress, as pre-incubation of neurons with the antioxidant N-acetyl cysteine prevented Aβ-induced decreases in CREB mRNA (Pugazhenthi et al.,

2011).

5.1.3 Activation of PKA, but not inactivation of GSK3β, prevented Aβ-induced down-regulation of BDNF

While others have shown that both the activating phosphorylation of CREB (Vitolo et al., 2002) and inactivating phosphorylation of CREB (DaRocha-Souto et al., 2012) play an important role in Aβ-induced toxicity, we show here that in the absence of cell stimulation, Aβ has no effect on the phosphorylation of CREB. This importantly distinguishes that basal and activity-induced BDNF down-regulation rely on different mechanisms. Furthermore, while inactivating GSK3β and thus reducing pCREB129 has no effect on Aβ's ability to down-regulate basal BDNF, increasing the levels of pCREB133 using forskolin prior to Aβ addition can prevent Aβ-induced BDNF down-regulation. However, if administered after Aβ-induced BDNF down-regulation, forskolin is unable to rescue BDNF expression. This result supports the view that altering CREB phosphorylation after Aβ down-regulates CREB transcriptionally is not sufficient to rescue Aβ-induced basal BDNF down-regulation.

5.1.4 Significance

While brain-derived neurotrophic factor is significantly down-regulated in Alzheimer's disease and the amount of BDNF decrease is directly correlated with the degree of cognitive decline (Peng et al., 2005), the mechanism of basal BDNF down-regulation remained unclear. However, our current findings reveal a novel mechanism of

BDNF down-regulation, which could lead to new methods to combat BDNF decline in Alzheimer's disease. Increasing BDNF levels has been shown to greatly improve learning and memory deficits in animal models (Ando et al., 2002; Blurton-Jones et al., 2009; Fahnestock et al., 2012; Nagahara et al., 2013; Nagahara et al., 2009). Further, increasing CREB activity via viral delivery of CREB activators, CBP (Caccamo et al., 2010; Espana et al., 2010) and CRTC1 (Espana et al., 2010), has successfully reversed synaptic atrophy and learning and memory impairments in transgenic mice. These findings highlight the possibility that increasing BDNF expression by modulating CREB mRNA levels in AD, even after clinical onset of the disease, could rescue memory impairments and cognitive function.

5.2 OBJECTIVE 2

In AD, although the primary insult may be Aβ over-expression, ultimately cognitive dysfunction and neurodegeneration are thought to be a result of alterations in tau (Gotz, 2001; Iqbal and Grundke-Iqbal, 2008; Lewis et al., 2001; Masliah et al., 2001; Pennanen and Gotz, 2005; Roberson et al., 2011). However, the mechansims that lead from alterations in tau to the cognitive symptoms of AD are not clear. Our lab has previously shown that there is decreased BDNF mRNA and protein in the parietal cortex of human tauopathy (PiD and CBD) patients (Belrose et al., 2014). In tauopathies, we reported down-regulation of BDNF transcript IV, which is largely regulated by CREB-mediated transcription (Pruunsild et al., 2011; Shieh et al., 1998; Tao et al., 1998). In the present study, we demonstrate that soluble, wild-type tau significantly down-regulates

BDNF transcription *in vitro* and *in vivo*. Furthermore, we determined that tau down-regulates BDNF transcript IV *in vitro*.

5.2.1 Over-expression of wild-type tau in transgenic mice down-regulates BDNF

Both 8c-het and hTau transgenic mice significantly down-regulate BDNF mRNA compared to non-transgenic control animals. These results importantly demonstrate that the over-expression of wild-type tau devoid of mutations is sufficient to cause neurotrophin dysregulation. Historically, the majority of research into understanding tau toxicity has focused on both transgenic animal and cell culture models harboring disease-causing tau mutations, such as transgenic mice expressing the P301L tau mutation (Lewis et al., 2000) or mutated Tau441 cell lines (Loffler et al., 2012). In contrast, the current study focuses on wild-type tau toxicity, which highlights that without any mutation, the over-expression of wild-type tau, whether induced by Aβ or some other toxic insult, is capable of down-regulating BDNF, which is highly relevant to understanding the pathophysiology of sporadic AD, by far the most common form of this neurodegenerative disease.

