THE COMBINATION OF AGING AND INFLAMMATION INTERACT TO PRODUCE SPECIFIC CHANGES IN BDNF PROTEIN ISOFORM EXPRESSION, BDNF-DEPENDENT SIGNALING AND HIPPOCAMPAL SYNAPTIC PLASTICITY

by

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The combination of aging and inflammation interact to produce specific changes in BDNF protein isoform expression, BDNF-dependent signaling and hippocampal synaptic plasticity

Thesis directed by Assistant Professor Susan L. Patterson

The goals of this research were to investigate the combined effects of aging and inflammation on brain-derived neurotrophin (BDNF) protein biology and BDNF-dependent synaptic plasticity in the hippocampus. For the past two decades neurotrophin research has generated a large body of evidence supporting the role for neurotrophins in facilitating multiple forms of synaptic plasticity and memory in the hippocampus. However, little is known about the effects that both aging and neuroinflammation may impose on these neurotrophin-related pathways.

To carry out the experiments in this study we utilized multiple models: *(1)* A rodent model of aging and inflammation using young (3-month-old) and aged (24-month-old) Fisher344/Brown Norway rat F1 crosses (F344xBN). An inflammatory response in the central nervous system (CNS) was peripherally-induced in these animals following an intraperitoneal (i.p.) injection of live *E.coli*. *(2)* A murine model of Alzheimer's disease (AD) with inflammation. APP/PS1 transgenic mice were given an i.p. injection of lipopolysaccharide (LPS) to induce an inflammatory

response. *(3)* An *in vitro* model of AD and inflammation using a human neuroblastoma SH-SY5Y cell line that received treatment of amyloid beta protein (Aß) and recombinant interleukin-1 beta (IL-1ß). Data was collected using the following experimental methods: (a) hippocampal synaptoneurosomal fractionationation to enrich for synaptic proteins of interest; (b) co-Immunoprecipitation for protein-protein interactions in hippocampal tissue; (c) Western Blot to determine protein expression levels; (c) electrophysiology to measure LTP and LTD.

The results of these works indicated that the combination of aging and inflammation produce a shift in the ratio of BDNF protein isoforms (pro : mature); and this shift in the ratio of BDNF protein isoforms are consistent with a shift in hippocampal synaptic plasticity from LTP to LTD. Furthermore, these data showed that inflammatory markers have the capacity to act at the level of synapses to induce changes in BDNF protein expression, as well as normal synaptic events that include exocytosis and cell signaling. Recommendations from these findings suggest that further research examining the effects of aging and neuro-inflammation on hippocampal function may provide insight into the phenotypic symptomology associated with some neurodegenerative disorders, which include hippocampal dysfunction and memory impairments.

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CHAPTER I:

INTRODUCTION

I.1. Aging and cognitive function

Aging has long been thought to be the primary cause of cognitive decline in humans; presumably responsible for 90% of memory related neurodegenerative disorders. Disorders that span from age-related mild-cognitive impairment (MCI) and early-stage dementia to later-stage clinical Alzheimer's disease (AD) all have a common link to age and age-associated cognitive decline contributing to the clinical symptomology of memory loss and learning impairments [106]. Currently, there is extensive work being done to further understand the onset and/or progression of the cognitive decline associated with aging. Unfortunately, necessary work attempting to elucidate the cellular mechanisms underlying this phenomenon has yet to be performed.

Environmental factors have a strong contribution to, the potential onset, and severity of aging-associated cognitive decline. Psychological stress, surgical procedures and infections are common life events that are shown to activate the innate immune system and negatively influence cognitive function in the aged brain [15,156,162]. This phenomenon is quite interesting; as high functioning aged individuals often show significant cognitive decline following an inflammatory event. Therefore, it is important to understand how, and why the aging brain

becomes vulnerable to challenging life events that compromise the innate immune system.

I.2. The innate immune system and neuroinflammation in aging brain

Microglia are the predominant immune cell in the brain responsible for regulating the inflammatory response. The inflammatory response is characterized by the production, processing and release of the pro-inflammatory cytokines interleukin-1 beta (IL-1ß), tumor necrosis factor alpha (TNFα) and interleukin-18. The interleukins, IL-1ß and IL-18, are synthesized as inactive precursor proteins that are cleaved by the cysteine protease caspase-1 following activation of the NALP1 (NAcht leucine-rich-repeat protein 1) inflammasome [36]. These events occur in both the peripheral (PNS) and central nervous systems (CNS); with recent evidence identifying a communicative link between the two systems [14]. Inflammation in the CNS occurs through peripheral activation of microglia in the brain, therefore having pronounced effects on neural activity and brain function [14, 87]. The mechanism through which glia affect brain function is not clear, however it is hypothesized that the physiological state of microglia cells in the brain may contribute to the overall differences.

Aging alters the basal state of microglia in the brain. Microglia in the aged brain are in a primed, or sensitized state at rest; as indicated by the upregulation of glial activation markers MHCII (major histocompatibility complex II and CD11b (complement receptor 3) [14, 45, 110, 118, 122, 129, 144.]. In contrast, microglia in

young brain is in a quiescent state, and express low basal levels of glial activation markers [111]. Having a primed glial cell means that the cell has a reduced threshold for activation, as seen in the aged brain [14]. This is one speculation in support of aged brain vulnerability – microglia can easily be activated. A number of peripheral immune challenges, such as infection, surgery and stress can trigger a neuroinflammatory response, and this response is significantly exaggerated with aging, as indicated by heightened levels, and prolonged expression of pro-inflammatory cytokines in the aged brain [1, 22, 51, 121, 137, 138]. Moreover, these age-related increases in the level and expression of pro-inflammatory cytokines in the brain can significantly impact brain function.

Age can impact both the severity and duration of the neuroinflammatory response. Following an immune challenge, young animals mount a rapid immune response and recovery whereas aged animals generate a response more severe, and much longer than young animals [1, 13, 22, 51, 121, 137, 138]. The time course for the neuroinflammatory response was further characterized by Barrientos and colleagues using young (3-month-old) and aged (24-month-old) Fischer 344 x Brown-Norway (F344xBN) rats. Following a peripheral infection with live *E. coli*, IL-1ß levels in young animals, specifically in the hippocampus, were elevated 2 hours post-infection with levels returning to baseline at 24 hours. Aged animals also exhibited elevated levels of hippocampal IL-1ß 2 hours post-infection, however heightened IL-1ß was most pronounced at day 4 post-infection with sustained elevations through day 8, and eventually returning to baseline at day 14. There

were significant functional consequences resulting from increased IL-1ß in the hippocampus. Deficits in hippocampal function were exacerbated and prolonged in the aged animals following infection; a result that paralleled the time course for elevated levels of IL-1ß [11, 13]. Thus, there is a strong correlation between infection-evoked upregulation of IL-1ß and hippocampal dysfunction, and aging appears to play a large role in regulating these behaviors.

I.3. Hippocampal function: BDNF and neuroinflammation

Pro-inflammatory cytokines are specifically linked to cognition and memory. Increased levels of cytokines, as a result of inflammation can contribute to the cognitive deficits and memory impairments associated with neurodegenerative disorders, like Alzheimer's disease (AD) [35, 61]. Furthermore, elevated levels of the pro-inflammatory cytokine IL-1ß are strongly associated with memory impairments when elevated in the hippocampus – a key brain region responsible for learning and memory [8-10, 49, 57-59, 112, 113]. Therefore, the hippocampus and hippocampal processes are being studied to measure the deleterious effects of inflammation on memory and cognition.

The hippocampus is the primary brain structure involved in learning and memory. These phenomena are easily identified behaviorally; however hippocampal synaptic plasticity, specifically long-term potentiation (LTP), is thought to represent the cellular and molecular correlate underlying learning and memory acquisition [86, 89]. Brain-derived neurotrophic factor (BDNF) is shown to

regulate LTP, as well as other forms of synaptic plasticity in the hippocampus [85, 96]. Furthermore, BDNF drives synaptic signaling mechanisms that are thought to play a critical role in initiating and maintaining synaptic processes that underlie some enduring forms of long-term learning and memory [21, 23, 152]. Thus, it is important to understand how BDNF functions on a cellular and molecular level in order to fully understand the mechanisms of synaptic plasticity that underlie memory. All of which may be target by aging and neuroinflammation.

BDNF is highly expressed in the hippocampus and is shown to be important in the induction and facilitation of multiple forms of synaptic plasticity. It is now well established that BDNF, and its interaction with the tropomyosin receptor kinase B (TrkB) receptor induces, and facilitates LTP in a naturalisitc and physiological manner [25, 66, 72]. BDNF enhances LTP by increasing NMDA (N-methyl-D-aspartate) phosphorylation [40, 79] and currents [32, 76, 134], and by decreasing inhibitory post-synaptic currents (IPSCs) [47, 146]. More interestingly, BDNF supports synaptic remodeling and promotes intracellular signaling necessary for the maintenance of LTP. Therefore, the expression of hippocampal LTP relies heavily on the presence, and function of BDNF and TrkB at synapses [3, 96].

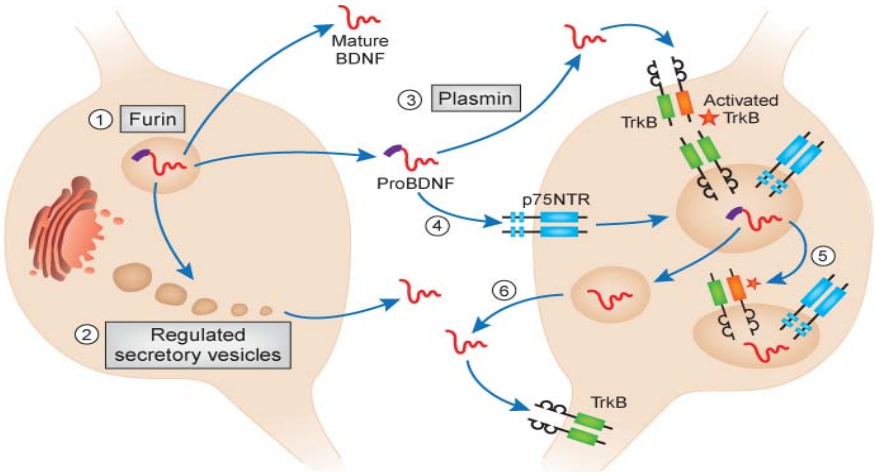

Diagram D.1: Full-length proBDNF is synthesized in cell body and can be released (3+4) or undergo intracellular or extracellular cleavage producing a postsynaptic effect via TrkB or p75 receptors.

BDNF is first synthesized as a precursor protein containing a pro-domain (proBDNF), which later undergoes proteolytic cleavage by either plasmin – an extracellular protease activated by cleavage of plasminogen by tissue plasminogen activator (tPA) (Pang 2004), or furin – an intracellular Golgi-associated enzyme (Mowla 2001). ProBDNF cleavage results in the production of the mature form of the BDNF protein (mBDNF). Diagram 1 (D.1) illustrates the production and proteolytic processing of the full-length BDNF protein (Barker 2009). The mBDNF protein isoform binds to, and activates the TrkB receptor; an event important for facilitating LTP.

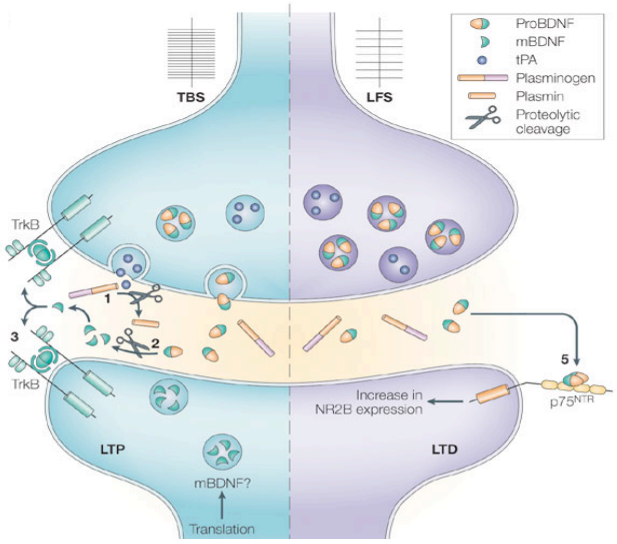

Diagram D.2: Following TBS proBDNF is released and processed producing mBDNF which can facilitate LTP following TrkB binding **(LEFT).** LFS promotes release of proBDNF, which is not processed and facilitates LTD following p75 binding **(RIGHT).**

If proBDNF is not cleaved into mBDNF, proBDNF has the capacity to bind to, and activate the $p75^{NTR}$ receptor. The consequence of this interaction has an opposing effect on signaling and plasticity to that of the mature isoform. ProBDNF activation of $p75^{NTR}$ can induce, and facilitate long-term depression (LTD); a form of hippocampal synaptic plasticity that is correlated with memory impairments – an opposition to LTP [85, 93]. Thus, processing of the BDNF protein plays an important role in determining its cellular functions [5], and may contribute differentially to multiple forms of memory-related synaptic plasticity [85, 164].

One compelling model illustrating the bi-directional modulation of synaptic plasticity by different BDNF protein isoforms is shown in Diagram 2 (D.2) [85]. Both high-frequency stimulation (HFS) and low-frequency stimulation (LFS) can

cause proBDNF release [85]. It has been hypothesized that LTP induced by HFS not only promotes proBDNF release, but also initiates extracellular cleavage of proBDNF by plasmin. This results in mBDNF production and the resulting activation of the TrkB receptor necessary to faciliate LTP and promote cell signaling necessary for learning and memory [85, 105, 140]. In contrast, the induction of LTD following LFS promotes proBDNF release, but in the absence of plasmin. This prevents proBDNF cleavage and mBDNF production; resulting in a ratio shift of protein isoforms at synapses that leads to $p75^{NTR}$ receptor binding, LTD induction and activation of cell-death mediated pathways resulting in cell death [85, 164]. A change in the ratio of BDNF protein isoforms has a significant impact on memory. For example, cleavage of proBDNF in the hippocampus is positively correlated with acquisition of contextual fear memory, whereas decreased cleavage is associated with extinction [7]. Furthermore, aged Wistar rats with memory impairments are reported to have lower levels of total BDNF protein, but higher ratios of proBDNF to mBDNF relative to controls [132]. Similarly, training in a spatial learning task increases levels of proBDNF in both the young and aged rats, but only the young rats show a corresponding increase in mBDNF [131]. Thus, BDNF protein isoforms play a large role in regulating opposing forms of synaptic plasticity and memory in the hippocampus.

I.4. IL-1ß and hippocampal function

Elevated levels of hippocampal IL-1ß can completely block several forms of LTP in hippocampus [16, 24, 29, 30, 67]. Recent work done by Chapman and colleagues (2010) found that only theta-induced L-LTP recorded from Schaeffer collateral-CA1 synapses in hippocampus was impaired in aged F344xBN rats following a peripheral infection, while other forms of L-LTP (4 train stimulation) and E-LTP (1 train stimulation) were unaffected [24]. Administration of interleukin-1 receptor antagonist (IL-1Ra) immediately before a peripheral infection ameliorated age and infection-evoked deficits in hippocampus-dependent memory [46], and hippocampal L-LTP [24]; suggesting the involvement of IL-1 signaling. The cell biological mechanisms by which IL-1ß interferes with hippocampal function are not fully understood, however due to the large role BDNF protein isoforms play in regulating multiple forms of synaptic plasticity that underlie memory, it is plausible that IL-1ß may interfere with the processing of BDNF, and/or alter BDNF-dependent signaling pathways.

There is recent evidence stating that IL-1ß can produce overall changes in BDNF biology, however these conclusions are few and controversial. Infusion of IL-1ß into hippocampus decreases BDNF transcription [10] while blocking IL-1 signaling with IL-1Ra prevents it [9]. In addition, elevated levels of IL-1ß in the neurons directly disrupt downstream targets of BDNF-TrkB signaling [149], while others have shown that some these downstream targets are unaffected in rodent brain [31]. Furthermore, similar shifts in synaptic plasticity that require BDNF for

full expression, such as deficits in LTP and enhancement of LTD) seen with age and inflammation also occur in hippocampus of AD brain [24, 27, 68, 127, 167]. These data suggest that changes in hippocampal synaptic plasticity driven by BDNF signaling may give rise to the memory impairments observed with age and inflammation, as well as AD. Thus, IL-1ß may target BDNF and BDNF-dependent signaling pathways to alter hippocampal function

I.5. Neurodegenerative disorders: neurotrophins and inflammation

Neurotrophins, both nerve-growth factor (NGF) and BDNF, are first synthesized as precursor pro-proteins that later undergo proteolytic cleavage to produce the mature forms of the protein. Both pro forms of NGF and BDNF can bind to $p75^{NTR}$, and activate its associated signaling pathways [117]. The clinical symptomology of AD is associated with neural dysfunction in the basal forebrain and hippocampus that includes deficits in synaptic plasticity, synaptic function and apoptotic cell death [70, 78, 86, 89, 125-127, 166]. Furthermore, the neurodegeneration associated with AD is strongly correlated with activation of the $p75^{NTR}$ receptor and initiation of its related apoptotic pathways downstream [70, 166]. Full-length neurotrophins, NGF and BDNF, are ligands of the $p75^{NTR}$ receptor, and it is through this interaction that perturbations in synaptic function and initiation of apoptotic pathways occur [85, 117]. Interestingly, the toxic amyloid beta fragment (Aß) of the processed amyloid-precursor protein (APP); a pathological hallmark of AD [37, 50, 94], is also found to be a direct ligand and activator of the $p75^{NTR}$ receptor; acting on, and activating similar downstream

synaptic signaling pathways [38, 70, 136, 166]. These data suggest that there may be an intriguing link between pro-neurotrophins, their post-synaptic receptors and their downstream synaptic signaling mechanisms, and the mechanisms underlying the early-stage changes in synaptic function associated with AD.

I.6. Purpose of the thesis

The main goal of this dissertation is to provide evidence for a BDNF-dependent cellular signaling mechanism by which the combination of age and inflammation interact to produce specific deficits in long-term forms of hippocampus-dependent memory and synaptic plasticity. In addition, these works aim to identify a common mechanism involved in the phenotypic changes associated with age and inflammation, with that of Alzheimer's disease by way of a common signaling pathway through the $p75^{NTR}$ receptor.

Chapter one introduces the topics of discussion, and provides background information necessary to understand the work that was performed for the thesis projects. The second chapter discusses work that identified an age and infection-evoked change in the ratio of BDNF protein isoforms at hippocampal synapses; a significant decrease in the amount of mBDNF protein, but not proBDNF. Furthermore, reduced levels of mBDNF at synapses perturbed mBDNF – TrkB downstream signaling. In the third chapter, a time course for the age and infection-evoked reductions in mBDNF was identified. Decreases in mBDNF protein levels and mBDNF – TrkB signaling events persisted for 8 days post-infection before

returning to baseline levels at day 14; a result that paralleled elevated levels of IL-1ß following infection. Chapter three unveiled another form of hippocampal synaptic plasticity, LTD, that is affected by age and inflammation. The combination of age and infection caused an enhancement of LTD in hippocampal slices following LFS. LTD can be facilitated by proBDNF – $p75^{NTR}$ receptor-ligand interactions in the hippocampus. This interaction was increased in hippocampal tissue from aged animals with a recent history of infection; however, the pathways downstream of $p75^{NTR}$ activation were unchanged. Finally, the fourth chapter provides evidence for the deleterious effects of Aß and IL-1ß both independently and co-occurring. Aß and IL-1ß upregulate proBDNF protein expression but does not affect mBDNF expression. Interestingly, the introduction of IL-1ß to an Aß rich environment increases proBDNF levels beyond that of Aß and IL-1ß alone.

The research questions addressed in this dissertation are as follows:

Research Question 1:

Does the combination of age and a peripheral infection interact to produce changes in the expression of BDNF protein isoforms and the activation of BDNF-dependent signaling pathways at hippocampal synapses?

Research Question 2:

How long do age and infection-evoked deficits in mBDNF and mBDNF-dependent signaling persist?

Research Question 3:

Does the combination of age and infection alter other forms of hippocampus-dependent synaptic plasticity, such as LTD?

Research Question 4:

Do Aß and IL-1ß interact to alter expression of BDNF protein isoforms?

CHAPTER II

AGING AND A PERIPHERAL IMMUNE CHALLENGE INTERACT TO REDUCE MATURE BRAIN-DERIVED NEUROTROPHIC FACTOR AND ACTIVATION OF TRKB, PLCγ-1 AND ERK IN HIPPOCAMPAL SYNAPTONEUROSOMES

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II.1. Abstract

For reasons that are not well understood, aging significantly increases brain vulnerability to challenging life events. High-functioning older individuals often experience significant cognitive decline after an inflammatory event such as surgery, infection, or injury. We have modeled this phenomenon in rodents and have previously reported that a peripheral immune challenge (intraperitoneal injection of live *Escherichia coli*) selectively disrupts consolidation of hippocampus-dependent memory in aged (24-month-old), but not young (3-month-old), F344xBN rats. More recently, we have demonstrated that this infection-evoked memory deficit is mirrored by a selective deficit in long-lasting synaptic plasticity in the hippocampus. Interestingly, these deficits occur in forms of long-term memory and synaptic plasticity known to be strongly dependent on brain-derived neurotrophic

factor (BDNF). Here, we begin to test the hypothesis that the combination of aging and an infection might disrupt production or processing of BDNF protein in the hippocampus, decreasing the availability of BDNF for plasticity-related processes at synaptic sites. We find that mature BDNF is markedly reduced in Western blots of hippocampal synaptoneurosomes prepared from aged animals following infection. This reduction is blocked by intra-cisterna magna administration of the anti-inflammatory cytokine IL-1Ra (interleukin 1-specific receptor antagonist). Levels of the pan-neurotrophin receptor p75NTR and the BDNF receptor TrkB (tropomyosin receptor kinase B) are not significantly altered in these synaptoneurosomes, but phosphorylation of TrkB and downstream activation of PLCγ-1 (phospholipase Cγ-1) and ERK (extracellular response kinase) are attenuated— observations consistent with reduced availability of mature BDNF to activate TrkB signaling. These data suggest that inflammation-evoked reductions in BDNF at synapses might contribute to inflammation-evoked disruptions in long-term memory and synaptic plasticity in aging.