5.2.2 Tau-induced BDNF down-regulation is independent of age

The results presented here further demonstrate that tau-induced BDNF down-regulation is an early event. The down-regulation of BDNF in both the 8c-het and hTau animals observed was independent of the ages examined. An intentionally large age range was used for these transgenic animals to determine whether BDNF down-regulation was

correlated with age. Previously our lab reported that BDNF down-regulation is an early event in the progression of AD (Peng et al., 2005). This is supported here by findings that BDNF was down-regulated in tau over-expressing transgenic mice from the earliest ages examined. Specifically the down-regulation of BDNF in hTau mice younger than 15 months, the age at which NFT formation is initiated, supports that tau down-regulates BDNF prior to the formation of NFTs. This is consistent with a previous report of cell death in the hTau model preceding the formation of NFTs (Andorfer et al., 2005). The present study is the first to demonstrate early and persistent neurotrophin dysregulation by soluble tau.

5.2.3 Tau-induced BDNF down-regulation is independent of the formation of NFTs

By examining the age of BDNF down-regulation in hTau animals we determined that BDNF is down-regulated prior to the formation of NFTs. However, we also determined more directly that tau-induced BDNF down-regulation is independent of the formation of NFTs by examining tau-induced BDNF down-regulation in 8c-het mice, which do not develop NFT pathology. It has previously been shown that significant synaptic loss can occur prior to NFT formation (Yoshiyama et al., 2007). Furthermore, in AD before the formation of NFTs, neuronal loss can be detected along with the presence of tau oligomers (Gomez-Isla et al., 1996), suggesting that soluble, aggregated tau species are neurotoxic. In addition, both 8c-het mice (Andorfer et al., 2005) and a Drosophila tauopathy model (Wittmann et al., 2001) exhibit neurodegeneration despite their lack of NFT-like tau pathology. Lastly, hTau mice begin to experience neuronal cell loss prior to the onset of NFT pathology, supporting the notion that NFTs are not the primary cause of

neurodegeneration (Andorfer et al., 2005). However, the mechanism of soluble tau induced cell death is not clear. In this study, we have shown that both 8c-het and hTau mice down-regulate BDNF, and further that their reduction of BDNF is roughly equal. These findings provide further evidence that NFTs are not required for toxicity. Rather, soluble, hyperphosphorylated tau exerts its toxicity, at least in part, via reduction in BDNF expression.

5.2.4 Over-expression of wild-type tau in transfected human cells down-regulates BDNF and BDNF transcript IV

To build upon results obtained using transgenic mice, human neuroblastoma SH-SY5Y cells were transfected with human wild-type tau to determine if tau is capable of down-regulating BDNF in a human culture system. As discussed in *Section 5.1.2*, differentiated SH-SY5Y cells are an excellent model for human cortical neurons, and determining the effect of soluble wild-type tau over-expression on these cells is an ideal way to understand the effect of tau on BDNF regulation in AD. Similar to tau over-expression in transgenic mice, tau over-expressing human SH-SY5Y cells significantly down-regulate BDNF mRNA compared to non-transfected control cells. Further, BDNF transcript IV was also significantly down-regulated in cells over-expressing wild-type human tau.

BDNF transcript IV is down-regulated in human AD cortical tissue (Garzon et al., 2002), in mouse models of AD (Peng et al., 2009) and in SH-SY5Y cells treated with Aβ (Garzon and Fahnestock, 2007). We know that BDNF transcript IV accounts for

approximately half of the total BDNF mRNA found in the cortex (Garzon and Fahnestock, 2007; Pruunsild et al., 2007) and is transcriptionally regulated by CREB (Pruunsild et al., 2011; Shieh et al., 1998; Tao et al., 1998). The current investigation demonstrates that over-expression of wild-type tau alone is capable of down-regulating BDNF and that it does so via transcript IV. Together with our previous findings that amyloid-β down-regulates BDNF transcript IV (Garzon and Fahnestock, 2007), these results suggest that Aβ and tau may down-regulate BDNF via the same pathway.

5.2.5 Significance

The current investigation has established that over-expression of wild-type tau, devoid of pathological mutations and prior to the formation of NFTs, down-regulates BDNF. This is the first direct report of tau-induced dysregulation of trophic support. While the previous investigation from our lab was the first to provide evidence for a link between tau and BDNF (Belrose et al., 2014), it was confounded by the limitations of post-mortem tissue. A number of contributing factors could result in BDNF down-regulation in post-mortem tissue, including: the cause of death, post-mortem interval, and use of medications or comorbid disorders. The current investigation has eliminated these confounds by examining tau over-expression in both transgenic animals and human neuroblastoma cells. We demonstrated in both systems that the over-expression of soluble, wild-type tau alone causes significant BDNF down-regulation. Taken together with our previous findings that amyloid-β down-regulates BDNF (Garzon and Fahnestock, 2007; Rosa and Fahnestock, 2015), and findings that tau is a down-stream

effector of Aβ (Ittner et al., 2010; Lewis et al., 2001; Masliah et al., 2001; Pennanen and Gotz, 2005), these results suggest that Aβ and tau may down-regulate BDNF via the same pathway.