II.2. Introduction

Aging is associated with increased variability in cognitive functioning, in part because aging brains are more vulnerable to negative life events such as infections, surgery, and stress [15, 156, 162]. We have recently developed a rodent model to study the mechanisms involved in this vulnerability [11]. Twenty four-month-old Fischer-Brown Norway rats are aged, but not senescent; they generally do not show significant physical or cognitive impairments. However, in response to signals

triggered by activation of the peripheral innate immune system (by an intraperitoneal injection of *Escherichia coli*), they show an exaggerated inflammatory response in the brain. Following the immune challenge, hippocampal production of the pro-inflammatory cytokine interleukin-1ß (IL-1ß) is potentiated and prolonged in the aging rats relative to their younger (3-month-old) counterparts [12]. This exaggerated elevation in IL-1ß does not compromise initial learning or formation of short-term memories, nor does it disrupt basal synaptic function or short-term synaptic plasticity—instead it is paralleled by specific deficits in hippocampus-dependent long-term memory tasks (e.g., contextual fear and place learning) and theta burst-evoked late-phase long-term potentiation (L-LTP) [12,24]. Conversely, blocking IL-1 signaling in the brain with IL-1-specific receptor antagonist (IL-1Ra) ameliorates the deficits in memory [46] and L-LTP [24]. It is not clear how aberrantly elevated levels of IL-1ß in the hippocampus may produce limited, selective impairments in long-lasting forms of synaptic plasticity and memory. However, one intriguing possibility is suggested by the observation that infusion of IL-1ß into the hippocampus decreases its capacity for transcription of brain-derived neurotrophic factor (BDNF) [10], and infusion of IL-1Ra protects it [9]. BDNF plays a critical role in forms of long-lasting synaptic plasticity thought to be associated with consolidation of hippocampus-dependent memory [21, 23, 84, 152]. BDNF is synthesized as a precursor protein proBDNF that is post-translationally cleaved to produce mature BDNF (mBDNF). This processing of the BDNF protein appears to play a key role in determining its cellular functions [5,

53]. Pro-BDNF binds preferentially to the pan-neurotrophin receptor p75NTR, activates apoptosis-related signaling pathways, and may facilitate long-term depression in the hippocampus. In contrast, cleaved mBDNF binds to TrkB (tyrosine kinase B) tyrosine kinase receptors, promotes cell survival, and facilitates some forms of long-term potentiation. In the experiments presented here, we have examined the combined effects of aging and an infection on levels of pro- and mature BDNF protein isoforms and their receptors in the hippocampus. The infection appears to produce limited, relatively subtle synaptic deficits rather than large-scale, nonspecific disruptions in hippocampal function [24], and there is increasing evidence that some important pro-plasticity effects of BDNF are exerted locally at synapses [65, 124]. We have therefore prepared hippocampal synaptoneurosomes, enriching for peri-synaptic proteins, and increasing the probability of detecting subtle changes in proteins at synaptic sites.

II.3. Materials and Methods

Animals. The rats used were 3- and 24-month old male Fischer344/Brown Norway F1 crosses from the National Institute on Aging Aged Rodent Colony. Animals were pair housed, on a 12 h light/dark cycle, with *ad libitum* access to food and water. Upon arrival, rats were allowed to acclimate to the animal facility for 2 weeks before experiments were begun. All experiments conformed to protocols approved by the University of Colorado Animal Care and Use Committee.

E. coli infection model. Stock *E. coli* cultures (ATCC 15746; American Type Culture Collection) were thawed and cultured overnight in 40ml of brain-heart infusion (Difco) in an incubator (37°C, 95% air plus 5% CO2). The bacterial content in individual cultures was quantified by extrapolating from previously determined growth curves. Cultures were centrifuged for 15 min at 3000 rpm, the supernatants were discarded, and the bacteria were resuspended in sterile PBS, yielding a final dose of 2.5 x 10^9 colony-forming units in 250 µl. All animals received an intraperitoneal injection of 250µl of either *E. coli* or the vehicle (sterile PBS).

Blocking CNS consequences of the peripheral infection. IL-1RA was injected into the cisterna magna, rather than into the cerebral ventricles or the hippocampus, because this procedure does not require surgery (which can itself produce memory impairments in aging animals). Rats were briefly anesthetized with halothane. The dorsal aspect of the skull was shaved and swabbed with 70% EtOH; then a 27 gauge needle attached via PE50 tubing to a 25µl Hamilton syringe was inserted into the cisterna magna. The IL-1RA (112µg; Amgen) by intra-cisterna magna administration in a total volume of 3µl; the animals received an intraperitoneal injection of either *E. coli* or vehicle immediately after.

Synaptoneurosome preparation. All tissue was collected 5 d after the injections. This time point was selected because of the following: **(1)** all of the animals have completely recovered from the acute infection (e.g., fever has subsided) [13]; **(2)** the aging, but not the young *E. coli*-injected rats show significant impairments in long-term memory [11] and L-LTP [24]; and **(3)** levels of IL-1 protein in the hippocampus

are still significantly elevated in the aging rats, but not in the young rats [12]. Rats underwent rapid decapitation, and hippocampi were extracted. Tissue was minced in 500µl of homogenization buffer (HB) with protease and phosphatase inhibitors [1MTris, 1Msucrose, 0.5MEDTA, 0.25MEGTA, 0.5M NaF, 1 M benzamidine, and 100 mM AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride)] and homogenized using a glass tissue grinder with a Teflon pestle. Nuclear material and unbroken cells were removed by centrifugation at 960 x *g* for 15 minutes. The remaining supernatant was centrifuged at 10,000 x *g* for 15 minutes, yielding an S2 cytosolic fraction and a P2 crude synaptoneurosomal fraction containing both pre-synaptic and postsynaptic material. The P2 synaptoneurosomal pellet was washed gently in 100µl of HB. The P2 pellet was then homogenized using a 0.5ml plastic pestle in 100µl of HB with 10µl of 10x sodium chloride-TRIS-EDTA (1x final concentration) and sonicated. The P2 fraction obtained using this protocol is enriched for peri-synaptic components including pre-synaptic and postsynaptic proteins, terminal mitochondria and cytoplasm and synaptic vesicles [20, 160]. Synaptic enrichment of the P2 fraction was confirmed using synaptophysin and postsynaptic density 95 (PSD95), common synaptic markers. Protein content was quantified using the BCA protein assay (Bio-Rad).

Western blots. Samples were prepared under reducing conditions in 4x Laemmli buffer and heated at 70°C for 5 minutes. For Western blotting, 40µg of protein sample was loaded onto 4–12% NuPage (Invitrogen) Bis-Tris SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Millipore).

Membranes were blocked in 5% milk/PBST (PBS with Triton) for 30 minutes at room temperature; all primary antibody incubations were at 4°C overnight followed by 3 x 10 minute washes with PBST; secondary antibody incubations were at room temperature for 1 hour and washed 3 x 10 minutes. The following primary antibodies (and dilutions) were used: mature BDNF (1:1000; sc-546; Santa Cruz Biotechnology), proBDNF (1:500; ab72440; Abcam), phospho-TrkB (1:700; pTrkBY816, antisera gift from Moses Chao, New York University School of Medicine, New York, NY) and total TrkB (1:1000; sc-8316; Santa Cruz Biotechnology), p75 (1:500; gift from Mark Bothwell, University of Washington, Seattle, WA), phospho-PLCγ1 (phospholipase Cγ-1) (1:1000; 07–2134) and total phosphorylated and unphosphorylated) PLCγ-1 (1:500; 05–366; Millipore), phospho-ERK (extracellular response kinase) (1:1000; 9101) and total ERK (1:1000; 9102; Cell Signaling Technology), and phospho-AKT (1:1000; 4058) and total AKT (1:1000; 9272; Cell Signaling Technology). Blots were probed with synaptic markers synaptophysin (1:1000; sc-12737; Santa Cruz Biotechnology) and PSD95 (1:1000; United Biomedical) to validate synaptoneurosomal fractions, and ß-tubulin (1:100,000; MAB1637; Millipore Bioscience Research Reagents) and ß-actin (1:5000; sc-47778; Santa Cruz Biotechnology) as loading controls. Identity of the BDNF isoform bands in synaptoneurosomes was confirmed by comparison with extracts from HeLa cells transfected with a plasmid that overexpresses BDNF, producing both the pro- and mature form. Secondary antibodies were purchased from GE Healthcare and Bio-Rad, and were diluted in the range of 1:5000–1:10000;

SuperSignal West Pico Chemiluminescent was purchased from Pierce. Following chemiluminescent application, blots were exposed to autoradiography film (Denville Scientific). Blots were stripped using Restore Western Blot Stripping Buffer (Pierce) for 15 minutes, washed 3 x 10 min in PBST, and subjected to standard Western blotting conditions.

Analysis. Protein bands were quantified using ImageJ software, and all bands were normalized to their actin controls. Because we had previously shown that the combination of age and infection uniquely disrupts BDNF-dependent plasticity and memory, we hypothesized that it might also uniquely reduce BDNF (and related proteins). We therefore used an unpaired *t* test to determine whether the level of the protein of interest in the aged + *E. coli* group differed from the level of the protein in the other groups. The *p* value listed for each protein (or phosphorylation state ratio) is for an unpaired *t* test comparing the mean of the aging + *E. coli* group to the mean of the summed values of the other test groups.

II.4. Results

Levels of the mature BDNF protein isoform are significantly reduced in hippocampal synaptoneurosomes prepared from aged animals with a recent history of infection.

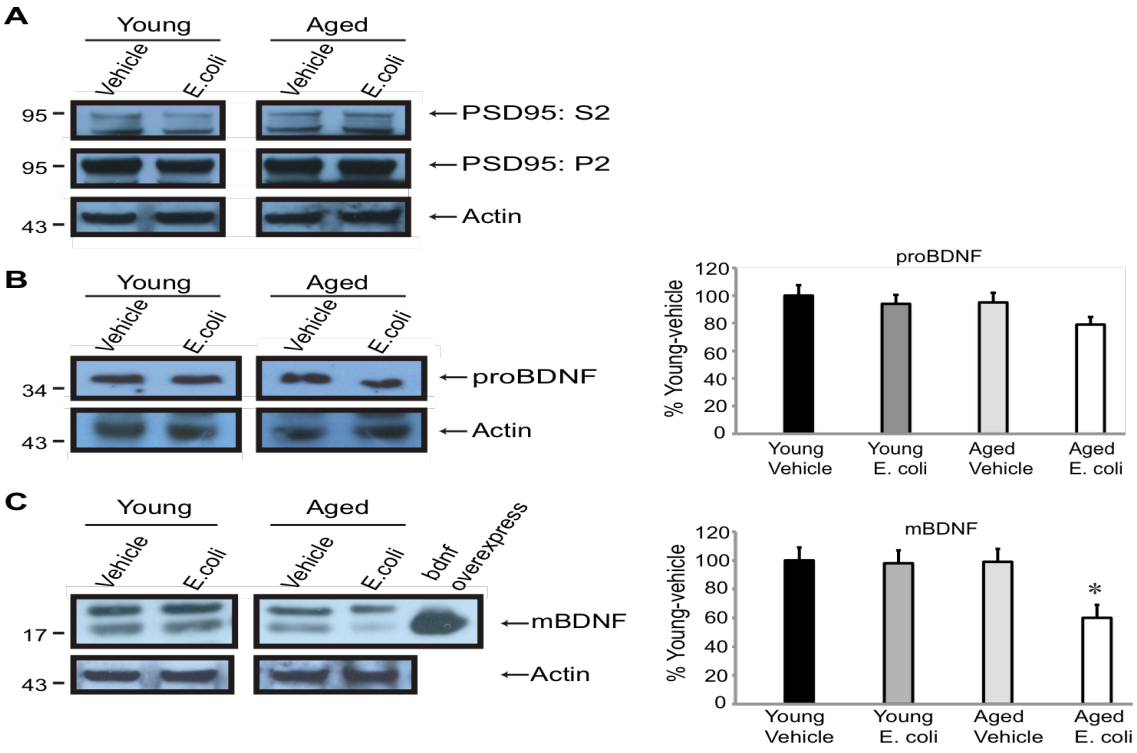

Figure 1. Infection differentially affects BDNF protein isoforms in aged vs. young animals. Western blot analysis showing enrichment of synaptic material in synaptoneurosomes, and levels of pro- and mature BDNF in hippocampal synaptoneurosomes prepared from young and aged rats, with and without a recent history of infection. **(A)** Representative examples of blots probed with a PSD95 antibody to confirm enrichment of synaptic material in the synaptoneurosomal (P2) vs. the cytosolic (S2) fractions. **(B)** Levels of proBDNF were not significantly reduced by the combination of aging and infection. **(C)** Infection markedly reduced mature BDNF in synaptic fractions from aged animals. Band intensities were quantified (NIH-ImageJ), normalized to actin controls, and expressed as percentages of mean protein levels from young vehicle-injected rats. Error bars indicate SEM. All graphs (here and below) represent a minimum of three independent experiments with 1–2 animals per group in each experiment. Asterisks indicate statistical significance from all other groups; individual P-values are reported in the text.

We hypothesized that aging and a recent history of infection might interact to disrupt biosynthesis or processing of BDNF protein in the hippocampus, decreasing the availability of BDNF for plasticity-related processes at synaptic sites. To begin investigating this possibility, we prepared synaptoneurosomes from hippocampi collected from young and aging rats 5 days after a vehicle or *E. coli* injection. This procedure produces a significant enrichment in synaptic proteins, making it possible to identify subtle experience-dependent changes in the protein composition of synapses [160]. Western blot analysis detected a specific proBDNF signal (ab72440 antibody; Abcam) in the 30–35 kDa range (Fig. 1*B*). The combination of age and infection produced a trend toward a slight (10–15%) reduction in the proBDNF signal, but it did not reach significance ($t(18) = 1.234$, $p = 0.232$). In contrast, further analysis with an antibody for the mature domain of BDNF (sc-546 antibody; Santa Cruz Biotechnology) [75] revealed that age and infection together reduced levels of mBDNF by more than half ($t(22) = 2.615$, $p = 0.0158$) (Fig. 1*C*).

Levels of receptors for BDNF are not significantly altered by aging or a history of infection.

In contrast to its effects on BDNF protein, the combination of age and infection produced no detectable changes in levels of BDNF receptors in hippocampal synaptoneurosomes prepared 5 days after the initial *E. coli* injection. Expression of the $p75^{NTR}$ receptor was unchanged (antibody gift from Mark Bothwell, University of Washington, Seattle, WA) (Fig. 2*B*). Similarly, total levels of TrkB did not vary significantly across conditions, nor was there a shift in the

relative proportions of full-length versus truncated (lacking the tyrosine kinase) TrkB receptor isoforms (sc-8316; Santa Cruz Biotechnology) (Fig. 2*A*).

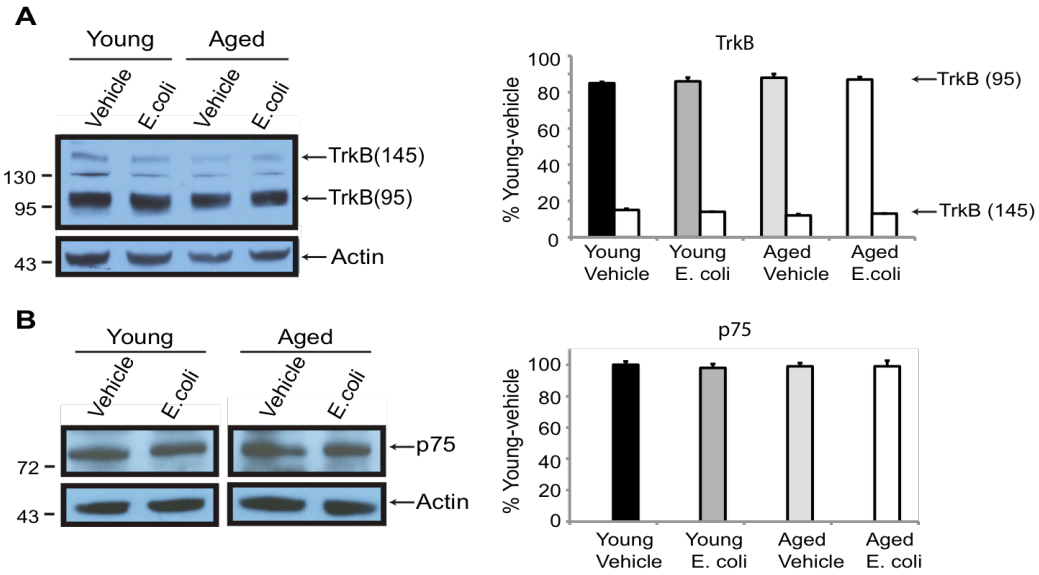

Figure 2. Age and infection do not alter levels of BDNF receptors under the conditions of the study. Western blot analysis was performed on hippocampal synaptoneurosomes prepared from young and aged rats, with and without a recent history of infection. Levels of the **(A)** pan neurotrophin receptor p75NTR, and **(B)** principal TrkB receptor isoforms were unchanged. Quantification was as above.

Age and infection interact to reduce activation of TrkB and downstream signaling systems.

Activation of TrkB by mBDNF triggers a series of phosphorylation events, beginning with the receptor, which can activate proteins in the three major growth factor-regulated signaling pathways: the PLCγ-1 pathway, the Ras/ERK pathway, and the PI3K (phosphatidyl inositol 3-kinase)/Akt pathway [54, 62]. We found that the ratio of phospho-TrkB (antisera gift from Moses Chao) to total TrkB (sc-8316; Santa Cruz Biotechnology) was significantly reduced by the combination of age and

infection ($t(14) = 4.680$, $p = 0.0004$) (Fig. 3*A*). We next asked whether this was associated with reduced activation of PLCγ-1, ERK, and or Akt. We found that the ratio of phospho-PLCγ-1 (07–2134; Millipore) to total PLCγ-1 (05–366; Millipore) was significantly reduced in synaptoneurosomes from aged rats following infection ($t(10) = 4.468$, $p = 0.0012$) (Fig. 3*B*). This was also true of the ratio of phospho-ERK (9101; Cell Signaling Technology) to total ERK (9102; Cell Signaling Technology) ($t(10) = 5.581$, $p = 0.0002$) (Fig. 3*C*). In contrast, we found that the ratio of phospho-Akt to total Akt was not significantly reduced under the conditions examined ($t(10) < 1$, $p = 0.6568$) (Fig. 3*D*).

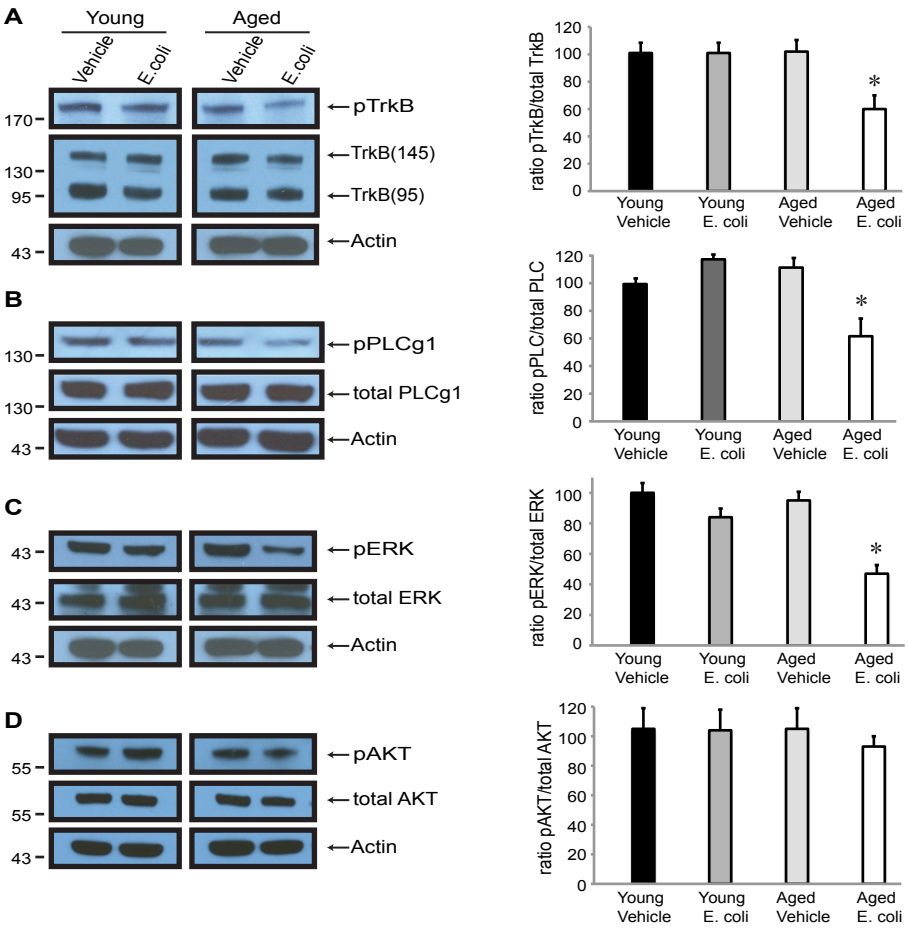

Figure 3. Aging and infection reduce activation of TrkB and downstream activity in major TrkB-signaling pathways. Levels of phosphorylated TrkB (**A**), PLCγ-1 (**B**), and ERK (**C**) were significantly lower in synaptoneurosomes prepared from aged animals 5 days following infection; levels of phosho-Akt (**D**) were not. Quantification was as above.

Central administration of the anti-inflammatory cytokine IL-1Ra ameliorates the infection-induced reductions in mBDNF and activated TrkB in synaptoneurosomes from aged rats.