5.3 OBJECTIVE 3

5.3.1 Tau partially mediates Aβ-induced BDNF down-regulation

Results here demonstrate that Aβ-induced BDNF down-regulation is at least partially rescued by the depletion of tau in APP23xTauKO mice. Prior to the current investigation, it was known that increased Aβ down-regulates BDNF in both cell culture (DaRocha-Souto et al., 2012; Garzon and Fahnestock, 2007; Rosa and Fahnestock, 2015) and transgenic mice (Peng et al., 2009). The latter is further supported by the current findings that APP23 transgenic mice, which over-express Aβ, also experience a significant reduction in BDNF mRNA compared to non-transgenic control animals. While this finding was expected based on previous reports of Aβ-induced BDNF down-regulation, this was the first report that this well-studied model of AD does significantly diminish BDNF expression. While this is a significant finding in itself, tau is now thought to be down-stream of Aβ and therefore a more direct mediator of neurodegeneration (Ittner et al., 2010; Lewis et al., 2001; Masliah et al., 2001; Pennanen and Gotz, 2005). Specifically, it has been shown that NFT-like changes can be induced by Aβ *in vivo* (Gotz et al., 2001) and *in vitro* (Busciglio et al., 1995). Furthermore, Aβ-induced neurodegeneration is prevented in primary neuronal cultures from TauKO mice (Rapoport et al., 2002), and knocking out tau in a transgenic AD mouse model can block Aβ-

induced cognitive impairments (Roberson et al., 2007). Whether the rescue of cognitive impairment is mediated by BDNF was not tested. In this study we demonstrate that tau mediates Aβ-induced BDNF down-regulation, as knocking out tau in APP23 mice restores BDNF levels to control values. BDNF levels in APP23xTauKO animals are not statistically significantly different from controls; however, they are intermediate between APP23 and control animals. Thus, while our findings demonstrate that tau mediates Aβ-induced BDNF down-regulation, we cannot rule out the possibility that Aβ also affects BDNF levels independently of tau.

5.3.2 Significance

This is the first report to show that tau is required for Aβ-induced neurotrophin dysregulation. These results suggest that BDNF loss may mediate tau neurotoxicity down-stream of Aβ, which greatly increases our understanding of BDNF regulation in AD. Additionally, other neurodegenerative diseases are also characterized by pathological accumulations of aggregated protein, including α-synuclein in Parkinson's disease (PD) (Polymeropoulos et al., 1997; Spillantini et al., 1997), huntingtin in Huntington's disease (HD) (Davies et al., 1997), and TDP-43 in amyotrophic lateral sclerosis (ALS) (Kwong et al., 2007; Neumann et al., 2006). In addition to AD, these neurodegenerative diseases also result in a significant down-regulation of BDNF mRNA (Howells et al., 2000; Mogi et al., 1999; Zuccato et al., 2001; Zuccato et al., 2008) and protein (Ferrer et al., 2000; Hock et al., 2000; Parain et al., 1999; Zuccato et al., 2008). Further, recent evidence demonstrates that accumulation of tau is a downstream consequence of protein

aggregation and a feature of all these disorders (Constantinescu et al., 2011; Fernandez-Nogales et al., 2014; Lei et al., 2010; Sengupta et al., 2015; Yang et al., 2003). These findings suggest that the current investigation focusing on tau-induced BDNF down-regulation, which mediates Aβ-induced BDNF down-regulation may have important implications for a variety of neurodegenerative diseases.

CHAPTER 6: CONCLUSIONS & FUTURE DIRECTIONS

6.1 CONCLUSIONS

Brain-derived neurotrophic factor is essential for cognition and memory and is decreased early in the progression of a number of neurodegenerative diseases including Alzheimer's disease. The amount of BDNF decrease in Alzheimer's disease is directly correlated with the degree of cognitive decline (Peng et al., 2005). The findings presented here have revealed a novel mechanism of Aβ-induced BDNF down-regulation, proven that wild-type soluble tau, devoid of any pathological mutations can down-regulate tau and determined that tau mediates Aβ-induced BDNF down-regulation. Taken together, these findings have greatly improved our understanding of BDNF down-regulation in AD and could have profound implications for therapeutic interventions in AD and tauopathies, primarily by suggesting that current treatments used to alleviate AD symptoms by targeting Aβ pathology alone may not be sufficient. Rather, targeting tau as a therapeutic approach in AD may be a more direct way to interrupt BDNF down-regulation, and also the subsequent impairments in memory and cognition. Further, evidence of the accumulation of tau as a downstream consequence of protein aggregation in other neurodegenerative diseases, such as HD, PD and ALS and the subsequent down-regulation of BDNF in these diseases, suggest that the understanding of tau-induced BDNF down-regulation revealed here may not only be important for therapeutic intervention in AD, but in a variety of neurodegenerative disorders.