IL-1ß is a major mediator of inflammatory responses in the brain as well as in the periphery. We have previously shown that blocking the actions of IL-1ß in the brain, by injecting IL-1Ra into the cistern magna at the time of the intraperitoneal *E.coli* injection, blocks infection-evoked deficits in long-term synaptic plasticity and memory in aged rats [24, 45]. Here we report that administration of IL-1Ra also blocks *E. coli*-evoked reductions in mBDNF ($t(10) = 0.3511$, $p = 0.7328$) (Fig. 4*A*) and phospho-TrkB ($t(10) = 1.339$, $p = 0.2102$) (Fig. 4*B*).

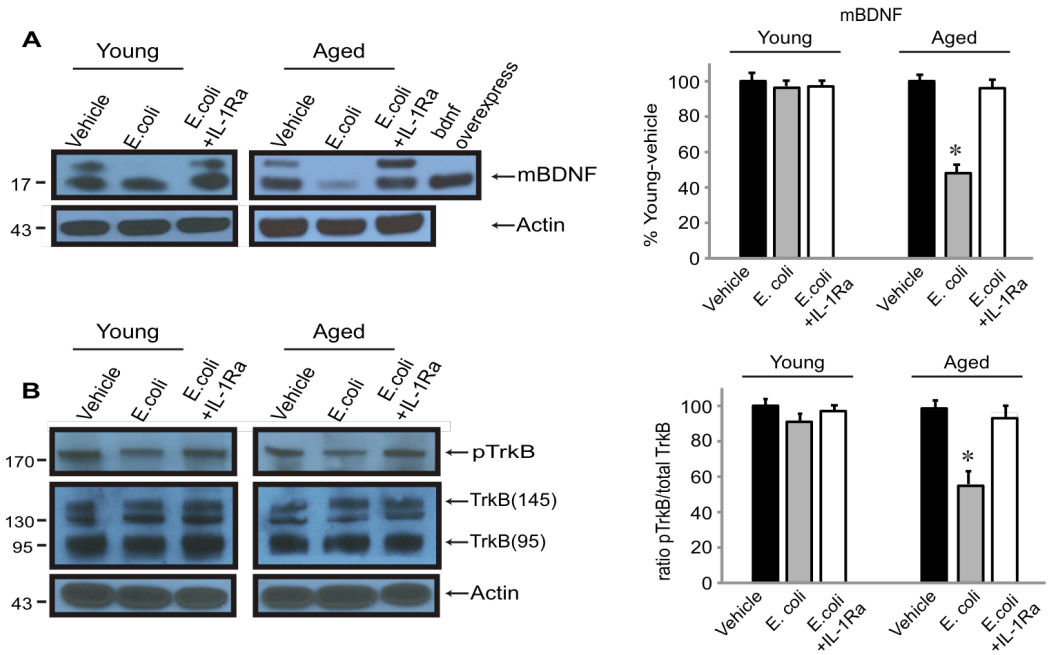

Figure 4. Blocking IL-1ß signaling in the CNS blocks the *E. coli*-evoked reduction in synaptic levels of mBDNF protein **(A)** and phospo-TrkB in aged rats **(B)**. *E. coli* injected rats received a concurrent injection of the anti-inflammatory cytokine IL-1Ra or vehicle into the cisterna magna. Hippocampi were collected, and synaptoneurosomes prepared 5 days after the injections. Quantification was as above.

II.5. Discussion

We have previously demonstrated that a peripheral immune challenge produces profound disruptions in forms of hippocampus-dependent long-term memory and synaptic plasticity known to be BDNF dependent in aged, but not young, F344xBN rats [11, 24]. Here, we have extended these observations, examining for the first time the combined effects of aging and infection on levels of BDNF protein isoforms and their receptors at synaptic sites in the hippocampus. Our key findings are that an immune challenge in aging rats **(1)** triggered a minimal reduction in proBDNF and a much larger reduction in mature BDNF detectable in hippocampal synaptoneurosomes prepared 5 days after the injections, after all the rats had recovered from the acute infection; **(2)** had no significant effects on levels of BDNF receptors; but **(3)** significantly reduced phosphorylation of TrkB, and downstream activation of PLCγ-1 and ERK, consistent with decreased availability of mBDNF for activation of TrkB; and **(4)** no longer reduced mBDNF and phospho-TrkB if IL-1 receptors in the brain were blocked with a selective antagonist.

These new data are consistent with the hypothesis that the interaction of aging and an infection might decrease availability of BDNF at hippocampal synapses, and thus might contribute to selective deficits in forms of long-lasting plasticity and memory that require BDNF for their full expression. We found that the interaction of aging and infection reduced levels of mBDNF at synaptic sites by

> 50%. Mouse models of BDNF haploinsufficiency have provided evidence that a critical threshold level of BDNF is necessary for full function in memory-related plasticity. Heterozygous BDNF (+/-) mice with approximately half the normal levels of BDNF in their brains have significant impairments in long-lasting synaptic plasticity [71, 108, 105] and in hippocampus-dependent learning and memory [80].

The consequences of reduction of BDNF protein isoforms are not yet so well studied, but mBDNF appears to play an important role in some forms of long-lasting synaptic plasticity in the hippocampus. Mature BDNF can be generated by cleavage of proBDNF by plasmin, an extracellular protease activated by tissue plasminogen activator (tPA)-dependent cleavage of plasminogen [104]. Theta burst stimulation is reported to induce secretion of tPA, and to increase extracellular conversion of proBDNF to mBDNF in cultures of hippocampal neurons [102]. Application of mBDNF, but not a cleavage-resistant proBDNF, can rescue deficits in theta burst L-LTP hippocampal slices from mice lacking tPA or plasminogen [105]. Mature BDNF can also rescue the impairment of theta-burst L-LTP caused by inhibition of protein synthesis in wild-type mice [105], suggesting that the mBDNF isoform may be one of the proteins whose production is required for long-lasting enhancement of synaptic efficacy. There is now some corresponding evidence that production of adequate amounts of mBDNF may be important for hippocampus-dependent memory. A recent study indicates that increased cleavage of precursor proBDNF in the hippocampus is positively correlated with acquisition of contextual fear memory, while decreased cleavage is associated with extinction [7].

The effects of aging on BDNF mRNA and protein have been extensively studied and generally found to be modest [105]. However, a few studies have now examined the impact of aging on BDNF protein isoforms. Aged Wistar rats with memory impairments are reported to have lower levels of total BDNF, but higher ratios of proBDNF to mBDNF than controls from a related strain known to have better preservation of cognitive function [132]. Perhaps not surprisingly, training in a spatial learning task increased levels of proBDNF in both young and aged Wistar rats, but only the young rats showed a corresponding increase in mBDNF [131].

Several studies have examined the effects of immune challenge or pro-inflammatory cytokines on BDNF in the brain. Lipopolysaccharide (LPS) is a component of the cell wall of Gram-negative bacteria. A potent endotoxin, it leads to the release of pro-inflammatory cytokines such as IL-1ß and tumor necrosis factor α [44]. BDNF mRNA in the principle neurons of the hippocampus was strongly downregulated 4 hours after an intraperitoneal injection of LPS or IL-1ß [73]. Depolarization-induced release of BDNF from slices of dentate gyrus was not altered by administration of LPS several days earlier [128]. However, when levels of BDNF protein were examined 7 hours after intraperitoneal injection of LPS, LPS was found to produce a dose-dependent reduction in BDNF in the cortex and hippocampus [56].

Much less is known about the effects of immune challenge on BDNF protein isoforms, but intraperitoneal injection of a single very high dose of LPS (3 mg/kg) is reported to produce a small (~15%) reduction in both proBDNF and mature BDNF

in crude synaptoneurosomes prepared from the brains of young mice 3 days after the injection [123]. We have previously shown that a peripheral *E. coli* infection produces an exaggerated inflammatory response in the brains of aging animals, paralleled by specific deficits in forms of long-term memory and synaptic plasticity known to be strongly dependent on BDNF [12, 13, 24]. We now report that this inflammation also gives rise to a large reduction in mBDNF and TrkB signaling at synapses in the hippocampus.

It is not yet clear how exaggerated CNS inflammation may impact levels of BDNF protein isoforms. The precise circumstances and sites of BDNF production, processing, and release, and the stability of the resulting isoforms is currently a very active, complex, and controversy-filled area of investigation [5]—and rather beyond the scope of this initial report. However, the work presented here provides new insights into naturalistic events that can affect BDNF production and processing *in vivo* and should provide a basis for further investigation of the interactions of aging, inflammation, and BDNF biology.

Chapter III

A time course for IL-1ß and its effects on BDNF

III.1. Abstract

There is a strong communicative link between the innate immune system and the central nervous systems (CNS). A peripheral infection (injection of live *E. coli*) produces CNS inflammation that is characterized by the upregulation of pro-inflamamtory cytokines interleukin-1 beta (IL-1ß), tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) in the brain of F344xBN rats [12]. More interestingly, aged (24-month-old) animals show an exaggerated and prolonged CNS immune response compared to young (3-month-old) counterparts. Levels of pro-inflammatory cytokines, specifically IL-1ß in the hippocampus of aged animals were significantly elevated compared to young animals at day 4 post-infection; and increase that persisted through day 8 before returning to baseline levels at day 14 [13]. We previously report that the combination of aging a peripheral infection produces a significant decrease in levels of mature, but not pro- BDNF (brain-derived neurotrophic factor) protein at hippocampal synapses 4-5 days post-infection; a result consistent with decreases in mBDNF-dependent downstream phosphorylation targets ERK (extracellular-signal-regulated kinase) and PLCγ-1 (phospholipase C gamma-1) [31]. Here, we show that decreases in mBDNF and

pPLCγ-1, but not pERK persists for 8 days post-infection returning to baseline levels at day 14; a result that parallels the time course of heightened IL-1β following a peripheral infection. Together, our findings suggest that the pro-inflammatory cytokine IL-1β directly alters BDNF protein levels and interferes with BDNF-dependent signaling in hippocampus.

III.2. Introduction

Aging significantly renders the brain more vulnerable to challenging life events such as infections, surgery, and psychological stress [15, 156, 162]. Higher functioning aging individuals have shown significant cognitive decline following an inflammatory event, such as infection or injury [15, 156, 162]. It is well documented that triggering the immune response, through activation of the NALP1 (NAcht leucine-rich-repeat protein 1) inflammasome and its constituent caspase-1, results in increased production of pro-inflammatory cytokines such as IL-1β, TNFα and IL-6; providing clear evidence for the presence of cytokines in the central inflammatory response [14, 36, 95]. Although this phenomenon is evident in many behavioral analyses, very little is known about the underlying cellular mechanisms.

Aging-associated neuroinflammation has now been modeled in rodents. From this, we learned that aged brain vulnerability to immune challenges (like infection, stress and surgery) is quite evident and pronounced; as indicated by an exaggerated inflammatory response accompanied by cognitive decline in aged, but not young animals [11, 12, 15, 22, 51, 52, 121, 156, 162]. Until recently, little was known

about the relationship between peripheral immune challenges and the CNS inflammatory response; let alone changes that exist with aging. However, there is now experimental data showing that elevated levels of pro-inflammatory cytokines are produced in the brain (*de novo*) following peripheral infections; an effect more pronounced in aged animals with IL-1ß found to be most abundant in key areas of the brain responsible for memory, such as the hippocampus [8-10, 14]. Therefore, IL-1ß in the brain, as a result of CNS inflammation, has the capacity to significantly influence neural function, and these effects can be influenced with aging.

Heightened levels of IL-1ß in the hippocampus is shown to be strongly correlated with significant impairments in hippocampal function; as seen with deficits in hippocampus-dependent memory and impairments in hippocampus-dependent forms of synaptic plasticity, like long-term potentiation (LTP) [8, 10, 34, 154, 155]. Barrientos and colleagues have modeled this phenomenon of aging-associated cognitive decline following an immune challenge using young (3-month-old) and aged (24-month-old) Fischer-Brown Norway (F344xBN) rats [11]. They found that deficits in long-term forms hippocampus-dependent memory in aged animals following infection paralleled elevated levels of IL-1ß both in time (24 hour, 4 day and 8 day post-infection) and severity, as shown by greater and prolonged memory deficits in the aged animals following contextual-fear conditioning and Morris Water Maze tasks [11, 13]. In addition, impairments in long-term memory were mirrored by deficits in hippocampal late-phase long-term potentiation (L-LTP)

4 days post-infection [24]; a form of long-lasting synaptic plasticity and memory known to require brain-derived neurotrophic factor (BDNF) [21, 23, 84, 152].

BDNF is a member of the neurotrophin family of proteins highly expressed in hippocampus and thought to play a large role in long-term forms of synaptic plasticity and memory [21, 23, 83, 84, 152]. BDNF is first synthesized as a precursor protein containing a pro-domain (proBDNF), which later undergoes proteolytic cleavage by plasmin, an extracellular protease activated by cleavage of plasminogen by tissue plasminogen activator (tPA) [104], or the intracellular Golgi-associated enzyme furin [98] to produce the mature form of the BDNF protein (mBDNF). This processing of the BDNF protein plays an important role in determining its cellular functions [5]. ProBDNF binds to, and activates the pan-neurotrophin receptor $p75^{NTR}$. The mature form of BDNF is shown to interact with the receptor tyrosine kinase B (TrkB) in order to facilitate long-term forms of synaptic plasticity, in addition to activating downstream signaling pathways that include PLCγ-1 and ERK [117]. Previously, we demonstrated that the combination of age and infection reduced levels of mBDNF protein in hippocampal synaptoneurosomes prepared from aged animals 4 days following a peripheral infection [31]. In addition, we showed that events downstream of mBDNF – TrkB interactions were also perturbed with aging and a 4-day infection, as seen with decreases in phosphorylated PLCγ-1 and ERK [31]. Thus, we began to test the hypothesis that reductions in mBDNF and mBDNF-dependent downstream

signaling will parallel elevations in IL-1ß in aged animals following infection; persisting for 8 days, but not 14 days post-infection.

Here we show that levels of mBNDF in hippocampal synaptoneurosomes from aged and infected animals are still reduced at 8 day post-infection, but recover to basal levels at day 14 compared to age and young counterparts. In addition, decreases in phosphorylated PLCγ-1, but not ERK, follow this same time frame. These data suggest that elevated levels of IL-1ß in the hippocampus directly parallel decreases in levels of mature BDNF. Also, the reductions in mBDNF are consistent with reductions in the phosphorylation of PLCγ-1 suggesting events downstream of mBDNF activity are also affected by elevated IL-1ß. Thus, this work provides further evidence for the direct influence of IL-1ß on BDNF and BDNF-dependent signaling in the hippocampus; a finding that may underlie the age and infection-evoked deficits in hippocampus-dependent memory and hippocampal synaptic plasticity previously observed.

III.3. Materials and Methods

Animals. The rats used were 3- and 24-month old male Fischer344/Brown Norway F1 crosses from the National Institute on Aging Aged Rodent Colony. Animals were pair housed, on a 12 h light/dark cycle, with *ad libitum* access to food and water. Upon arrival, rats were allowed to acclimate to the animal facility for 2 weeks before experiments were begun. All experiments conformed to protocols approved by the University of Colorado Animal Care and Use Committee. *E. coli infection*

model. Stock *E. coli* cultures (ATCC 15746; American Type Culture Collection) were thawed and cultured overnight in 40 ml of brain-heart infusion (Difco) in an incubator (37°C, 95% air plus 5% CO2). The bacterial content in individual cultures was quantified by extrapolating from previously determined growth curves. Cultures were centrifuged for 15 min at 3000 rpm, the supernatants were discarded, and the bacteria were resuspended in sterile PBS, yielding a final dose of 2.5 x 10^9 colony-forming units in 250µl. All animals received an intraperitoneal injection of 250µl of either *E. coli* or the vehicle (sterile PBS).

Synaptoneurosome preparation. Tissue was collected at days 8 and 10 post injections. Rats underwent rapid decapitation, and hippocampi were extracted. Tissue was minced in 500µl of homogenization buffer (HB) with protease and phosphatase inhibitors [1MTris, 1Msucrose, 0.5MEDTA, 0.25MEGTA, 0.5M NaF, 1 M benzamidine, and 100mM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride)] and homogenized using a glass tissue grinder with a Teflon pestle. Nuclear material and unbroken cells were removed by centrifugation at 960 x *g* for 5 minutes. The remaining supernatant was centrifuged at 10,000 x *g* for 15 minutes, yielding an S2 cytosolic fraction and a P2 crude synaptoneurosomal fraction containing both presynaptic and postsynaptic material. The P2 synaptoneurosomal pellet was washed gently in 100µl of HB. The P2 pellet was then homogenized using a 0.5ml plastic pestle in 100µl of HB with 10µl of 10x sodium chloride-TRIS-EDTA (1x final concentration) and sonicated. The P2 fraction obtained using this protocol was

previously characterized [31]. Protein content was quantified using the BCA protein assay (Bio-Rad).

Western blots. Samples were prepared under reducing conditions in 4x Laemmli buffer and heated at 70°C for 5 minutes. For Western blotting, 40μg of protein sample was loaded onto 4–12% NuPage (Invitrogen) Bis-Tris SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Millipore). Membranes were blocked in 5% milk/PBST (PBS with Triton) for 30 minutes at room temperature; all primary antibody incubations were at 4°C overnight followed by 3 x 10 minutes washes with PBST; secondary antibody incubations were at room temperature for 1 h and washed 3 x 10 minutes. The following primary antibodies (and dilutions) were used: mature BDNF (1:1000; sc-546; Santa Cruz Biotechnology), proBDNF (1:500; ab72440; Abcam), phospho-TrkB (1:700; pTrkBY816, antisera gift from Moses Chao, New York University School of Medicine, New York, NY) and total TrkB (1:1000; sc-8316; Santa Cruz Biotechnology), p75 (1:500; gift from Mark Bothwell, University of Washington, Seattle, WA), phospho-PLCγ-1 (phospholipase Cγ-1) (1:1000; 07–2134) and total (phosphorylated and unphosphorylated) PLCγ-1 (1:500; 05–366; Millipore), phospho-ERK (extracellular response kinase) (1:1000; 9101) and total ERK (1:1000; 9102; Cell Signaling Technology). Blots were probed with synaptic marker PSD95 (1:1000; United Biomedical) to validate synaptoneurosome fractions, and β-tubulin (1:100,000; MAB1637; Millipore Bioscience Research Reagents) and β-actin (1:5000; sc-47778; Santa Cruz Biotechnology) as loading controls. Identity of the BDNF

isoform bands in synaptoneurosomes was confirmed by comparison with extracts from HeLa cells transfected with a plasmid that overexpresses BDNF, producing both the pro- and mature form. Secondary antibodies were purchased from GE Healthcare and Bio-Rad, and were diluted in the range of 1:5000–1:10000; SuperSignal West Pico Chemiluminescent was purchased from Pierce. Following chemiluminescent application, blots were exposed to autoradiography film (Denville Scientific). Blots were stripped using Restore Western Blot Stripping Buffer (Pierce) for 15 minutes, washed 3 x 10 minute in PBST, and subjected to standard Western blotting conditions.

Analysis. Protein bands were quantified using ImageJ software, and all bands were normalized to their actin controls. Because it had previously shown that the combination of age and infection uniquely disrupts hippocampus-dependent memory and this deficit parallels upregulation of IL-1ß 8 days, but not 14 days post infection, we hypothesized that reductions in BDNF (and related proteins) may also follow this same time frame. We therefore used an unpaired *t* test to determine whether the level of the protein of interest in the aged + *E. coli* group differed from the level of the protein in the other groups. The *p* value listed for each protein (or phosphorylation state ratio) is for an unpaired *t* test comparing the mean of the aging + *E. coli* group to the mean of the summed values of the other test groups.

III.4. Results

Levels of mBDNF are reduced 8 days, but not 14 days post-infection in hippocampal synaptoneurosomes prepared from aged animals

To determine changes in synaptic levels of BDNF proteins we isolated synaptoneurosomal fractions from hippocampus of 3-month-old and 24-month-old animals 8 and 14 days following injection of vehicle saline or live *E. coli*. We previously reported that levels of mBDNF were significantly reduced (~60%) in aged animals 4 days post-infection [31]. We now find that this decrease still persists 8 days following infection of live E. coli (Fig 1a; p=0.02), and return back to baseline levels at day 14 post-infection (Fig1b) in aged, but not young animals. Furthermore, we did not observe any change in the levels of proBDNF with age (Fig 1a, panel 2) or infection (Fig 1b, panel 2) at day 8 and 14 post-infection (p=0.7).

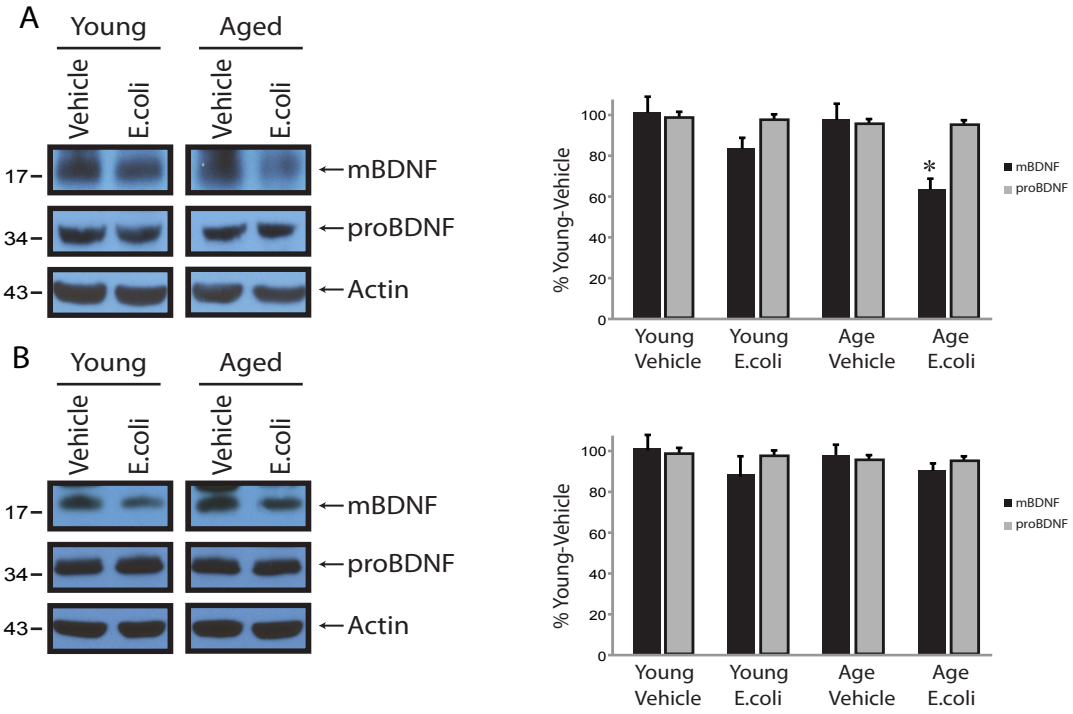

Figure 1. Infection differentially affects BDNF protein isoforms in aged vs. young animals 8 days, but not 14 days pos-infection. Western blot analysis showing levels of pro- and mature BDNF in hippocampal synaptoneurosomes prepared from young and aged rats, with and without a recent history of infection. **(A)** Infection markedly reduced mature BDNF in synaptic fractions from aged animals 8 days post-infection (panel 1, lane 2). ProBDNF was unchanged (panel 2). **(B)** Levels of mBDNFand proBDNF were not significantly reduced by aging and 14 days following infection. Band intensities were quantified (NIH-ImageJ), normalized to actin controls, and expressed as percentages of mean protein levels from young vehicle-injected rats. Error bars indicate SEM. All graphs (here and below) represent a minimum of three independent experiments with 1–2 animals per group in each experiment. Asterisks indicate statistical significance from all other groups; individual P-values are reported in the text.

Levels of BDNF receptors are not altered by age or infection at day 8 and 14 post-injection.

In contrast to its effects on BDNF protein, the combination of age and infection produced no detectable changes in levels of BDNF receptors in hippocampal synaptoneurosomes prepared 8 and 14 days after the initial *E. coli* injection. Levels of TrkB expression, both the full-length (145kDa) and truncated (95kDa) forms are not changed by age and infection at day 8 post-infection (Fig 2a, panel 1; p=0.7) and day 14 post-infection (Fig 2b, panel 1; p=0.6). In addition the proBDNF receptor $p75^{NTR}$ is unaffected by age, and infection at day 8 (Fig 2a, panel 2 [p=0.9]) and 14 post-infection (Fig 2b, panel 2; p=0.5). These results suggest that the combination of age and infection specifically acts on BDNF protein levels and not on post-synaptic receptor expression.

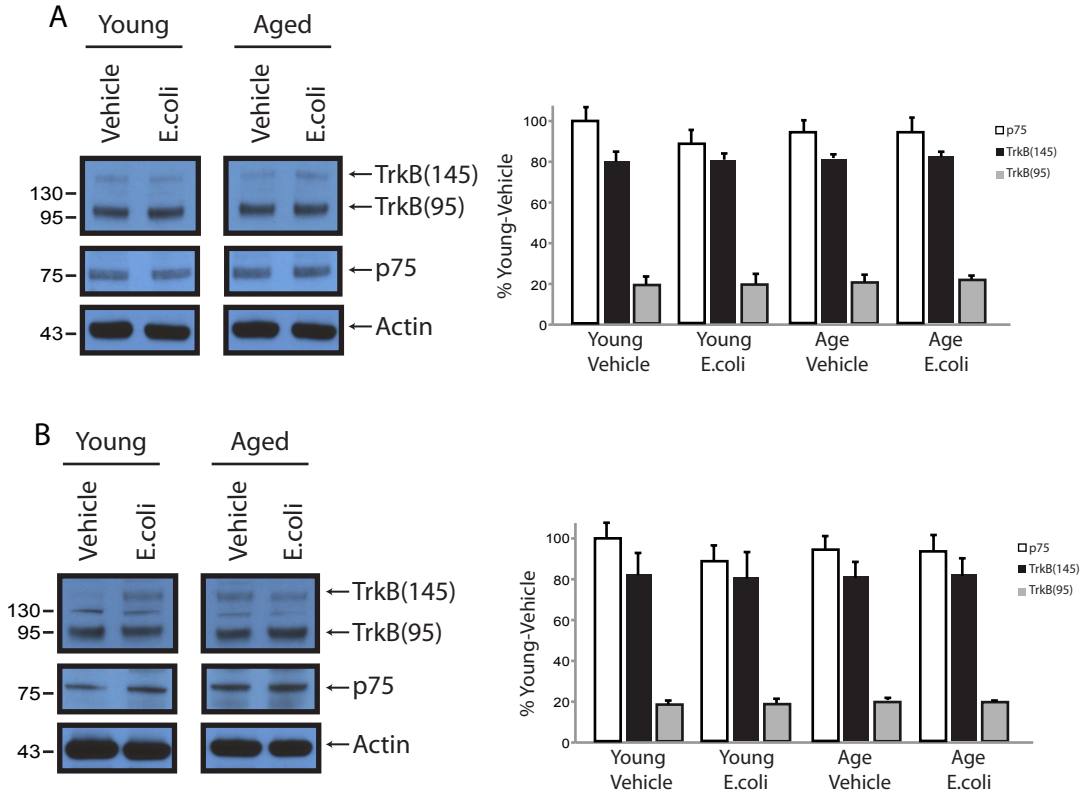

Figure 2. Age and infection do not alter levels of BDNF receptors. Western blot analysis was performed on hippocampal synaptoneurosomes prepared from young and aged rats, with and without a recent history of infection. Levels of the pan neurotrophin receptor p75NTR and principal TrkB receptor isoforms were unchanged (A) 8 days and (B) 14 days post-infection. Quantification was as above.

Age and infection decrease TrkB activation downstream signaling systems are 8 days post-injection.

To determine if limited availability of mBDNF at hippocampal synapses reduces activation of TrkB , and downstream mBDNF – TrkB events we explored expression levels of phosphorylated TrkB, PLCγ-1 and ERK. The TrkB receptor is autophosphorylateed following neurotrophin binding, specifically mBDNF [117]. Here we report that phospho- TrkB is significantly reduced in hippocampal synaptoneurosomes prepared from aged animals 8 days following a peripheral infection (Fig 3a; p=0.02). This reduction in pTrkB did not persist for 14 days post-infection (Fig 4a; p=0.3). To examine if reductions in TrkB activation affected downstream events we explored two related signaling pathways, PLCγ-1 and ERK. Reductions in pTrkB were consistent with reductions in the amounts of pPLCγ-1 (Fig 3b; p=0.02) and pERK (Fig 3c; p=0.02) in synaptic fractions prepared from aged animals 8 days post-infection. However, levels of phospho- PLCγ-1 and ERK were not significantly altered in aged animals 14 days post-infection (Figs 4b [p=0.1] and 4c [p=0.6]).

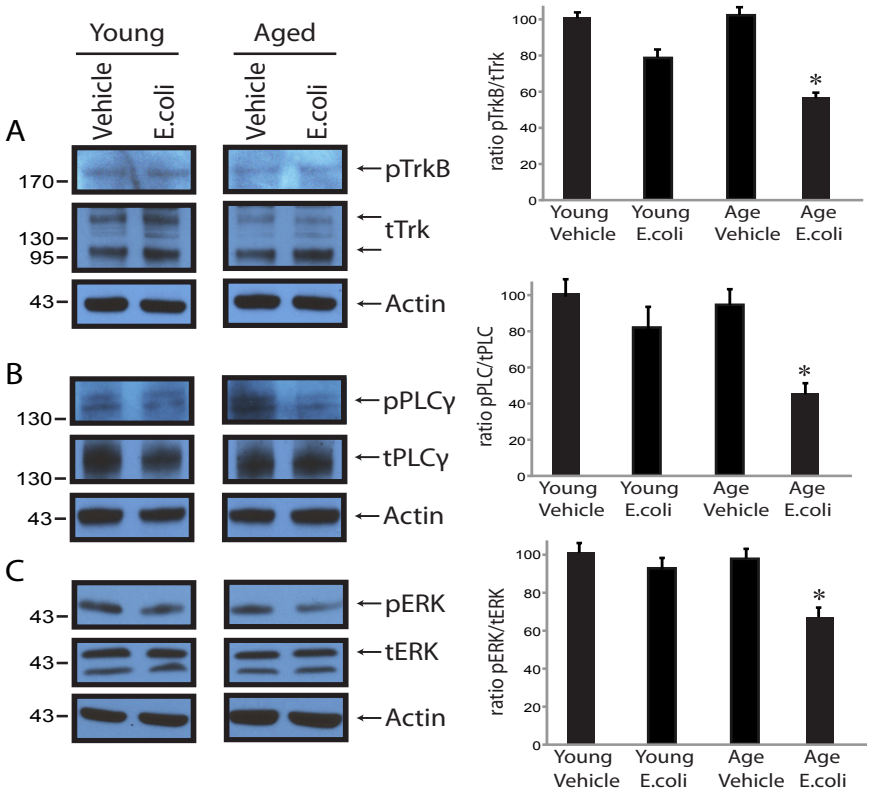

Figure 3. Aging and infection reduce activation of TrkB and downstream activity in major TrkB-signaling pathways 8 days post-infection. Levels of phosphorylated TrkB (**A**), PLC-γ1 (**B**), and ERK (**C**) were significantly lower in synaptoneurosomes prepared from aged animals 8 days following infection. Quantification was as above.

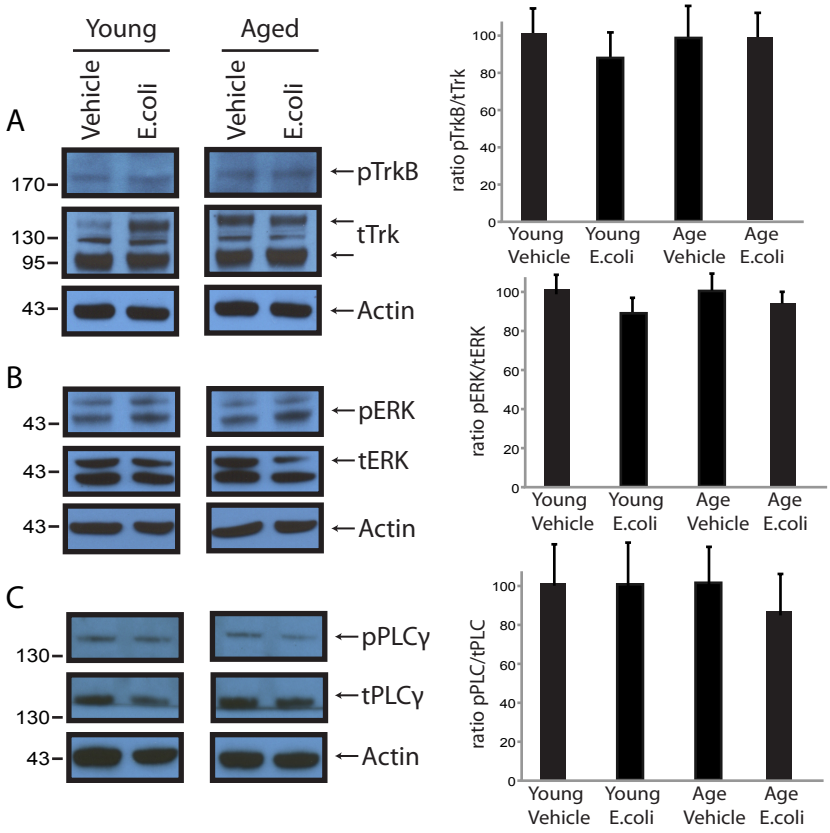

Figure 4. Reductions in phospho- TrkB and downstream TrkB-signaling pathways do not persist for 14 days post-infection. Levels of phosphorylated TrkB (**A**), PLC-γ1 (**B**), and ERK (**C**) were unchanged in synaptoneurosomes prepared from aged animals 14 days following infection. Quantification was as above.

III.5. Discussion

A single peripheral immune challenge, i.p. injection of live *E. coli*, into aged rats produces a prolonged and exaggerated inflammatory response in the CNS [11]. Following a peripheral infection, levels of the pro-inflammatory cytokine IL-1β are significantly upregulated in the hippocampus; causing pronounced deficits in hippocampus-dependent memory and synaptic plasticity [13, 24]. Levels of

hippocampal IL-1ß were most abundant 4-5 days following infection [13], and deficits in memory and plasticity were observed at this time point [13, 24]. It was previously reported that peripherally-induced increases in IL-1ß in hippocampus persists up to 8 days following a single injection of live *E. coli*, but returns to baseline levels by day 14 [13]. More interestingly, impairments in hippocampus-dependent memory parallel these increases in hippocampal IL-1ß; with observed deficits in behavioral performance on memory tasks at day 8, but not day 14 post-infection [11].

The mature BDNF protein isoform plays a large role in facilitating forms of synaptic plasticity that underlies some forms of memory [18, 21, 152]. BDNF is shown to facilitate these forms of plasticity via TrkB signaling; an event that causes autophosphorylation of the receptor kinase itself, as well as phosphorylation of downstream proteins PLC-γ and ERK [117]. We have previously reported that synaptic levels of mBDNF protein are significantly reduced in hippocampal synaptoneurosomes from aged animals 5 days following a peripheral infection [31]. Furthermore, reductions in synaptic levels of mBDNF were consistent with decreases in autophosphorylation of the TrkB receptor (pTrkB), pPLCγ-1 and pERK at the same time point [31]. Here, we extend the initial findings and show that reductions in levels of mBDNF protein persist for 8 days post-infection, but return to baseline levels at day 14. We observed a similar decreasing trend in protein expression of pPLCγ-1 and pERK at day 8, but not day 14 post-infection; suggesting

that these decreases are consistent with infection-evoked upregulation of IL-1β in hippocampus.

It is becoming clearer that the aged brain is vulnerable to inflammatory events that can have precipitous declines in normal cognitive function; the likes of which include severe memory impairments and the potential onset of memory-associated neurodegenerative disorders. We now have mechanistic evidence suggesting that age and infection-evoked hippocampal dysfunction occurs by targeting BDNF and BDNF-dependent signaling at synapses [31]. We see that hippocampus-dependent memory impairments follow, in concert the heightened levels of IL-1 following a peripheral infection; at days 4-5 post-infection, persisting through day 8, and returning to baseline levels at day 14 [13]. Thus, in order to fully understand the prolonged effects of inflammation in the aged brain on synaptic mechanism, we have extended on our 4-5 day time point and determined that a single inflammatory event is capable of have persistent effects on BDNF protein expression and BDNF-dependent signaling in the hippocampus.

This work is the first to report that a single, acute peripheral inflammatory event can have prolonged effects on neural function. It is quite remarkable that an acute infection can have persistent effects on neural function up to 8 days following the event. More specifically, we have identified a specific target (BDNF protein expression at hippocampal synapses), and a potential mechanism (BDNF-dependent synaptic signaling pathways), by which the pro-inflammatory cytokine IL-1β acts on to potentially cause cognitive impairments and memory deficits.

We found that decreases in the phosphorylation of PLCγ-1 and ERK occurred 4-5 days post-infection [31], however only reductions of pPLCγ-1, but not pERK extended to 8 days post-infection in aged animals. One possibility for this may be due to the fact that phosphorylation of PLCγ-1 is an event closely associated with activation of TrkB; a synaptic target of mBDNF [117]. In addition, ERK phosphorylation occurs farther downstream of PLCγ-1, and ERK activation is not specific in that it can be phosphorylated by a number of cellular events; including Ca^{2+} influx thorough a number of post-synaptic receptors aside from Trks [146].

Altering BDNF protein isoforms and BDNF-dependent can affect learning and memory acquisition [131, 132]. Our results indicate that these effects on BDNF occur as a result of aging and infection. Thus, this may be one potential mechanism that underlies the cognitive decline and memory impairments with aging. Synaptic failure is a phenomenon thought to underlie the early-stage hippocampal inefficiencies contributing to the memory impairments associated with Alzheimer's disease (AD) [125]. In addition to aging, neuroinflammation significantly influences the onset and severity of AD pathologies [61]. In addition, there is a strong correlation between increases in IL-1ß and hippocampal dysfunction observed in AD [35, 61, 119]. Therefore, it is possible that a common mechanism underlying cognitive dysfunction associated with aging, and potentially AD may be dependent on BDNF protein at synapses, as well as activation of downstream signaling cascades.

CHAPTER IV

Aging and CNS Inflammation Arising from a Peripheral Infection Enhances LTD and Increases proBDNF – $p75^{NTR}$ Interactions

IV.1. Abstract

Older individuals often experience precipitous declines in cognitive function after events (Ex. surgery, infection, or injury) that trigger activation of the peripheral immune system. Aging sensitizes the hippocampal inflammatory response to peripheral infection, increasing the size and duration of the resulting spike in interleukin-1beta (IL-1ß). We have previously demonstrated that in aging (24-month-old), but not in young (3-month-old) F344xBN rats, a peripheral immune challenge (i.p. injection of live *E. coli*) triggers an exaggerated elevation in hippocampal IL-1ß, which in turn disrupts forms of hippocampal long-term memory and synaptic plasticity known to be BDNF-dependent [11, 24].

Hippocampal memory processes are thought to involve shifts in the balance of plasticity processes - LTP and LTD (long-term potentiation and depression of excitatory synaptic transmission). Numerous studies have shown that disruptions in hippocampal LTP [4, 6, 39, 42, 114, 116, 148], and enhancements of hippocampal LTD [93, 163] are strongly correlated with memory impairments. In addition, shifts in the direction of hippocampal synaptic plasticity have been reported in rodent models of neurodegenerative diseases associated with memory loss [77].

Interestingly, BDNF modulates both LTP and LTD. BDNF is synthesized as a precursor protein (proBDNF), and cleaved to produce the mature BDNF protein isoform (mBDNF). Pro-BDNF binds preferentially to the pan-neurotrophin receptor $p75^{NTR}$, activates apoptosis-related signaling pathways, and facilitates long-term depression (LTD) in the hippocampus. In contrast, mBDNF binds to TrkB receptors, promotes cell survival, and is required for some forms of long-term potentiation (LTP).

We have previously found that mBDNF, but not pro-, is significantly reduced in hippocampal synaptoneurosomes prepared from aged animals following an infection [31] - an observation consistent with reduced theta burst L-LTP at Schaffer collateral-CA1 synapses in these animals [24]. Here, we report that the combination of age and infection enhance hippocampal LTD and increase proBDNF - $p75^{NTR}$ interactions in hippocampal tissue. Furthermore, we do not observe changes in sortilin and pJNK, proteins associated with $p75^{NTR}$ signaling; suggesting a non-apoptotic role for proBDNF – p75 activity. This work begins to examine the functional role of endogenous BDNF protein isoforms in memory-related plasticity processes. It may also provide novel insights into the early stages of synaptic failure in a variety of disorders associated with dysregulated brain inflammatory responses (Ex. post-operative cognitive dysfunction, autoimmune diseases, depression, PTSD and some neurodegenerative disorders).

IV.2. Introduction

The combination of aging and a peripherally-induced CNS inflammatory response, characterized by elevated levels of the pro-inflammatory cytokine IL-1ß, impairs hippocampus-dependent memory consolidation in 24-month, but not 3-month old F344xBN rats [11]. These age and infection-evoked declines in memory are mirrored by specific deficits in theta-burst evoked hippocampal late-phase long-term potentiation (L-LTP) [24]. Theta burst L-LTP involves mBNDF-dependent activity at hippocampal synapses, and this form of long-term synaptic plasticity is associated with long-lasting forms of memory in the hippocampus [21, 23, 84, 152]. However, the mechanisms through which age and infection interact to produce specific deficits in plasticity and hippocampus-dependent memory are still poorly understood.

BDNF is first synthesized as a precursor protein containing a pro-domain (proBDNF), which later undergoes post-translational cleavage to produce the mature form of the BDNF protein (mBDNF) [98, 104]. This processing is critical in determining the cellular function of BDNF because each isoform has distinct and reciprocal functions at hippocampal synapses. Mature BDNF facilitates multiple forms of LTP in the hippocampus important for the acquisition and consolidation of multiple forms of memory [85, 96, 104, 140]. In addition, mBDNF – Trk receptor activity activates downstream signaling pathways that include PLCγ-1 (phosphor-lipasce C gamma-1) and MAPK (Ras-mitogen-activated protein kinase), which are necessary for facilitating plasticity and promoting cell survival [96, 117]. ProBDNF, on the other hand, has been shown to facilitate LTD following binding and

activation of the pan-neurotrophin receptor $p75^{NTR}$; an event linked to memory impairments and memory-related neurodegenerative diseases [93, 164]. $P75^{NTR}$ is necessary for facilitating synaptic plasticity in the hippocampus, however, $p75^{NTR}$ activation by pro-neurotrophins also initiates downstream signaling pathways that regulate apoptosis: these include JNK (c-Jun NH(2)-terminal kinase and NF-κB (nuclear factor-kappa B) [85, 117]. To regulate apoptosis, $p75^{NTR}$ requires the formation of a complex with sortilin, a type I transmembrane glycoprotein that is a member of the mammalian vacuolar protein sorting 10p domain [133, 143]. Additionally, sortilin is necessary for regulating apoptosis and neurodegeneration associated with AD [145]. The apoptotic function occurring through $p75^{NTR}$ signaling is more pronounced, however, its role in regulating synaptic plasticity is less known. Mechanisms driven by distinct BDNF protein isoforms can have entirely different effects on synaptic function. They can play a large role in regulating synaptic plasticity and memory in the hippocampus, as well as initiate cell death and associated with memory impairments. Thus, it is critical to determine which BDNF isoforms and signaling pathways are at play that may contribute to changes in plasticity and memory associated with age, inflammation and potentially neurodegenerative disorders.