6.2 FUTURE DIRECTIONS

This work has exposed a novel mechanism of Aβ-induced BDNF down-regulation and determined that soluble, wild-type tau is capable of down-regulating BDNF. Beyond expanding our current understanding of BDNF down-regulation in AD, this work has opened up several avenues for future experimentation. At the forefront is to determine what pathological modification of tau is required for tau-induced BDNF down-regulation and to begin to elucidate a mechanism of tau-induced BDNF down-regulation as was done for Aβ. As such, the most direct future directions to expand on this work would answer the following questions: 1) which pathological modification of tau is required for tau-induced BDNF down-regulation? 2) Does dysregulated CREB mediate tau-induced BDNF down-regulation? 3) Does tau interfere with BDNF axonal transport?

6.2.1 Which pathological modification of tau is required for tau-induced BDNF down-regulation?

There are a number of post-translational modifications of tau that may be required for tau toxicity, and specifically for tau-induced BDNF down-regulation. Namely, hyperphosphorylation, aggregation and truncation of tau are all increased in AD (Buee et al., 2000; Hrnkova et al., 2007; Kolarova et al., 2012; Sergeant et al., 2008). As such, it would be a suitable follow-up from the current investigation to determine which of these modifications of tau is required for tau-induced BDNF down-regulation. It is hypothesized here that hyperphosphorylation, aggregation and/or truncation are required for BDNF-down-regulation. To address this hypothesis, human neuroblastoma SH-SY5Y

cells will be transfected with mutated tau plasmids following the same protocol used in Objective 2 of the presented research. These tau plasmids will be mutated either at specific epitopes that are known to be hyperphosphorylated in AD, or sites required for tau aggregation and/or truncation. The mutations at these specific epitopes will prevent the hyperphosphorylation, aggregation and/or truncation of tau. Therefore, if any of these pathological modifications of tau are required for tau-induced BDNF down-regulation, BDNF levels will be rescued in cells expressing mutated tau when compared to cells expressing wild-type tau, which I have shown here significantly down-regulate BDNF. Overall, this future direction will help to determine which pathological modifications of tau mediate tau-induced BDNF down-regulation. This information will significantly impact our ability to intervene with tau-induced BDNF down-regulation. For instance, if the aggregation of tau alone mediates BDNF down-regulation, inhibiting the aggregation of tau alone may rescue BDNF levels.

6.2.2 Does dysregulated CREB mediate tau-induced BDNF down-regulation?

In order to understand how wild-type, soluble tau is capable of down-regulating BDNF, another future direction is to determine if CREB mediates tau-induced BDNF down-regulation. Objective 1 of the presented work highlighted the importance of CREB in Aβ-induced BDNF down-regulation, Objective 2 demonstrated that tau down-regulates BDNF transcript IV, transcription of which is predominantly controlled by CREB and in Objective 3 it was determined that tau mediates Aβ-induced BDNF down-regulation. As such, the next step should be to investigate the role of CREB in tau-induced BDNF down-

regulation. It is hypothesized here that the inactivation of CREB or decreased transcription of CREB mediates tau-induced BDNF down-regulation. To address this hypothesis, CREB mRNA and phosphorylation levels (both of activating pCREB133 levels and inactivating pCREB129 levels) can be quantified from the hTau-transfected human SH-SY5Y cells developed in the current investigation. This suggested future direction would help to elucidate the mechanism of tau-induced BDNF down-regulation.

6.2.3 Does tau interfere with BDNF axonal transport?

Another possible mechanism of tau toxicity is its ability to impair axonal transport. This may result from a gain-of-toxic function whereby aggregated tau physically interferes with intracellular transport (Wolfe, 2012) or a loss-of-function resulting from tau detachment from MTs and consequent MT instability (Alonso et al., 1996). In AD, the loss of cholinergic neurons in the basal forebrain and their projections to the cortex and hippocampus underlie learning and memory impairments (Bierer et al., 1995; Coyle J., 1983; Fahnestock, 2011). While the cause basal forebrain cholinergic neuron (BFCN) death is not clear, it is known that BFCNs receive trophic support via connections with the hippocampus and cortex (DiStefano et al., 1992; Lapchak et al., 1993; Seiler and Schwab, 1984) and that these connections are critical for learning, memory and attention (Baxter and Chiba, 1999). As such, it is hypothesized here that pathological forms of tau result in neuronal and synaptic degradation of cholinergic neurons by specifically impeding BDNF axonal transport, resulting in learning and memory impairments. To address this hypothesis, we will grow BFCNs from hTau mice