We have previously reported that levels of the mature BDNF protein isoform, but not the full-length pro BDNF protein are significantly reduced in hippocampal synaptoneurosomes prepared from aged, but not young animals with a recent history of infection [31]. The reduction in the mBDNF protein isoform is consistent

with the age and infection-evoked declines in hippocampal L-LTP and hippocampus-dependent memory previously observed [13, 26]. However, additional effects on hippocampal function that may arise from shifting the ratio of the endogenous proBDNF and mBDNF protein isoforms have not yet been identified.

In the present study, we observed enhanced LTD in hippocampal slices prepared from aged animals with a recent history of infection. Furthermore, we observed an increase in proBDNF – $p75^{NTR}$ interactions; suggesting a potential cellular signaling mechanism which may be facilitating this enhancement of LTD. Interestingly, sortilin and $p75^{NTR}$ downstream activator protein JNK were unaffected by age and infection. These effects are dependent on IL-1 signaling, as administration of the IL-1ß-receptor antagonist (IL-1RA) blocks the enhancement of LTD, as well as the increase in proBDNF – $p75^{NTR}$ receptor – ligand interaction. These results extend our initial findings, demonstrating that the combination of aging and a peripherally-triggered CNS inflammatory response can influence other forms of BDNF-associated synaptic plasticity in the hippocampus. Furthermore, these data provide further support for the idea that aging and inflammation can shift levels of endogenous BDNF protein isoforms at hippocampal synapses; a result that is consistent with shifts in the polarity of hippocampal synaptic plasticity.

IV.3. Materials and Methods

Animals. The rats were 3- and 24-month old male Fischer344/Brown Norway F1 crosses from the NIA Aged Rodent Colony. Animals were group housed, on a 12-hour light dark cycle, with *ad libitum* access to food and water. Upon arrival, all rats were allowed to acclimate to the animal facility for at least a week before experiments are begun. All experiments were conducted in accordance with protocols approved by the University of Colorado Animal Care and Use Committee.

Infection. One day prior to the start of experimentation, stock *E. coli* cultures (ATCC 15746; American Type Culture Collection, Manassas, VA) were thawed and cultured overnight (15–20 hour) in 40mL of brain-heart infusion (BHI; DIFCO Laboratories, Detroit, MI) in an incubator (37 ^{O}C, 95% air + 5% CO_2). The number of bacteria in individual cultures was quantified by extrapolating from previously determined growth curves. Cultures were then centrifuged for 15 minutes at 3000 rpm, the supernatants were discarded, and the bacteria were resuspended in sterile phosphate buffered saline (PBS), to achieve a final dose of 2.5 X 10 ^{9} CFU in 250µl. All animals received an intraperitonneal (i.p.) injection of 250µl of either *E. coli* or the vehicle (sterile PBS).

IL-1RA Administration. IL-1RA was administered immediately before i.p. injections of vehicle saline or live *E. coli*. Rats were briefly anesthetized with halothane. The dorsal aspect of the skull was shaved and swabbed with 70% ETOH. A 27-gauge needle attached via PE50 tubing to a 25µl Hamilton syringe was

inserted into the cisterna magna. To verify entry into the *cisterna magna*, ~ 2µl of CSF was drawn. In all cases, CSF was clear of red blood cells indicating entry into the cisterna magna. Vehicle (0.9% saline) or hIL-1RA (100µg) was administered in 3µl total volume.

Slice Preparations. Physiology experiments were performed 4-5 days after the initial infection This time point was chosen based on several observations: **(1)** all of the animals have completely recovered from the infection and **(2)** the 24 month old rats, but not the 3 month old rats have significantly higher levels of proBDNF present at hippocampal synapses [31].

Hippocampi were collected from rats following decapitation. Transverse hippocampal slices (400µm) were prepared using conventional techniques (E.g. [107, 108]). Slices were maintained in an interface chamber at 28ºC, and perfused with an oxygenated saline solution (124.0mM NaCl, 4.4mM KCl, 26.0mM $NaHCO_3$, 1.0mM NaH_2PO_4, 2.5mM $CaCl_2$, 1.3mM $MgSO_4$, 10mM glucose). Slices were permitted to recover for at least 90 minutes before recording. Field excitatory postsynaptic potentials (fEPSP) were recorded from Schaffer collateral–CA1 synapses by placing both stimulating and recording electrodes in the *stratum radiatum*. All stimuli were delivered at intensities that evoked field EPSP slopes equal to 40% of the maximum in each slice. Test stimuli were delivered once every minute, and test responses were recorded for 15-30 minutes prior to beginning the experiment to assure stability of the response.

Stimulation Protocol. LTD was induced in hippocampal slices using the following low-frequency stimulation protocol: 1Hz for 15 minutes (900 pulses total). The same stimulus intensity was used for tetanization and evoking test responses.

Co-Immunoprecipitation and Crosslinking. Hippocampi from young and aged animals with and without a recent history of infection were collected 4-5 days post injection. Tissue was homogenized using a glass Teflon pestle in cold RIPA buffer (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS) and centrifuged at 15,000 x g for 10 minutes to remove insoluble material and unbroken cells. *Co-IP:* All steps were done at 4^0C and/or on ice; 1.5mg of protein brought to 1mL total volume was used for the assay. The tissue lysate was pre-cleared by adding 25μL of protein agarose beads and disrupted for 3 hours. proBDNF was immunoprecipated by an antibody specifically raised against the pro-domain of full-length BDNF Antibody was added to the cleared lysate at a concentration of 1:100 and allowed to incubate for 1 hour while neutating. After, 50μL protein agarose beads were added and the complex was allowed to form overnight. All beads were spun at 20,000 x g for 30 seconds. Beads were washed 3 x in washing buffers (high to low salt and with and without lysate detergent) for 25 minutes. After the final wash, beads were dried and 15 μL of 4x Laemmli sample buffer was added and samples were heated at 70^0C to dissociate the complex. Interactions were analyzed using Western Blot analysis.

Western Blot Analysis. Fractionated samples obtained were prepared under reducing conditions in 4x Laemmli sample buffer and heated at 70˚C for 10

minutes. For Western blotting, samples were loaded onto NuPage Novex (Invitrogen) Bis-Tris pre-cast gels, transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) and probed for BDNF (sc-546; Santa Cruz Biotechnology, Santa Cruz, CA, USA) pJNK (9251; Cell Signaling, Danvers, MA, USA), sortilin (612101; BD, San Jose, CA, USA) and TrkB (sc-8316; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were probed with β-tubulin (MAB1637; Millipore, Billerica, MA, and actin (sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for loading controls. Secondary antibodies were purchased from GE Healthcare (Buckinghampshire, UK) and SuperSignal West Pico Chemiluminescent was purchased from Pierce (Rockford, IL, USA). Following chemiluminescent application, blots were exposed to autoradiography film (Midsci, St. Louis, MO, USA). Blots were stripped using Restore Western Blot Stripping Buffer (Pierce) for 15 minutes and washed 3 x 10 minutes in 0.1% PBS/Tween and subjected to standard Western blotting conditions.

IV.4. Results

LTD is enhanced in aged animals with a recent history of infection

It has been previously reported that the combination of age and infection impairs hippocampus-dependent memory [8-10, 49, 57-59], and these memory deficits are consistent with impairments in hippocampal LTP [24, 33, 154, 155]. Therefore, we examined whether this combination of age and infection can alter other forms of hippocampal synaptic plasticity, such as LTD.

For these experiments hippocampal slices from young and aged animals with and without a recent history of injection, as well aged animals that received IL-1RA *icm* immediately before *E. coli* injection were tetanized using low-frequency stimulation (1Hz for 15 minutes; a total of 900 pulses) to induce LTD. We observed an enhancement of LTD in 24-month-old animals 4 days post-infection with live *E. coli* (Fig 1), compared to young and age-matched vehicle-injected controls (Fig 1). Also, IL-1RA blocked the enhancement of LTD in aged and infected animals. The aged vehicle-injected animals, as well as young vehicle and E. coli injected animals produced a steady LTD response (exhibited 25% drop in slope from baseline), whereas the aged-infected animals exhibited an enhancement of LTD (dropping about 60% from baseline and 40% from control animals [p_{age}=f(3,56)=662.584, p<0.001; $p_{infection}$=f(3,56)=989.512, p<0.001]).

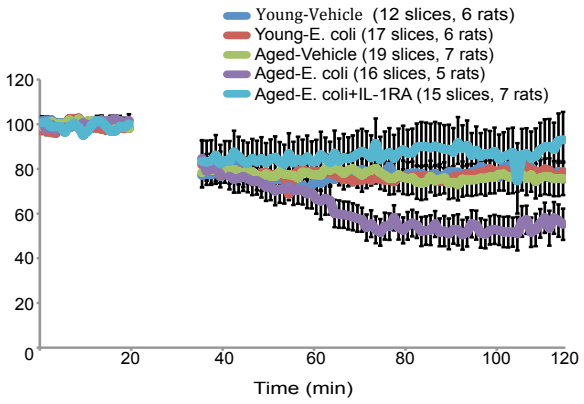

Figure 1. LTD is significantly enhanced in aged rats following infection. LTD enhancement is blocked with administration of IL-1RA into *icm* of rats before injection of *E. coli.* Slices were prepared from animals 4 d after the *E. coli,* IL-1RA and vehicle injections. LTD was elicited at the Schaffer collateral–CA1 synapse using the following stimulus protocols: low-frequency stimulation (1 Hz stimulus delivered for 15 minutes, 900 pulses total). LFS produced a moderate LTD response in age and young vehicle (blue & green) and young infected rats (red). Age and infection significantly enhanced the LTD response (purple), which was blocked by IL-1RA. Error bars indicate SEM.

proBDNF – p75 interactions increase in hippocampal tissue from aged animals following infection

The proBDNF protein isoform is reported to be involved in facilitating LTD in the hippocampus through its interaction with the $p75^{NTR}$ receptor [164]. We have previously reported that levels of mBDNF, but not proBDNF are decreased in hippocampal synaptoneurosomes prepared from aged, but not young animals with a recent history of infection. This suggests that neurons in these animals have an increased ratio of pro – to – mature BDNF protein isoform expression.

We therefore began to test the hypothesis that increases in proBDNF – $p75^{NTR}$ interactions parallel an enhancement in hippocampal LTD. To do this, proBDNF and $p75^{NTR}$ proteins were crosslinked and co-immunoprecipated from hippocampal tissue from 3-month-old and 24-month-old animals with and without a previous history of infection. Age and infection had no impact on basal levels of $p75^{NTR}$ and proBDNF respectively (Fig 2a). We observed a 2.5-fold increase in the amount of proBDNF – $p75^{NTR}$ interaction as indicated by immunoblotting (Fig 2b, lane 4). Basal levels of receptor – ligand interactions were consistent amongst the young and aged counterparts (Fig 2, panel 3, lanes 1-3). ProBDNF – $p75^{NTR}$ interactions were significantly increased with age and infection ($p<0.001$) while no change was detected in aged vehicle animals, as well as young vehicle and E. coli injected animals; suggesting that neither aging- nor infection alone have a detectible effect on proBDNF – $p75^{NTR}$ interactions.

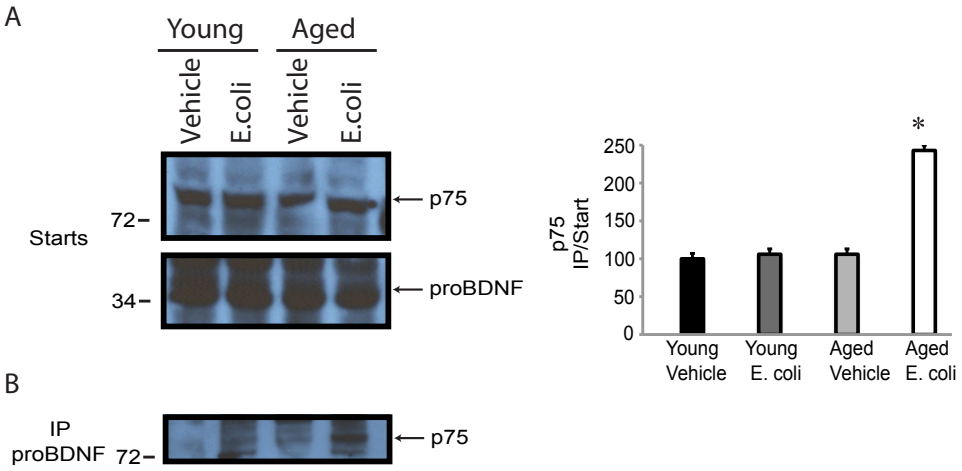

Figure 2. proBDNF – p75 interactions increase with age and infection. Co-immunoprecipitation of proBDNF follwed by Western blot analysis of showing levels of $p75^{NTR}$ in hippocampal lysates prepared from young and aged rats, with and without a recent history of infection. **(A)** Starting lysate showing no change in levels of proBDNF and $p75^{NTR}$ **(B)** proBDNF was pulled down in lysates and immunoblot for $p75^{NTR}$ reveals an increase in receptor – ligand interaction with age and infection. Error bars indicate SEM. All graphs (here and below) represent a minimum of three independent experiments with 1–2 animals per group in each experiment. Asterisks indicate statistical significance from all other groups; individual P-values are reported in the text.

Levels of sortilin and downstream activation of JNK were unaffected by age and infection.

A $p75^{NTR}$ – sortilin complex is necessary for $p75^{NTR}$ activation and induction of downstream apoptotic signaling [133, 143]. The phosphorylation of JNK occurs following pro-neurotrophin binding to $p75^{NTR}$ [117]. To test the involvement of sortilin in our system, and determine if downstream events are affected by age and infection we used Western Blot analysis to look at levels of sortilin and pJNK in hippocampal tissue. Surprisingly, we find that protein levels of sortilin ($p=0.6$) and pJNK ($p=0.8$) are unchanged in all groups; young and aged animals with and without infection (Fig 3). These results suggest an anti-apoptotic role for proBDNF – $p75^{NTR}$ activity; one that may be important for regulating synaptic plasticity, like LTD, which does not require apoptosis.

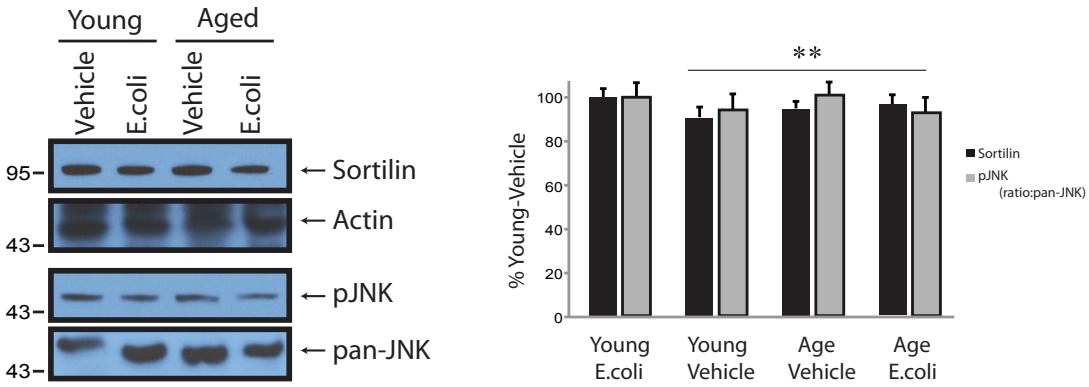

Figure 3. Aging and infection does not alter downstream activity in major p75-signaling pathways. Levels of Sortilin (panel 1) and phosphorylated JNK (panel 3) were uchanged in hippocampal lysates prepared from aged animals 5 d following infection; levels. Phosphorlated proteins were normalized to their respective total levels. Error bars indicate SEM. All graphs (here and below) represent a minimum of three independent experiments with one to two animals per group in each experiment. ** indicates no statistical significance from all other groups; individual *p* values are reported in the text.

IL-1RA blocks the enhancement of LTD and the increases in proBDNF – 75^{NTR} interactions in aged and infected animals.

A peripheral injection of live *E. coli* (i.p.) produces an inflammatory response in the CNS as indicated by upregulation of IL-1ß in the hippocampus [13]. It has been previously reported that this peripheral infection produces specific deficits in hippocampus-dependent memory, impairments in hippocampal L-LTP, and significant decreases in mBDNF protein expression at synapses [24, 31]. Even more interestingly, blocking IL-1 signaling with IL-1RA prevented the deficits in memory, the impairments in L-LTP and the decreases in mBDNF protein expression [24, 31].

To determine if the observed enhancement of LTD, as well as increases in proBDNF – $p75^{NTR}$ interactions are dependent on IL-1 signaling we injected the IL-1 receptor antagonist IL-1RA into the *cistern magna* of the aged rats immediately before an i.p. injection of vehicle saline or *E. coli*. We found that blocking IL-1 signaling in the CNS also blocked the enhancement of LTD (Fig 4a, purple). In fact we see an improvement of plasticity in aged vehicle animals, as indicated by a reduced expression of LTD compared to other controls; suggesting IL-1RA may block low-level endogenous inflammatory responses (Figure 4a, purple). Injections into the *cisterna magna* did not seem to cause additional changes in the expression of LTD in aged vehicle animals – LTD expression was consistently ~25% below baseline as seen in Fig 1 where animals only received i.p. injections (Fig 4a, red & blue). Furthermore, a vehicle injection *icm* did not change the enhancement of LTD in the aged animals follwling *E. coli* infection (as seen in Fig 1 [Fig 4a, green]). Consistent with the electrophysiological data, we find that that the increased proBDNF – $p75^{NTR}$ interactions were also blocked by IL-1RA (Fig4b); suggesting that proBDNF may regulate LTD via $p75^{NTR}$ in the hippocampus, and can be significantly effected by IL-1 regulated inflammation.

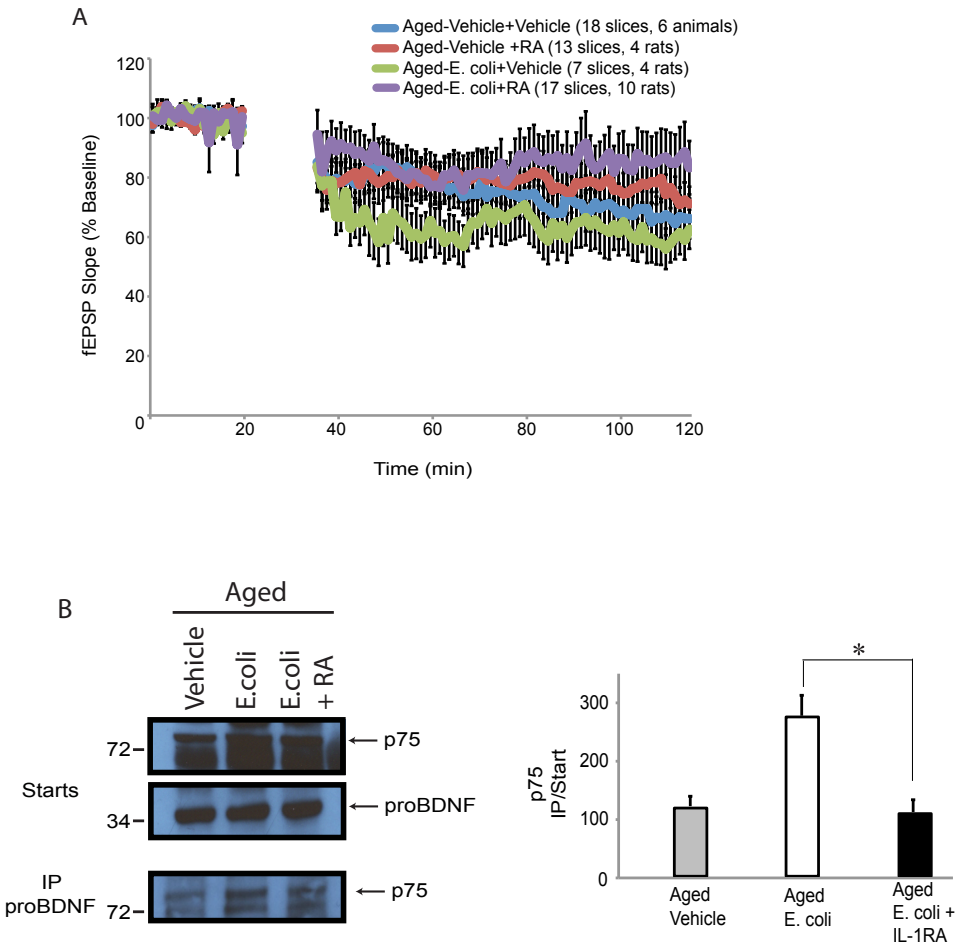

Figure 4. Enhancement of LTD and increase in proBDNF – $p75^{NTR}$ are blocked by administration of IL-1RA into icm of aged animals immediately before *E. coli* injection. LTD stimulation protocol and co-immunoprecipitation wer discussed in Figs 1 & 2. (A) IL-1RA blocks infection-evoked increases in LTD (green) in hippocampal slices prepared from aged and infected animals (purple). Vehicle treatments icm and i.p. had no effect on LTD expression (red & blue). (B) proBDNF was pulled down in lysates and immunoblot for $p75^{NTR}$ reveals an increase in receptor – ligand interaction with age and infection (panel 3, lane 2) which is blocked with administration of IL-1RA (panel 2, lane 3). E.coli and IL-1RA did not alter protein expression in the starting material (panels 1 & 2).

IV.5. Discussion

In the experiments presented here, we have examined the effects of aging and a peripheral immune challenge (i.p. injection of live *E. coli*) on hippocampal LTD, as well as the synaptic role of the proBDNF protein and $p75^{NTR}$ receptor in hippocampus. The principle findings of this study are that the combination of age and infection **(1)** enhance hippocampal LTD following low-frequency stimulation in Schaffer collaterals in area CA1 of hippocampus, **(2)** produce increased proBDNF – $p75^{NTR}$ receptor-ligand interaction at hippocampal synapses; a potential mechanism for facilitating enhanced LTD and (3) these events depend on IL-1 signaling in the brain as administration of IL-1Ra icm blocks these effects.

These findings are consistent with our previous results indicating that the combined effects of aging and infection produce significant memory impairments in the hippocampus, as well as significant reductions in hippocampal L-LTP [11, 24]. Furthermore, this combination produces a shift in the ratio of mBDNF protein expression to that of proBDNF [31]. The present results extend these initial behavioral, electrophysiological and protein biochemical findings by identifying another form of hippocampal synaptic plasticity (LTD) affected by age and infection. In addition, these works have identified a potential cellular mechanism through proBDNF – $p75^{NTR}$ signaling that may be responsible for facilitating the observed enhancements of hippocampal LTD.

The facilitation of LTD through proBNDF and its interaction with $p75^{NTR}$ is not fully understood and its role in normal physiological systems remains controversial. The current evidence in support of the hypothesis that proBDNF is involved in regulating LTD through $p75^{NTR}$ activation, is reported in murine models involving complex genetic manipulations [85, 120, 164]. For example, surgical procedures and viral delivery methods used in the development of genetic models are essentially peripheral immune challenges and compromising the immune system; an event that we now know can have a significant impact on brain function. In addition, the studies done in the field of neurotrophin biology have utilized multiple models of overexpression, which renders the true endogenous system compromised and physiologically challenged.

The work presented here is the first to investigate the endogenous interactions between proBDNF and $p75^{NTR}$, and further correlate this interaction with the regulation of hippocampal LTD in a naturalistic rodent model of aging and peripherally induced CNS inflammation. We report that proBDNF – $p75^{NTR}$ interactions are significantly increased in hippocampal tissue from aged animals with a recent history of infection [Fig 2]. Furthermore, we find that increased receptor – ligand interactions are specifically correlated to enhancements in hippocampal LTD in aged and infected animals compared to young and aged counterparts [Fig 1]. Young vehicle and infected animals, and aged vehicle animals displayed consistent LTD [Fig 1] and detectable levels of basal receptor – ligand interactions [Fig 2]. We find these effects to be specifically linked to the

inflammatory response; as IL-1Ra prevents both the enhancement of LTD and increases in proBDNF – $p75^{NTR}$ interactions [Fig 4]. This work is significant in that these findings provide evidence for a potential mechanism via proBDNF activation of $p75^{NTR}$ for the facilitation of LTD in a naturalistic model; a finding that contributes to the initial reports on BDNF-dependent modulation of plasticity in the hippocampus.

Multiple forms of synaptic plasticity (LTP and LTD) regulated by BDNF and BDNF-dependent signaling pathways play a large role in the expression of learning and memory [21, 23, 85, 96, 163, 164]. Changes in hippocampal synaptic plasticity produce significant changes in memory expression. For example, alterations in hippocampal LTP [4, 6, 39, 42, 114, 116, 148] and enhancement of hippocampal LTD [93, 163] are strongly correlated with significant memory impairments. More interestingly, shifts in hippocampal synaptic plasticity are commonly observed, yet still controversial, in rodent models of Alzheimer's disease (AD) [27, 68, 127]. LTD is enhanced in models of AD, however other groups have shown that LTD is unaffected, and that memory problems arise more so from deficits in LTP [78]. The mechanisms through which changes in memory-related synaptic plasticity occur both naturally and in AD are not yet clear. BDNF plays a large role in regulating multiple forms of synaptic plasticity important for memory. Thus, BDNF and its related signaling pathways may be responsible for the underlying changes in synaptic plasticity observed naturally, as well as with memory-associated neurodegenerative disorders.

Biological changes associated with AD include shifts in BDNF and NGF (nerve-growth factor) expression [61, 109], activation of $p75^{NTR}$ receptor [38, 136], changes in hippocampal synaptic plasticity [70, 78, 166] and the presence of pro-inflammatory cytokines following an inflammatory response [35, 61]. These events have all been identified and targeted independently with AD. Similarly, inflammation-induced upregulation of IL-1ß has profound and individualistic effects on LTP [16, 24, 30, 67, 150], BDNF protein expression [31, 135] and BDNF-dependent signaling pathways [31, 135, 149]. Thus, it is important to identify a potential link between the effects of neuroinflammation, and the signaling pathways necessary for the synaptic plasticity underlying memory in order to better understand the pathologies associated with memory-related disorders, and BDNF may be a potential link.

LTD occurs following the activation of $p75^{NTR}$ by pro-neurotrophins [85, 164]. LTD is a correlate of memory impairments [93], and activation of $p75^{NTR}$ initiates downstream cellular events involved in cell death [117]. It is hypothesized that the occurrence of these events, in combination with aging underlies the memory impairments, cognitive decline and neurodegeneration associated with AD, however, it is unclear how this occurs.

We have previously reported that the combination of aging and inflammation impairs hippocampal L-LTP [24] and shifts the ratio of pro- and mature BDNF protein isoforms at synapses [31]. We now report that LTD is enhanced, and proBDNF – $p75^{NTR}$ interactions are increased in aged animals with a recent history

of infection [Figs1 & 2]. Furthermore, we did not observe changes in $p75^{NTR}$ downstream activators of apoptosis, such as pJNK and sortilin [Fig 3]. Since markers of apoptosis are unchanged, we propose that the combination of aging and inflammation in the brain may be an early event promoting synaptic inefficiency and synaptic failure; a process thought to be occurring in the early-stage pathologies of MCI and AD [125]. In conclusion, our findings provide support that the endogenous proBDNF – $p75^{NTR}$ system may facilitate LTD in the hippocampus, and further providing implications for a mechanism potentially underlying the early synaptic events that occur in memory-related disorders.

CHAPTER V

ProBDNF expression changes with Aß and IL-1ß treatment

V.1. Abstract

Alzheimer's disease (AD) is characterized by severe memory loss resulting from the aggregation of monomeric Aß in entorhinal cortex and hippocampus. Aging still remains the primary process underlying AD onset; however inflammatory components, such as interleukin-1 beta (IL-1ß) can dictate the severity of AD pathologies. IL-1ß and Aß independently impair memory, produce deficits in synaptic plasticity and alter BDNF (brain-derived neurotrophic factor) biology in the hippocampus. BDNF is synthesized as precursor pro protein (proBDNF) that later undergoes proteolytic cleavage to produce the mature form of BDNF (mBDNF). ProBDNF interacts with the pan-neurotrophin receptor $p75^{NTR}$ and activates cell death-mediated pathways. Increase in proBDNF and $p75^{NTR}$ expression and activity correlate with the neurodegeneration associated with AD. Here we show that levels of proBDNF, but not mBDNF are significantly increased in BDNF-expressing human neuroblastoma SH-SY5Y cells following treatment with IL-1ß and Aß alone. Increases in proBDNF were further enhanced with the combination of Aß and IL-1ß. To determine the effects of inflammation in AD we

injected 6-month-old and 10-month-old APP/PS1 transgenic mice with lipo-polysaccharide (LPS) and found that levels of the proBDNF receptor $p75^{NTR}$ are increased in an age and infected manner. Levels of proBDNF, but not mBDNF were increased in an aged dependent manner only, unaffected by infection.

V.2. Introduction

Clinical symptomology associated with Alzheimer's disease (AD) includes early stage delirium, mild cognitive impairment (MCI), dementia, and memory loss [48, 63]. Upregulation of the amyloid precursor protein (APP), along with the accumulation of the secretase-dependent production of insoluble-oligomeric amyloid-beta protein (Aβ) are primary biochemical characteristics of AD [37, 50, 94]. These behavioral and biochemical changes associated with AD are significantly influenced by compromising the innate immune system [35, 61]. The effects of inflammation on AD pathologies have been well studied in a clinical setting; however the cell biological mechanisms underlying them are poorly understood. Here we explore several synaptic proteins and signaling pathways in the rodent brain and a neuronal cell line that may be targeted by AD and neuroinflammation; potentially giving rise to the cognitive decline and memory impairments commonly observed.

Central and peripheral infections have the capacity to initiate the innate immune response, which is characterized by the production and release of pro-

inflammatory cytokines interleukin-1 beta (IL-1ß) and tumor necrosis factor-alpha (TNFα), to name a few [14, 43]. Following an infection, or immune challenge, production and processing of pro-inflammatory cytokines occurs – a process dependent on the activation of the NLRP1 inflammasome (nucleotide binding domain, leucine-rich repeat (LRR)-containing receptor-1) [95]. Activation of the NLRP1 inflammasome promotes the cleavage of pro-caspase 1 that produces initiates the maturation and release of IL-1ß [95]. Elevating levels of cytokines following an inflammatory event can exacerbate the cognitive impairments caused by Alzheimer's disease, as demonstrated in both animal models of the diseases as well as human patients [35, 61]. Furthermore, upregulation of IL-1ß and TNFα can cause excessive release and accumulation of the Aß protein in the brain; a process known as oligomerization and plaque formation [60].

Aside from the evident clinical symptoms of AD (including significant cognitive dysfunction and severe memory loss), there seems to be a critical cellular event that occurs which may give rise to AD-associated impairments – the targeting of neuronal synapses by Aß plaques. Individuals with AD-associated dementia report having significant cortical atrophy that includes neuronal loss, decreased spine density and neurite degeneration [55, 69, 79, 147]. Furthermore, synaptic targeting by oligomeric Aß in the basal forebrain and hippocampus is correlative with significant deficits in synaptic plasticity – forms of plasticity known to underlie memory [70, 86, 89, 127, 166]. Because of this strong correlation, extensive work has gone into examining the effects of Aß plaques on multiple forms of synaptic

plasticity, specifically in the entorhinal cortex and hippocampus – primary brain regions affected by AD due to abundant inputs from entorhinal cortex into hippocampus for facilitating learning and memory [101, 126, 140]. However, the cellular and biochemical mechanisms behind changes in memory and plasticity have yet to be identified.

BDNF is a member of the neurotrophin family of proteins that is highly expressed in the hippocampus and is important for the induction and facilitation of multiple forms of synaptic plasticity [93, 96]. Forms of hippocampal synaptic plasticity, like long-term potentiation (LTP) and long-term depression (LTD), have long been accepted as a model for the cellular and molecular mechanisms underlying the synaptic changes associated with learning and memory [21, 23, 96, 152]. Deficits in synaptic plasticity are commonly observed in models of AD [78, 127] and neuroinflammation [11, 24, 147] alone, but whether these forms of plasticity rely on BDNF and BDNF-dependent signaling is still not fully known.

BDNF is first synthesized as a precursor protein containing a pro-domain (proBDNF), which later undergoes proteolytic cleavage to produce the mature form of the BDNF protein (mBDNF) [98, 104]. Mature BDNF binds to TrkB (tyrosine kinase B) tyrosine kinase receptors, promotes cell survival, and facilitates some forms of long-term potentiation. In contrast, proBDNF binds preferentially to the pan-neurotrophin receptor $p75^{NTR}$, activates apoptosis-related signaling pathways, and may facilitate long-term depression in the hippocampus. Recent work has provided some insight into the mechanisms that may underlie AD-associated, as

well as inflammation-evoked changes in synaptic plasticity. It is reported that IL-1ß can directly interfere with BDNF-dependent signal transduction and BDNF-associated forms of synaptic plasticity [24, 149, 150]. Furthermore, Aß is shown to directly bind to- and activate the $p75^{NTR}$, thereby altering synaptic plasticity and activating downstream apoptotic pathways that are thought to underlie the neurodegeneration associated with AD [38, 70, 78, 136, 166]. Thus, we hypothesize that levels of BDNF protein isoforms and BDNF-associated synaptic target proteins will be altered in an in vitro model of AD and neuroinflammation, as well as in the APP/PS1 transgenic mice following LPS infection.

V.3. Materials and Methods

Cell culture, plasmids and transfection methods. PC-12 cells (ATCC) were cultured in Dulbecco's Modified Eagle's Media (DMEM) with 10% fetal bovine and 5% horse serum serum with penicillin (100 U/ml), Streptomycin (100µg/ml) and L-Glutamine. Human SH-SY5Y neuroblastoma cells (generous gift of Dr. Matt LaVoie) were cultured in DMEM with 10% fetal bovine serum with penicillin and streptomycin. BDNF was cloned into lentiviral expression vector pCMV-IRES mCherry (generous gift of Dr. Kevin Jones). Transient transfections were performed using Lipofectomine 2000 plus reagent (Invitrogen) according to the manufacturer's protocol. Transfections were performed using 10µg plasmid DNA per 10cm dish in Opti-MEM media for 18 hours. Following transfections treatments were administered.

Pharmacological agents and Treatments. Recombinant rat IL-1ß was purchased from R&D Systems (Minneapolis, MN) and reconstituted in sterile phosphate buffer saline (PBS) with 0.1% BSA. Synthetic Aß was prepared 24 hours prior to treatment and reconstituted in NaOH (generous gift of Dr. Michael Stowell). SH-SY5Y cells were treated with 10ng/mL of rrIL-1ß for 4 hours. Cells were treated with synthetic Aß at a concentration of 500nM for 24 hours. Following whole-cell lysates and synaptoneurosomes were prepared. For lysates, plates were washed in ice cold PBS to remove residual media and lysed in 1x RIPA Buffer [50mM Tris-HCl; 150mM NaCl; 0.1% SDS; 0.5% Na-Deoxycholate; 1% Triton X-100; 1mM PMSF] (300µL buffer per 10cm dish) on ice for 25 minutes. Lysate was centrifuged for 10 minutes at 14,000 x g in a cold microfuge producing soluble fraction (supernatant) and insoluble fraction (pellet). Pellet was lysed in 250µL 2% SDS and sonicated. Protein levels were assessed using Western Blot analysis. Both IL-1ß and Aß were dissolved in sterile phosphate-buffered saline prior to dilution of the doses in the tissue culture media

V.4. Results

Aß and IL-1ß independently increase levels of pro-, but not mature BDNF protein

Pro-forms of neurotrophins are upregulated, and their related signaling pathways are altered in Alzheimer's disease [82]. Similar effects are also observed with inflammation [31, 149]. To determine whether Aß and IL-1ß change levels of

BDNF protein isoform expression we analyzed human neuroblastoma SH-SY5Y cell lysates following treatment with synthetic Aß and rrIL-1ß. Lysates collected from cells treated with Aß (500nM for 24 hours) displayed a 2-fold increase in levels of proBDNF [Figure 1a, panel 1, lane 2; p=0.006]. Levels of mBDNF consistently trended upwards, however this was not found to be significant [Figure 1a, panel 2, lane 2; p=0.7]. Similar results were observed following treatment with rrIL-1ß (10ng/mL for 4 hours). Levels of proBDNF protein also increased 2-fold compared to controls [Figure 1b, panel 1, lane 2; p=0.02], while levels of mBDNF trended upwards without significance [Figure 1b, panel 2, lane 2; p=0.7].

The pro-inflammatory cytokine IL-1ß exacerbates the Aß-dependent alterations in BDNF protein expression

Inflammatory events significantly increase the deleterious effects associated with Alzheimer's disease in humans [35, 61]. To emulate this in vitro, we first treated SH-SY5Y cells with Aß (500nM for 24 hours) followed by IL-1ß at 20 hours post-Aß for the final 4 hours. We find that the introduction of pro-inflammatory IL-1ß to a 'pre-Aß – *in vitro*' environment significantly enhances the increase in proBDNF of Aß alone (Fig 1b, panel 1, lane 3 [p=0.006]. To determine if the reverse treatment scheme also had significant effects on BDNF levels we pre-treated cells with IL-1ß (10ng/mL for 4 hours) followed by treatment with Aß (500nM for 24 hours). We found that the increase in proBDNF was no different to that IL-1ß or Aß treatment alone (Fig 1a, panel 1, lane 3). All single and co-treatments had no significant effect on mBDNF protein levels (p=0.7).

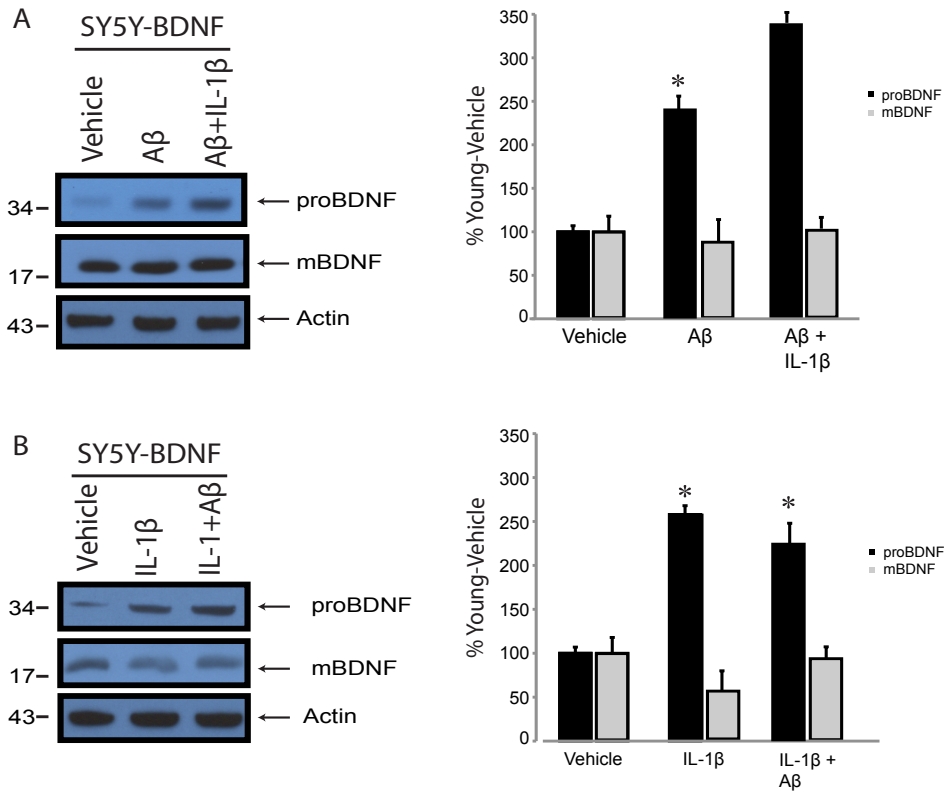

Figure 1. Aß and IL-1ß differentially affect proBDNF protein expression. Western blot analysis showing levels of pro- and mature BDNF in SH-SY5Y lysates following individual and co-treatment of Aß and IL-1ß. **(A)** Representative examples of blots probed with pro- and mature BDNF. Levels of proBDNF were increased with Aß treatment (panel 1, lane 2), and this increase was further enhanced when IL-1ß was administered for 4 hours after 24 hours of Aß treatment (panel 1, lane 2). This represents an inflammatory event in an Aß-rich environment. Levels of mBDNF were unchanged (panel 2) **(B)** Levels of proBDNF were also increased with IL-1ß treatment for 4 hours (panel 1, lane 2). four hour pre-treatment with IL-1ß followed by 24 hour treatment of Aß increased proBDNF from vehicle, but did not exacerbate this effect. Levels of mBDNF were also unchanged (panel 2). Band intensities were quantified (NIH-ImageJ), normalized to actin controls, and expressed as percentages of mean protein levels from vehicle lysates. Error bars indicate SEM. All graphs represent a minimum of three independent experiments. Asterisks indicate statistical significance from vehicle samples; co-treated were compared to single treated; individual P-values are reported in the text.

V.5. Discussion

Aggregation of amyloid beta protein is a key hallmark of Alzheimer's disease [126]. Many hypotheses have been advanced regarding the effects of Aß oligomerization on early stage synaptic failure, synaptic plasticity, and cell signaling. One common hypothesis, the 'Aß hypothesis', states that there is an imbalance in the rate of production and clearance of Aß, causing Aß aggregation that leads to gradual synaptic failure and neuronal dysfunction [125, 126]. This is supported by a number of studies that have identified specific deficits in synaptic plasticity (like LTP), synaptic transmission and cell signaling pathways, including neurotrophin-based pathways (like TrkB and $p75^{NTR}$) that are affected by Aß oligomers [78, 82, 136, 151]. Further studies have provided additional evidence for an inflammatory component that may be at play in regulating the effects of Aß in the AD brain. Glial cells, the primary innate immune cell responsible for regulating the immune response, are activated in the presence of Aß plaques. Whether inflammation acts independently to influence functional deficits observed in AD, or occurs as a result of having the disease is not yet clear and still in debate. However, there is convincing evidence supporting the hypothesis that activation of the immune response, characterized by the upregulation of pro-inflammatory cytokines (such as IL-1ß and TNFα), acts independently on the AD brain to influence the pathologies associated with the disease [35, 61, 119].

Although the role of inflammation on governing the multiple effects of Aß oligomers on synaptic events still warrants clarification, it is well appreciated that both pro-inflammatory IL-1ß and AD-associated Aß share common cellular pathways that target synaptic plasticity in the brain. There are multiple forms of synaptic plasticity currently known, and they have long been accepted as a model for the cellular and molecular mechanisms underlying the synaptic changes associated with learning and memory [21, 23, 96, 152]. There are two isoforms of the BDNF protein, and each isoform has a distinct bi-directional modulation of synaptic plasticity at synapses. ProBDNF is shown to facilitate LTD following $p75^{NTR}$ binding and activation, whereas mBDNF facilitates LTP through the TrkB receptor [85]. It is hypothesized that deficits in memory and plasticity associated with AD and inflammation independently occur through BDNF-dependent signaling – the common pathway shared. Experimental support for this stems from the fact that infection-evoked elevations in IL-1ß completely block LTP [14, 150]; a result consistent with perturbations in BDNF-dependent signaling [149]. Furthermore, similar deficits in LTP, as well as signaling through BDNF-dependent pathways are also observed in systems with abundant Aß oligomers present [78]; as seen by Aß-dependent upregualtion of proBDNF and activation of the proBDNF-associated receptor $p75^{NTR}$ [61, 82, 136].

Here we find that the pro-inflammatory cytokine IL-1ß exacerbates the effects of Aß on BDNF protein levels and BDNF-associated synaptic proteins *in vitro*. Neuronal administration of Aß and IL-1ß *alone* produces a significant

upregulation of the proBDNF protein isoform; an increase that is further that is enhanced when the presence of Aß and IL-1ß co-occur in a neural environment. In vitro analysis of BDNF and BDNF-dependent signaling pathways is not well studied in neuroinflamamtory models of AD; however our work further supports and generally compliments the current *in vivo* data.