in microfluidic chambers (Figure 19). The axon terminals of cells grown in microfluidic chambers are isolated from cell bodies, which allows for the study of retrograde transport within axons, without dendritic or somal contamination (Taylor et al., 2005). BFCNs from hTau or wild-type animals will be grown on the somal side of the chambers, and will extend axons through microgrooves that can be microscopically imaged. We will then compare the efficiency of BDNF trafficking in these tau over-expressing (hTau) neurons compared to wild-type cells. Efficiency of BDNF trafficking will be assessed by quantifying the speed, direction and amount of axonal transport of fluorescently labeled BDNF-GFP added to the axonal compartment of the chambers using Metamorph software. I have optimized conditions for growth of BFCNs as well as use of microfluidic chambers. Completing this future direction will help us to understand whether tau interferes with BDNF axonal transport as a mechanism of cellular and synaptic degeneration.

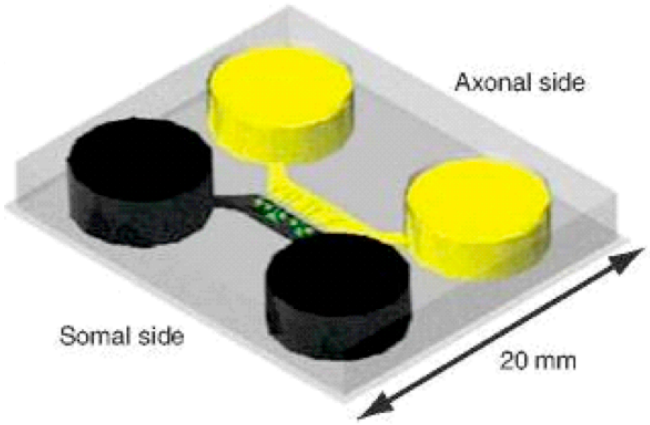


Figure 19: Microfluidic chambers allow for isolation of axon terminals from cell bodies. Cells are plated in the two wells on the somal side of the chamber and will grow in the channel connecting the two wells. Cells will then project axons through microgrooves to the axonal side of the chamber. The somal and axonal sides of the chamber are microfluidically isolated, as such transfer of proteins from one side of the chamber to the other is dependent on axonal transport. (Figure from Taylor et al., 2005).

PUBLICATIONS ARISING FROM THIS AND OTHER WORK:

Peer-Reviewed Journal Articles

Rosa E., Mahendram S., Ke Y.D., Ittner L.M., Ginsberg S.D., & Fahnestock M. (*submitted)* Tau modulates BDNF expression and mediates Aβ-induced BDNF down-regulation in animal and cellular models of Alzheimer's disease. *Journal of Neuroscience.*

Rosa E. & Fahnestock M. (2015) CREB expression mediates Aβ-induced BDNF down-regulation. *Neurobiology of Aging* 6(8): 2406-13.

Rosa E., Cha J., Bain J. & Fahnestock M. (2015) GDNF regulation in an *in vitro* model of denervated muscle. *Journal of Neuroscience Research* 93(3): 514-520.

Hutton C., Dery N., **Rosa E**., Lemon J., Rollo C.D., Boreham D., Fahnestock M., DeCatanzaro D., Wojtowicz J.M., Becker S. (*2015*). Synergistic effects of diet and exercise on hippocampal function in chronically stressed mice. *Neuroscience* 308:180-193.

Willand M.P., **Rosa E.,** Michalski B., Fahnestock M., Borschel G.H. & Gordon T. (*submitted*) Modulation of intramuscular trophic factors by daily application of electrical stimulation following peripheral nerve injury and repair. *Experimental Neurology*

Book Chapters:

Rosa E. & Fahnestock M. (2014). Amyloid-beta, BDNF and the mechanism of neurodegeneration in Alzheimer's disease. In R.M. Kostrzewa (Ed.), *Handbook of Neurotoxicity* (pp. 1597-1620). New York, NY: Springer.

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Rosa E., Mahendram S., Ginsberg S.D., Ke Y., Ittner L. & Fahnestock M. (2015) Tau-

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Rosa E. & Fahnestock M. (2015) CREB expression mediates Aβ-induced BDNF down-regulation. Poster Presentation. McMaster University 27th Annual Psychiatry Research Day. Hamilton, Ontario, Canada.

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