The functional consequences of neuroinflammation and AD are strikingly similar when analyzed independently, however the combination of AD and neuroinflammation is poorly understood and not well studied. Currently, there is human and animal data suggesting that cognitive deficits, as well as neurodegeneration and cellular dysfunction associated with mid – to – late stage AD are exacerbated following immune challenges (like illness and infection), but data is minimal [35, 61].

In conclusion, we provide an *in vitro* system modeling the presence of an inflammatory response on Aß abundant neurons – levels physiological to that of the AD phenotype. Our data is the first to report that BDNF protein isoforms and BDNF-associated synaptic proteins are significantly altered in Aß treated human neuroblastoma SH-SY5Y cells following additional treatment with IL-1ß. Thus, this system represents a pathway in which neuroinflammation, AD and BDNF function converges. Additional experiments will uncover more specific details about this mechanism, which in turn may provide targets for more effective therapeutics.

Chapter VI

DISCUSSION

The pertinent findings of the current study are summarized in this chapter. Research questions will be addressed and the significance of each finding will be concluded. Implications of the current research and understanding of the mechanisms underlying both aging and neurodegenerative disorders will be examined. Finally, implications for future studies and therapeutic approaches will be discussed.

VI.1. Topical Discussion

Understanding Memory

The classical experiments conducted with patient HM served as an important human model that provided significant insight into the numerous forms of memory present in the human brain. More specifically, we learned how different brain regions, such as hippocampus and entorhinal cortex for example, are responsible for regulating different forms of memory. Furthermore, in the last two decades a significant rise in neurodegenerative disorders associated with memory loss have

been identified and clinically observed. Because of this, numerous research groups have focused on understanding the molecular and cellular basis of memory in order to identify more effective therapeutics targeting the behavioral and cellular pathologies associated with these disorders.

The hippocampus has long been thought to be the primary brain structure involved in learning and memory consolidation; receiving inputs from several brain structures (like basal forebrain) and consolidating them as long-term memory. Extensive work on hippocampal function and hippocampal long-term potentiation (LTP) has provided a primary model for studying the cellular mechanisms underlying learning and memory acquisition [86, 89]. The most widely known and well-studied form of LTP requires NMDAR (N-methyl-d-aspartate receptor) activity at synapses in the CA1 Schaffer collaterals of the hippocampus [91]. It is through NMDA receptor Calcium (Ca^{2+}) influx that multiple forms of synaptic plasticity in the hippocampus can be initiated and facilitated [96]. These forms of hippocampal synaptic plasticity (LTP) are categorized as: ***a)*** early-phase LTP (E-LTP) and ***b)*** late-phase LTP (L-LTP). E-LTP, a physiological representation of short-term plasticity and memory, is shown to be a preface to LTP and is independent of gene transcription; however induction requires Ca^{2+} influx, and facilitation depends on activation of Ca^{2+}/calmodulin-dependent protein kinase II and protein kinase C (PKC). Activation of these kinases goes on to phosphorylate AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors that proceeds to regulate post-synaptic receptor membrane insertion and additional increased kinase activity

[17, 81, 86, 88, 90, 91, 96, 130, 142]. L-LTP, shown to be a molecular representation of long-term memory (LTM) has similar activity at synapses to that of E-LTP including AMPA and NMDA receptor activation and cation influx [17, 91]. However, L-LTP differs from E-LTP in that it requires nuclear transcription, *de novo* protein synthesis and local dendritic protein synthesis, all of which are shown to last for several hours [19, 64, 97, 141, 142, 159, 168]. These forms of plasticity are shown to occur in hippocampus and underlie several forms of memory. Glutamatergic-dependent receptor activity (NMDA and AMPA) causing cation influx in neurons is important for the induction and facilitation of several forms of LTP. Aside from ion influx however, there are a number of other molecules and cell signaling events that occur at synapses that are as equally important for regulating LTP.

A number of studies have shown that BDNF – TrkB signaling, as well as glutamate receptor-dependent ion influx are important cellular processes underlying learning and memory. Learning animals are found to have increased neurotrophin signaling at hippocampal synapses, as indicated by increased auto-phosphorylation of TrkB receptors following mBDNF binding [26]. This same group also showed that animal learning can be mimicked by theta-burst stimulation of hippocampal slices; and reported theta-dependent increases in TrkB phosphorylation and NMDA-dependent Src kinase (sarcoma tyrosine-protein kinase) activation; a downstream event of NMDA receptor activation [26]. In further support, several other groups have shown that disruptions in hippocampus-

dependent synaptic plasticity are consistent with specific declines in hippocampus-dependent learning and memory [18, 92]; suggesting that multiple forms of synaptic plasticity may give rise to multiple forms of memory. These works provide evidence that LTP is a physiological memory correlate, and these forms of plasticity rely not only on glutamatergic receptor activation, but also on BDNF and BDNF-dependent signaling at hippocampal synapses. It is important to note that there are multiple forms of synaptic plasticity that occur in the hippocampus aside from LTP, and BDNF is shown to be equally involved in facilitating these forms as well. Thus, we must discuss another form of hippocampal synaptic plasticity, long-term depression (LTD).

LTD, like LTP is another cellular and molecular model for synaptic changes associated with learning and memory. LTD has been well studied in multiple brain regions (including cerebellum, striatum and peri-rhinal cortex); however the most prominent work in understanding the mechanisms and behaviors of LTD has been done in the hippocampus [93]. It is not known whether LTD has an independent role in regulating forms of synaptic plasticity underlying learning and memory because it is unclear whether LTD occurs in parallel with LTP, or only serves as an opposition to LTP, thereby having a negative effect on memory (e.g. deleting stored memories and failure to acquire memory) [93]. The idea that LTD is a reciprocal phenomenon of LTP and may be associated with memory impairments is still in debate; however there has been abundant work in identifying independent mechanisms that regulate LTD which are found to be characteristically different

from LTP. Unlike physiological theta frequency [induced by high frequency stimulation (HFS) [4 pulses at 100Hz] in hippocampus to induce LTP, LTD follows patterns of activity that require prolonged low frequent activity in hippocampus [low frequency stimulation (LFS) [600-900 pulses at 1-5 hertz (Hz)] [17, 164]. As seen with LTP, both AMPA and NMDA receptor activity as well as ion influx are necessary for LTD, however Ca^{2+} influx through NMDA receptors is not required and often does not occur during LTD. Interestingly, there exists a compensatory mechanism to counter NMDA inactivity. In the absence of NMDA receptors, calcium influx can still occur through utilization of voltage-gated calcium channels (VDCCs) [99, 100]. Thus, LTD is a form of synaptic plasticity that follows the classical Hebbian requirement of pre- and post-synaptic activity, however the mechanisms of action are functionally different. Although synaptic plasticity still occurs (as seen by ion influx and changes in post-synaptic neurons), there seems to be an inherent difference in the expression of memory associated with LTP and LTD, and the LTD memory correlate is still not fully understood.

There have been a number of studies in support of the hypothesis that LTD is associated with memory impairments. Damage to hippocampus and hippocampal connections (such as the fornix) is shown to produce anterograde amnesia, as well as difficulties performing tasks that require the use of spatial information and memory [93]. Furthermore, activating a stress response in rats is shown to promote low-frequency firing patters in hippocampus (much like LFS-induced LTD), as well as alter LTP; suggesting a form of plasticity underlying both stress-association

memories and potential cognitive decline which may eventually lead to severe memory impairments [41, 93, 165].

In studies of memory-associated neurodegenerative disorders (such as Alzheimer's disease), long-term forms of synaptic plasticity as well as the molecular structure and function of synapses are shown to be markedly effected in brain of animals modeling these diseases [55, 69, 77, 147]. It is proposed that shifts in forms of synaptic plasticity, from LTP to LTD, occur in neurodegenerative disorders suggesting that the induction of LTD is consistent with the memory impairments that are characteristic of these disorders [27, 68, 127]. These works have been followed up in an attempt to identify the cell biological mechanisms underlying the changes in memory and synaptic plasticity commonly observed with the disease pathology. To answer this question, a number of researchers began to look at whether the key players and pathways involved in regulating memory-associated synaptic plasticity in the brain were responsible for the memory impairments observed in clinical cases of memory-associated neurodegenerative diseases. This led to the discovery that neurotrophins, NGF and BDNF specifically, and their related signaling pathways are significantly affected in Alzheimer's disease, therefore opening up a new and important area of investigation [2].

As previously mentioned we see that BDNF is strongly involved in facilitating several forms of synaptic plasticity in the hippocampus; both LTP and LTD [85]. More specifically and mechanistically, variants of the BDNF protein are shown to be primarily responsible for the facilitation of these different forms of

plasticity [85]. BDNF is first synthesized as a precursor protein that contains a pro-domain (proBDNF) that gets cleavage, and produces the mature form of the protein (mBDNF) [85]. Both protein isoforms are shown to be released at synapses in an activity-dependent manner; however each isoform interacts with distinct synaptic receptors having independent functional outcomes. The mature form of BDNF is shown to facilitate LTP and activate downstream signaling pathways PLCγ and ERK following TrkB receptor binding [85, 117]. In contrast, proBDNF binds to – and activates the $p75^{NTR}$ receptor, regulates LTD and activates cell death mediated pathways [85]. More interestingly, there are strong implications for AD-associated pathologies and alterations in BDNF-dependent forms of synaptic plasticity and signaling pathways [2]. Furthermore, activation of $p75^{NTR}$ by pro-neurotrophins is thought to underlie the synaptic changes and initiate the cell death that is observed in AD [2, 28, 78, 136]. Thus, there seems to be in intriguing, and potentially important link between neurotrophin biology and AD pathology.

Memory: Age and Neuroinflammation

Aging has long been associated with cognitive decline. In addition, aging still remains the primary culprit underlying memory-associated neurodegenerative disorders that include several forms of dementia, MCI and clinical Alzheimer's disease. Research in the last decade has provided some critical evidence linking aging-associated cognitive decline with the onset of dementia and AD.

One interesting link, data suggests, is that environmental factors and negative life events may contribute to and/or exacerbate the cognitive decline associated with aging. These events include psychological stress, surgeries and infections which occur commonly throughout life, but are shown to have negative effects on cognitive function in the aged brain [15, 156, 162]. These findings were both interesting and informative; however it was still unclear how these normal life events were capable of affecting cognition. Recent work has explored this phenomenon and the findings show that these negative life events can produce an inflammatory response in the CNS; and it is through these acute inflammatory responses that cognition may be altered. Furthermore, CNS inflammation is becoming an important area of investigation with great focus on elucidating the underlying mechanisms associated with the clinical symptomology associated with cognitive dysfunction and neurodegenerative disorders. To do this, it is critical to identify what additional factors (e.g. environmental) are acquired while aging.

We now know that aging significantly renders the brain more vulnerable to challenging life events such as infections, surgery, and psychological stress. As mentioned above, this phenomenon is evident in many behavioral analyses with aging and diseased individuals; however the molecular and cellular mechanisms which underlie these deficits are poorly understood. The use of a rodent model of aging-associated cognitive decline [11] has provided some insight into the molecular and cellular mechanisms that may underlie memory and plasticity deficits associated with aging and neuroinflammation. The initial findings outlined in

Chapter 2 of this dissertation are the first to identify a potential mechanism, through BDNF-dependent activity and pathways that may underlie hippocampal deficits observed in the aged brain following inflammation. In addition, these works provide convincing evidence that the combination of aging and peripherally-induced CNS inflammation may be a strong contributing factor to the early-stage synaptic failure associated with memory-related neurodegenerative diseases. The significance of these works identifies and targets specific cellular events and neuronal signaling mechanisms that may underlie the cognitive decline associated with the combined effects of age and neuroinflammation. Furthermore, these works provide a potential mechanistic link between early stage memory impairments and later onset of memory-associated neurodegenerative disorders that expand our understanding in the field and may lead to more specific and effective therapeutic targets.

VI.2. Discussion of the Results

Research Question 1

Does the combination of age and a peripheral infection interact to produce changes in the expression of BDNF protein isoforms and the activation of BDNF-dependent signaling pathways at hippocampal synapses?

Hippocampal synaptoneurosomes prepared from young (3-month-old) and aged (24-month-old) F344xBN rats with and without a recent history of a peripheral

infection (i.p. injection of live *E. coli*) were examined in order to detect changes in protein expression. A peripheral infection did not produce changes in synaptic levels of mBDNF in young animals with and without infection, in addition to aged vehicle animals. However, we observed ~60% decrease in the expression of mBDNF protein at hippocampal synapses in aged animals following infection. More interestingly, this deficit in mBDNF was consistent with decreases in levels of phosphorylated TrkB, ERK and PLCγ-1 [31]; events associated with mBDNF activity at synapses [117]. Interestingly, we detected a slight, but not significant (~20%) decrease in the expression of the full-length proBDNF protein isoform as a result of age and infection. These data suggest that a change in the ratio of BDNF protein isoforms occurs at hippocampal synapses in aged animals following infection. Interestingly, we did not see decreases in phosphorylated AKT, another downstream cellular pathway linked to mBDNF activity. These results will be discussed further later in this chapter.

Research Question 2:

How long do age and infection-evoked deficits in mBDNF and mBDNF-dependent signaling persist?

It is previously reported that a peripheral infection of live *E. coli* produces an exaggerated and prolonged inflammatory response in the brain, characterized by upregulation of IL-1ß in the hippocampus of aged F344xBN rats [12]. This exaggerated inflammatory response (upregulation of IL-1ß) in the hippocampus of

aged animals is shown to extend 8 days, but not 14 days following a single peripheral infection [13]. More interestingly, heightened levels of IL-1ß were consistent with hippocampus-dependent memory impairments and deficits. Deficits in hippocampus-dependent memory followed, in parallel the time course for heightened levels of IL-1ß in hippocampus following infection; memory deficits continuing through day 8 and restored by day 14 [13]. Thus, we have explored the 8 day and 14 day time-point in order to determine if these effects are consistent with decreases in mBDNF protein expression and mBDNF-dependent signaling at synapses.

Hippocampal synaptoneurosomes from aged F344xBN rats were prepared in order to identify changes in the expression of BDNF protein isoforms, as well as downstream target proteins of BDNF activity. We observed a moderate, yet significant decrease in the expression of mBDNF (~40%) 8 days following a peripheral infection. At day 14 post-infection levels of mBDNF recover to baseline levels of expression. There was no change in the expression levels of proBDNF or the $p75^{NTR+}$ receptor at day 8 or day 14 post-infection. Furthermore, levels of phosphorylated TrkB, ERK and PLCγ-1 were also decreased at day 8 post-infection, but not day 14, with no changes in total levels of the proteins at either time point. These data expand our previous results at day 4-5 post-infection, identifying changes in mBDNF protein isoforms and downstream mBDNF signaling proteins at day 8 following a peripheral infection in aged animals. This suggests that the residual effects of a single peripheral infection have the capacity to alter BDNF

biology at hippocampal synapses throughout the duration of the inflammatory response.

Research Question 3:

Does the combination of age and infection alter other forms of hippocampus-dependent synaptic plasticity, such as LTD?

Transverse hippocampal slices were prepared from aged animals that have received a peripheral infection of live *E. coli* (5 days post-infection), or an *icm* injection of IL-1 receptor antagonist (IL-1RA) immediately before *E. coli* injection to block IL-1 signaling. LTD was significantly enhanced in aged animals with a recent history of infection following low-frequency stimulation compared to young and aged counterparts. This infection-evoked enhancement of hippocampal LTD was blocked with administration of IL-1RA, suggesting that infection-induced IL-1 signaling is necessary for enhancement of LTD.

To investigate further we propose that age and infection may increase proBDNF – $p75^{NTR}$ interactions, a pathway that facilitates LTD in hippocampus [164]. Co-immunoprecipitation assays using hippocampal lysates from aged animals with a recent history of infection reveal that proBDNF – $p75^{NTR}$ receptor-ligand interactions are increased compared to lysates used from young and aged counterparts. Total levels of $p75^{NTR}$ are unchanged with aging alone and aged animals following infection. Interestingly, this increase of receptor-ligand

interactions is blocked in hippocampal lysates from aged animals that received IL-1RA immediately before *E. coli* injection.

These data suggest that the combination of age and infection enhance hippocampal LTD, as well as proBDNF – $p75^{NTR}$ interactions; a potential pathway underlying enhanced hippocampal LTD. Furthermore, these events are dependent on infection-evoked increases in IL-1ß and IL-1 signaling, as IL-1RA blocks both the enhancement of LTD and the increases in proBDNF – $p75^{NTR}$ interactions.

Research Question 4:
Do Aß and IL-1ß interact to alter expression of BDNF protein isoforms?

A key characteristic in the clinical symptomology of Alzheimer's disease is the accumulation of Aß peptides resulting from cleavage of the APP protein [37, 50, 94]. Excess Aß in the brain of AD models exhibit enhancements in hippocampal LTD, changes in the ratio of neurotrophin protein expression (pro:mature), and increased activation of the $p75^{NTR}$ receptor [27, 68, 70, 109, 127, 166]. These phenotypes are currently expressed in our model of aging and peripherally-induced CNS inflammation, and the effects of CNS inflammation in models of AD have yet to be investigated.

Human neuroblastoma SH-SY5Y cells overexpressing BDNF were used to determine the effects of Aß and IL-1ß on BDNF in neurons. Our *in vivo* findings (60% reduction in mBDNF expression [31]) were replicated in our *in vitro* model

following treatment of 10ng/mL of rat-recombinant IL-1ß for 4 hours. In addition, levels of proBDNF were significantly increased following IL-1ß treatment; a result we did not see in our previous *in vivo* findings. One possibility for this finding is that we lose the aging component in cells lines, and increase in proBDNF may not occur in aging brain, but do in culture. Treatment with Aß (500nM for 24 hours) only slightly reduced levels of mBDNF by about 20%. However, proBDNF was significantly upregulated following Aß treatment, a finding consistent with IL-1ß treatment. These data provide evidence for the effects of the inflammatory molecule IL-1ß and the AD-associated peptide Aß on BDNF expression alone.

To further examine the effects of inflammation on AD we prepared two *in vitro* systems to model this:

1) Inflammatory environment encounters AD oligomers: Neurons in an inflamed state (treatment of SH-SY5Y cells with the pro-inflammatory cytokine IL-1ß for 4 hours) followed by presence of AD-associated oligomers (treatment of cells with Aß for 24 hours)

2) Cells expressing AD phenotype encounter an inflammatory event (SH-SY5Y cells first treated with Aß for 24 hours, followed by treatment of IL-1ß for 4 hours).

From these experiments we found that neurons expressing an AD phenotype (oligomeric Aß) produce a sensitized response following an inflammatory event (increased IL-1ß). Levels of proBDNF significantly increased beyond levels of IL-1ß

and Aß alone, as well as co-treatment of IL-1ß followed by Aß. Furthermore, mBDNF protein expression was unchanged with all treatments: IL-1ß and Aß alone and both co-treatments (as described above). Thus, these results further support the hypothesis that the effects of increased AD-associated Aß on BDNF are significantly influenced by an inflammatory event.

VI.3. Significance of Findings

Age and inflammation interact to produce specific deficits in mBDNF and activation of downstream signaling pathways.

We have previously demonstrated that a peripheral immune challenge produces profound disruptions in forms of hippocampus-dependent long-term memory and synaptic plasticity known to be BDNF dependent in aged, but not young, F344xBN rats [11, 24]. The work completed and discussed in Chapter 2 have extended these observations, examining for the first time the combined effects of aging and infection on levels of BDNF protein isoforms and their receptors at synaptic sites in the hippocampus. The key findings of this work are that an immune challenge in aging rats **(1)** triggered a minimal reduction in proBDNF and a much larger reduction in mature BDNF detectable in hippocampal synaptoneurosomes prepared 5 d after the injections, after all the rats had recovered from the acute infection; **(2)** had no significant effects on levels of BDNF receptors; but **(3)** significantly reduced phosphorylation of TrkB, and downstream activation of PLCγ-1 and ERK, consistent with decreased availability of mBDNF for

activation of TrkB; and (4) no longer reduced mBDNF and phospho-TrkB if IL-1 receptors in the brain were blocked with a selective antagonist.

These data are consistent with the hypothesis that the interaction of aging and an infection might decrease availability of BDNF at hippocampal synapses, and thus might contribute to selective deficits in forms of long-lasting plasticity and memory that require BDNF for their full expression. We found that the interaction of aging and infection reduced levels of mBDNF at synaptic sites by > 50%. Mouse models of BDNF haploinsufficiency have provided evidence that a critical threshold level of BDNF is necessary for full function in memory-related plasticity. Heterozygous BDNF (+/-) mice with approximately half the normal levels of BDNF in their brains have significant impairments in long-lasting synaptic plasticity [68, 104, 108] and in hippocampus-dependent learning and memory [80].

The consequences of reduction of BDNF protein isoforms are not yet so well studied, but mBDNF appears to play an important role in some forms of long-lasting synaptic plasticity in the hippocampus. Mature BDNF can be generated by cleavage of proBDNF by plasmin, an extracellular protease activated by tissue plasminogen activator (tPA)-dependent cleavage of plasminogen [118]. Theta burst stimulation is reported to induce secretion of tPA, and to increase extracellular conversion of proBDNF to mBDNF in cultures of hippocampal neurons [102]. Application of mBDNF, but not a cleavage-resistant proBDNF, can rescue deficits in theta burst L-LTP hippocampal slices from mice lacking tPA or plasminogen [104]. Mature BDNF can also rescue the impairment of theta-burst L-LTP caused by

inhibition of protein synthesis in wild-type mice [104], suggesting that the mBDNF isoform may be one of the proteins whose production is required for long-lasting enhancement of synaptic efficacy. There is now some corresponding evidence that production of adequate amounts of mBDNF may be important for hippocampus-dependent memory. A recent study indicates that increased cleavage of precursor proBDNF in the hippocampus is positively correlated with acquisition of contextual fear memory, while decreased cleavage is associated with extinction [7].

The effects of aging on BDNF mRNA and protein have been extensively studied and generally found to be modest [105]. However, a few studies have now examined the impact of aging on BDNF protein isoforms. Aged Wistar rats with memory impairments are reported to have lower levels of total BDNF, but higher ratios of proBDNF to mBDNF than controls from a related strain known to have better preservation of cognitive function [132]. Perhaps not surprisingly, training in a spatial learning task increased levels of proBDNF in both young and aged Wistar rats, but only the young rats showed a corresponding increase in mBDNF [131].

Several studies have examined the effects of immune challenge or pro-inflammatory cytokines on BDNF in the brain. Lipopolysaccharide (LPS) is a component of the cell wall of Gram-negative bacteria. A potent endotoxin, it leads to the release of pro-inflammatory cytokines such as IL-1β and tumor necrosis factor α [44]. BDNF mRNA in the principle neurons of the hippocampus was strongly downregulated 4 h after an intraperitoneal injection of LPS or IL-1β [73]. Depolarization-induced release of BDNF from slices of dentate gyrus was not

altered by administration of LPS several days earlier [128]. However, when levels of BDNF protein were examined 7 h after intraperitoneal injection of LPS, LPS was found to produce a dose-dependent reduction in BDNF in the cortex and hippocampus [56].

Much less is known about the effects of immune challenge on BDNF protein isoforms, but intraperitoneal injection of a single very high dose of LPS (3 mg/kg) is reported to produce a small (~15%) reduction in both proBDNF and mature BDNF in crude synaptoneurosomes prepared from the brains of young mice 3d after the injection [123]. We have previously shown that a peripheral *E. coli* infection produces an exaggerated inflammatory response in the brains of aging animals, paralleled by specific deficits in forms of long-term memory and synaptic plasticity known to be strongly dependent on BDNF [12, 13, 24]. We now report that this inflammation also gives rise to a large reduction in mBDNF and TrkB signaling at synapses in the hippocampus.

It is not yet clear how exaggerated CNS inflammation may impact levels of BDNF protein isoforms. The precise circumstances and sites of BDNF production, processing, and release, and the stability of the resulting isoforms is currently a very active, complex, and controversy-filled area of investigation [5]—and rather beyond the scope of this initial report. However, the work presented here provides new insights into naturalistic events that can affect BDNF production and processing *in vivo* and should provide a basis for further investigation of the interactions of aging, inflammation, and BDNF biology.

Decreases in mBDNF and BDNF-dependent signaling pathways following a single peripheral infection in aged animals persists 8 days, but not 14 days post-infection

A single peripheral immune challenge, i.p. injection of live *E. coli*, into aged rats produces a prolonged and exaggerated inflammatory response in the CNS [11]. Following a peripheral infection, levels of the pro-inflammatory cytokine IL-1ß are significantly upregulated in the hippocampus, causing pronounced deficits in hippocampus-dependent memory and synaptic plasticity [13, 24]. Levels of hippocampal IL-1ß were most abundant 4-5 days following infection [13], and both deficits in hippocampus-dependent memory and plasticity were observed at this time point [13, 24]. It was previously reported that peripherally-induced increases in IL-1ß in hippocampus persists up to 8 days following a single injection of live *E. coli*, but returns to baseline levels by day 14 [13]. More interestingly, impairments in hippocampus-dependent memory parallel these increases in hippocampal IL-1ß; with observed deficits in behavioral performance on memory tasks at day 8, but not day 14 post-infection [11].

The mature BDNF protein isoform plays a large role in facilitating forms of synaptic plasticity that underlies some forms of memory [18, 21, 152]. BDNF is shown to facilitate these forms of plasticity via TrkB signaling; an event that causes autophosphorylation of the receptor kinase itself, as well as phosphorylation of downstream proteins PLCγ-1 and ERK [117]. We have previously reported that synaptic levels of mBDNF protein are significantly reduced in hippocampal synaptoneurosomes from aged animals 5 days following a peripheral infection [31].

Furthermore, reductions in synaptic levels of mBDNF were consistent with decreases in autophosphorylation of the TrkB receptor (pTrkB), pPLCγ-1 and pERK at the same time point [31]. Here, we extend the initial findings and show that reductions in levels of mBDNF protein persist for 8 days post-infection, but return to baseline levels at day 14. We observed a similar decreasing trend in protein expression of pPLCγ-1 and pERK at day 8, but not day 14 post-infection; suggesting that these decreases are consistent with infection-evoked upregulation of IL-1ß in hippocampus.

It is becoming clearer that the aged brain is vulnerable to inflammatory events that can have precipitous declines in normal cognitive function; the likes of which include severe memory impairments and the potential onset of memory-associated neurodegenerative disorders. We now have mechanistic evidence suggesting that age and infection-evoked hippocampal dysfunction occurs by targeting BDNF and BDNF-dependent signaling at synapses [31]. We see that hippocampus-dependent memory impairments follow, in concert the heightened levels of IL-1 following a peripheral infection; at days 4-5 post-infection, persisting through day 8, and returning to baseline levels at day 14 [13]. Thus, in order to fully understand the prolonged effects of inflammation in the aged brain on synaptic mechanism, we have extended on our 4-5 day time point and determined that a single inflammatory event is capable of have persistent effects on BDNF protein expression and BDNF-dependent signaling in the hippocampus.

This work is the first to report that a single, acute peripheral inflammatory event can have prolonged effects on neural function. It is quite remarkable that an acute infection can have persistent effects on neural function up to 8 days following the event. More specifically, we have identified a specific target (BDNF protein expression at hippocampal synapses), and a potential mechanism (BDNF-dependent synaptic signaling pathways), by which the pro-inflammatory cytokine IL-1ß acts on to potentially cause cognitive impairments and memory deficits.

We previously reported that decreases in the phosphorylation of PLCγ-1 and ERK occurred 4-5 days post-infection [31], and these reductions now extended to 8 days post-infection in aged animals. However, these reductions are not consistent between proteins and are not as pronounced as the reductions at day 4-5 post-infection. It may be possible that levels of IL-1ß following infection may be so closely related to BDNF activity that as cytokine levels decay over time, the reductions in downstream BDNF targets also decay. Furthermore, one possibility for the inconsistent reductions with PLCγ-1 and ERK may be due to the fact that phosphorylation of PLCγ-1 is an event closely associated with activation of TrkB; a synaptic target of mBDNF [117]. In addition, ERK phosphorylation occurs farther downstream of PLC, and ERK activation is not specific in that it can be phosphorylated by a number of cellular events; including Ca^{2+} influx thorough a number of post-synaptic receptors aside from Trk receptors (Wiegert&Bading 2011).

Altering BDNF protein isoforms and BDNF-dependent can affect learning and memory acquisition [131, 132]. Our results indicate that these effects on BDNF

occur as a result of aging and infection. Thus, this may be one potential mechanism that underlies the cognitive decline and memory impairments with aging.

Synaptic failure is a phenomenon thought to underlie the early-stage hippocampal inefficiencies contributing to the memory impairments associated with Alzheimer's disease (AD) [125]. In addition to aging, neuroinflammation significantly influences the onset and severity of AD pathologies [61]. In addition, there is a strong correlation between increases in IL-1 and hippocampal dysfunction observed in AD [35, 61, 119]. Therefore, it is possible that a common mechanism underlying cognitive dysfunction associated with aging, and potentially AD may be dependent on BDNF protein at synapses, as well as activation of downstream signaling cascades.

Age and inflammation enhances hippocampal LTD via proBDNF – p75 receptor-ligand binding.

In the experiments presented here, we have examined the effects of aging and a peripheral immune challenge (i.p. injection of live *E. coli*) on hippocampal LTD, as well as the synaptic role of the proBDNF protein and $p75^{NTR}$ receptor in hippocampus. The principle findings of this study are that the combination of age and infection **(1)** enhance hippocampal LTD following low-frequency stimulation in Schaffer collaterals in area CA1 of hippocampus, **(2)** produce increased proBDNF – $p75^{NTR}$ receptor-ligand interaction at hippocampal synapses; a potential mechanism for facilitating enhanced LTD and **(3)** these events depend on IL-1 signaling in the brain as administration of IL-1RA *icm* blocks these effects.

These findings are consistent with our previous results indicating that the combined effects of aging and infection produce significant memory impairments in the hippocampus, as well as significant reductions in hippocampal L-LTP [11, 24]. Furthermore, this combination produces a shift in the ratio of mBDNF protein expression to that of proBDNF [31]. The present results extend these initial behavioral, electrophysiological and protein biochemical findings by identifying another form of hippocampal synaptic plasticity (LTD) affected by age and infection. In addition, these works have identified a potential cellular mechanism through proBDNF – $p75^{NTR}$ signaling that may be responsible for facilitating the observed enhancements of hippocampal LTD.

The facilitation of LTD through proBNDF and its interaction with $p75^{NTR}$ is not fully understood and its role in normal physiological systems remains controversial. The current evidence in support of the hypothesis that proBDNF is involved in regulating LTD through $p75^{NTR}$ activation, is reported in murine models involving complex genetic manipulations [85, 120, 164]. For example, surgical procedures and viral delivery methods used in the development of genetic models are essentially peripheral immune challenges and compromising the immune system; an event that we now know can have a significant impact on brain function. In addition, the studies done in the field of neurotrophin biology have utilized multiple models of overexpression, which renders the true endogenous system compromised and physiologically challenged.

The work discussed in Chapter IV is the first to investigate the endogenous interactions between proBDNF and $p75^{NTR}$, and further correlate this interaction with the regulation of hippocampal LTD in a naturalistic rodent model of aging and peripherally induced CNS inflammation. This work is significant in that these findings provide evidence for a potential mechanism via proBDNF activation of p75 for the facilitation of LTD in a naturalistic model; a finding that contributes to the initial reports on BDNF-dependent modulation of plasticity in the hippocampus.

Multiple forms of synaptic plasticity (LTP and LTD) regulated by BDNF and BDNF-dependent signaling pathways play a large role in the expression of learning and memory [21, 23, 85, 96, 152, 163, 164]. Changes in hippocampal synaptic plasticity produce significant changes in memory expression. For example, alterations in hippocampal LTP [4, 6, 39, 42, 114, 116, 148] and enhancement of hippocampal LTD [93, 163] are strongly correlated with significant memory impairments. More interestingly, shifts in hippocampal synaptic plasticity are commonly observed, yet still controversial, in rodent models of Alzheimer's disease (AD) [27, 68, 127]. LTD is enhanced in models of AD, however other groups have shown that LTD is unaffected, and that memory problems arise more so from deficits in LTP [78]. The mechanisms through which changes in memory-related synaptic plasticity occur both naturally and in AD are not yet clear. BDNF plays a large role in regulating multiple forms of synaptic plasticity important for memory. Thus, BDNF and its related signaling pathways may be responsible for the

underlying changes in synaptic plasticity observed naturally, as well as with memory-associated neurodegenerative disorders.

Biological changes associated with AD include shifts in BDNF and NGF (nerve-growth factor) expression [61, 109], activation of $p75^{NTR}$ receptor [38, 136], changes in hippocampal synaptic plasticity [70, 78, 166] and the presence of pro-inflammatory cytokines following an inflammatory response [35, 61]. These events have all been identified and targeted independently with AD. Similarly, inflammation-induced upregulation of IL-1ß has profound and individualistic effects on LTP [16, 24, 29, 30, 67, 150], BDNF protein expression [31, 135] and BDNF-dependent signaling pathways [31, 135, 149]. Thus, it is important to identify a potential link between the effects of neuroinflammation, and the signaling pathways necessary for the synaptic plasticity underlying memory in order to better understand the pathologies associated with memory-related disorders, and BDNF may be a potential link.

LTD occurs following the activation of $p75^{NTR}$ by pro-neurotrophins [85, 164]. LTD is a correlate of memory impairments [93], and activation of $p75^{NTR}$ initiates downstream cellular events involved in cell death [117]. It is hypothesized that the occurrence of these events, in combination with aging underlies the memory impairments, cognitive decline and neurodegeneration associated with AD, however, it is unclear how this occurs.

ProBDNF activation of $p75^{NTR}$ involves a complex formation with sortilin that then activates downstream JNK and NFκB [117, 143]. Interestingly, we did not observe changes in the expression of $p75^{NTR}$, as well as its downstream activators of apoptosis, pJNK and sortilin. Since markers of apoptosis are unchanged, we propose that the combination of aging and inflammation in the brain may be an early event promoting synaptic inefficiency and synaptic failure; a process thought to be occurring in the early-stage pathologies of MCI and AD [125]. In conclusion, our findings provide support that the endogenous proBDNF – $p75^{NTR}$ system may facilitate LTD in the hippocampus, and further providing implications for a mechanism potentially underlying the early synaptic events that occur in memory-related disorders.

Abeta and IL-1 alter expression of BDNF protein isoforms.

There has since been a large separation between immunologists and neuroscientists. The neglect of glial cells, along with the idea that CNS function is immune to peripheral events cannot continue if we want to make significant strides in understanding neurodegenerative disorders. It was only in the last decade that an extensive communication between the peripheral immune system and the central nervous system was uncovered [87]. Immune responses in the periphery is capable of activating microglial cells in the CNS causing *de novo* cytokine production in the brain [74, 103, 153]. Since these discoveries, a number of research groups have successfully linked pro-inflammatory cytokine production in the brain to impairments in memory, deficiencies in synaptic plasticity, changes in

neurodendocrine function and alterations in cell signaling [14]. Furthermore, some of the underlying mechanisms responsible for the neural deficits following inflammation are found to involve BDNF and BDNF-associated signaling pathways [31, 149]. These works have provided firm evidence that neuroimmunity significantly influences brain function.

During this same time period (in the past decade) significant advancements were also being made in the field of Alzheimer's disease. In an attempt to understanding the cell biological mechanisms underlying AD pathologies, researchers have invested a significant amount of time and resources into uncovering the neuronal consequences of Aß accumulation. Like IL-1ß, Aß plaques are strongly associated with cognitive decline and memory impairments, deficits in synaptic plasticity and perturbations in synaptic transmission and cell signaling [78, 125, 126]. Here too, it was found that BDNF and BDNF-dependent signaling pathways were targeted by Aß [82, 136].

The functional consequences of neuroinflammation and AD are strikingly similar, yet poorly understood and not well studied. Currently, there is human and animal data suggesting that cognitive deficits associated with mid – to – late stage AD, as well as neurodegeneration and cellular dysfunction are exacerbated following immune challenges (like illness and infection), but data is minimal [35, 61].

The data discussed in Chapter V provide an *in vitro* system modeling the interactions between inflammatory responses and the presence of Aß on neurons – levels physiological to that of the AD phenotype. Our data is the first to report that BDNF protein isoforms and BDNF-associated synaptic proteins are significantly altered in Aß treated human neuroblastoma SH-SY5Y cells following additional treatment with IL-1ß. Thus, this system represents a pathway in which neuroinflammation, AD and BDNF function converges. Additional experiments will uncover more specific details about this mechanism, which in turn may provide targets for more effective therapeutics.

Aggregation of amyloid beta protein is a key hallmark of Alzheimer's disease [126]. Many hypotheses have been advanced regarding the effects of Aß oligomerization on early stage synaptic failure, synaptic plasticity, and cell signaling. One common hypothesis, the 'Aß hypothesis', states that there is an imbalance in the rate of production and clearance of Aß, causing aggregation that leads to gradual synaptic failure and neuronal dysfunction [125, 126]. This is supported by a number of studies that have identified Aß-associated deficits in memory-related synaptic plasticity (like LTP), synaptic transmission and cell signaling pathways; including neurotrophin-based pathways (like TrkB and $p75^{NTR}$) [78, 82, 136, 151]. Further studies have provided additional evidence for an inflammatory component that may be at play in the Aß-rich AD brain.

Glial cells, which are involved in the innate immune response as well as synaptic transmission in neurons, are activated in the presence of Aß plaques.

Whether inflammation acts independently to cause cognitive deficits associated with AD, or occurs as a symptom of AD is not yet clear and still in debate. However, there is convincing evidence supporting the hypothesis that activation of the immune response, characterized by the upregulation of pro-inflammatory cytokines (such as IL-1ß and TNFα), acts independently on the AD pathology to influence the deleterious effects of the disease [35, 61, 119]. These findings support our hypothesis that inflammation may work in combination with another unknown, possibly aging, in order to produce significant declines in brain function, and the effects of age and inflammation may be greater in an AD brain compared to a normal aging brain which still is affected.

Although the role of inflammation on governing the multiple effects of Aß oligomers on synaptic events still warrants clarification, it is well appreciated that both pro-inflammatory IL-1ß and AD-associated Aß share common cellular pathways that target synaptic plasticity in the brain. There are multiple forms of synaptic plasticity currently known, and they have long been accepted as a model for the cellular and molecular mechanisms underlying the synaptic changes associated with learning and memory [21, 23, 96, 152]. There are two isoforms of the BDNF protein, and each protein isoform has a distinct bi-directional modulation of synaptic plasticity at synapses. ProBDNF is shown to facilitate LTD following $p75^{NTR}$ binding and activation, whereas mBDNF facilitates LTP through the TrkB receptor [85]. It is hypothesized that deficits in memory and plasticity associated with AD and inflammation *independently* occur through BDNF-dependent

signaling – the common pathway shared. Experimental support for this stems from the fact that infection-evoked elevations in IL-1ß completely block LTP [14, 150]; a result consistent with perturbations in BDNF-dependent signaling [149]. Furthermore, similar deficits in LTP, as well as BDNF-dependent signaling are also observed in systems with abundant Aß oligomers present [reviewed in Li 2010]; as seen by Aß-dependent upregualtion of proBDNF and activation of the proBDNF-associated receptor $p75^{NTR}$ [61, 82, 136]. Now, our studies find that the pro-inflammatory cytokine IL-1ß exacerbates the effects of Aß on BDNF protein levels and BDNF-associated synaptic proteins *in vitro*.

What is additionally interesting about the results discussed in Chapters IV and V are that the impairments associated with age and infection share similar pathology to those observed in early stage dementia and memory-related neurodegenerative diseases like Alzheimer's disease. Ratio shifts in BDNF protein isoforms and their given association to specific signaling pathways that result from the combined effects of age and neuroinflammation may contribute to the early-stage neurodegeneration and synaptic failure associated with later disease onset. Both Alzheimer's disease (AD) and Parkinson's disease (PD) are found to have overlapping clinical symptomology that include early stage delirium, memory impairments and dementia [48, 63], however these diseases have distinct biochemical markers that underlie their individual pathology. Upregulation of the amyloid precursor protein (APP) along with the accumulation of the secretase-dependent production of insoluble-oligomeric amyloid-beta protein (Aß) are primary

characteristics of AD [37, 50, 94]. In contrast to Aß aggregation, Lewy body formation is a defining pathological characteristic of sporadic PD [139]. Although these markers are biochemically different, both Lewy body formation and Aß plaque production are found in association with clinical cases of PD patients with dementia following post-mortem tissue analysis [63], thus linking PD-associated dementia with Aß production. Aside from evident clinical symptoms, such as memory impairments (AD and PD) and motor dysfunctions (PD), there seems to be a common cellular component to this degeneration – the targeting of neurons and their synapses. Individuals with AD-associated dementia report having significant cortical atrophy including neuronal loss, decreased spine density and neurite degeneration [55, 69, 77, 147]. More interestingly, increased release of cytotoxic inflammatory mediators (i.e., cytokines) following an inflammatory event is shown to exacerbate the deleterious effects of neurodegenerative diseases, as demonstrated in both animal models of AD, as well as human AD subjects [35, 61]. Therefore it is suggestive that a common link between symptoms of dementia and neurodegenerative disorders may be Aß plaque accumulation in the brain; and these effects can be significantly influenced by inflammation. Due to the strong correlation between mechanisms of synaptic plasticity and memory in the brain, extensive work has gone into examining the effects of Aß plaque accumulation on synaptic plasticity, specifically in the entorhinal cortex and hippocampus – primary brain regions affected by AD due to abundant inputs from entorhinal cortex into hippocampus for facilitating learning and memory [101, 140]. Numerous studies

have shown that Aß plaque formation can inhibit LTP in the hippocampus [78, 127]. To our interest, however, there is additional evidence suggesting that Aß can also induce and facilitate LTD. For example, *in vivo* treatment with both synthetic Aß peptide and Aß extracts from human AD brain are shown to induce, and facilitate LTD in hippocampus region CA1 [27, 68, 127]. However, it is important to note that other groups have found no effect on LTD with Aß treatment [115, 157, 158]. Thus, the mechanisms by which Aß acts to influence synaptic plasticity are poorly understood, however researchers have begun exploring neurotrophins (specifically BDNF) and their signaling pathways because of its importance in regulating multiple forms of synaptic plasticity.

There is a global interest in developing therapeutics to combat both Alzheimer's disease and Parkinson's disease. Current therapeutic approaches have targeted the clinical symptomologies, including cognitive decline, memory impairments and motor dysfunction well into the mid stages of the disease and throughout its progression. However, current available therapies are designed to keep symptoms at bay and do not address the early stage events that may be causal in the development of neurodegenerative diseases. Thus, it seems important to identify early cellular events, as well as the cell biological mechanisms at play that may occur before the disease onset. From this, we open the possibility for the development of more effective therapeutic strategies.

From the collection of data discussed throughout this dissertation we can conclude that activation of the innate immune system has significant and

pronounced effects on normal brain function. We see that neural processes in brain regions important for memory and cognition are specifically targeted by pro-inflammatory cytokines following an inflammatory episode. On a molecular level, we see that the pro-inflammatory cytokine IL-1ß alters hippocampal function by targeting BDNF and BDNF-dependent signaling. More interestingly, the deleterious effects of pro-inflammatory molecules on memory, neural processes and BDNF biology are more pronounced in the aging brain. These works find commonalities between aging and inflammation and Alzheimer's disease that include cognitive decline, neural dysfunction and perturbations in BDNF biology. The cooperative function of IL-1ß and Aß on neural processes may provide the necessary information in determining the early cellular events and cell biological mechanisms that underlie Alzheimer's disease.

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