8-2019

Loss of Brain-Derived Neurotrophic Factor and Sex-Specific Disruption of Neuronal Function

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I am submitting herewith a thesis written by Blake Mitchell entitled "Loss of Brain-Derived Neurotrophic Factor and Sex-Specific Disruption of Neuronal Function." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Arts, with a major in Psychology.

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Loss of Brain-Derived Neurotrophic Factor and Sex-Specific Disruption of Neuronal Function

A Thesis Presented for the
Master of Arts
Degree
The University of Tennessee, Knoxville

Blake Austin Mitchell
August 2019

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Subimal Datta for giving me this wonderful opportunity and experience. I have learned so much over the last two years. You have helped me discover my potential, and for this, I am eternally grateful.

Tremendous thanks to Dr. Roshni Thakkar for her mentorship and friendship. Without you, Roshni, I could not have completed this degree. I have learned so much from you; from molecular lab work to the minutia of scientific rigor; from creating scientific presentations all the way to email etiquette—you have truly been instrumental to my development and success.

I would like to thank my committee members, Dr. Robert Craft and Dr. Debora Baldwin. I have fond memories of sitting in each of your offices, listening to your invaluable feedback and advice as I grappled with the road ahead (now behind).

Thank you to my best friend, Kate Burford, who was my lab mate and emotional rock. We have certainly been through a lot together, and I cannot imagine these last two years without you.

I would like to extend special thanks to Dr. Helen Baghdoyan and Dr. Ralph Lydic for their wonderful guidance and support. Thank you very much, and to all, my best wishes.

ABSTRACT

Brain-Derived Neurotrophic Factor (BDNF) is considered one of the most influential neurotrophins in the brain. It has known involvement in synaptic signaling and interaction with estrogen in the brain (E2) to perform neuroprotective functions. Although previous studies have examined the role of BDNF under pathologic conditions, the detailed molecular interactions that mediate BDNF action in the adult brain remain incompletely explored. In this study, we used protein expression analyses of hippocampal tissue to examine the effects of whole-brain BDNF knockdown ($BDNF^{+/-}$ KD) on the expression of various synaptic markers and estrogen receptors in otherwise healthy male and female rats. Relative to wildtype (WT) controls, only the $BDNF^{+/-}$ KD males showed increased levels of the presynaptic marker, synaptophysin, while exhibiting a decrease in the postsynaptic marker, PSD-95. Glutamatergic and cholinergic neuronal markers were not significantly changed in both the male and female rats after BDNF loss. $BDNF^{+/-}$ KD males also show a uniform upregulation in estrogen receptor expression, including ER-α, ER-β, and GPER1, which remained unchanged in the females. Taken together, our results suggest that BDNF loss leads to disruptions in synaptic signaling mechanisms in a sex-specific manner, and these sex differences may reflect the actions of estrogen receptors in the hippocampus. In conclusion, our study contributes to the understanding of sex differences in BDNF signaling in the brain. This will aid in development of novel therapeutic targets for various neuropsychiatric and neurodegenerative disorders, including the natural process of aging, where BDNF is a known player.

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Statement of the Problem

Disrupted synaptic signaling is a well-established mechanism of several disease states. Many authors have suggested that deficits in BDNF action play a key role in many neuropathological disorders. As such, characterizing BDNF signaling under pathologic conditions has been the focus of recent research. However, the action of this neurotrophin in the brain under normal conditions remains incompletely understood. BDNF function is also modulated by biological sex. Interestingly, sexual dimorphisms in brain structure and function are posited to reflect the differential actions of estrogen in the brain. Mounting evidence suggests that estrogen performs trophic actions as well, and, in collaboration with BDNF, may ameliorate the degradation of cells via defined signaling cascades. Therefore, to contribute to the field, this study hypothesizes that synaptic signaling is disrupted in a rodent model of BDNF loss, and that this disruption involves alterations to estrogen receptor signaling in a sex-specific manner. To test this hypothesis, we designed the following specific aims.

Specific Aims

Aim 1: To determine the basal relationship between BDNF expression levels and neuronal markers of synaptic signaling in the hippocampus of the rat brain. We will examine markers of pre- and postsynaptic nerve connectivity, synaptophysin and PSD-95, as well as markers of glutamatergic and cholinergic activity, VGLUT2 and ChAT.

Aim 2: To determine whether sex-differences play a role in BDNF regulation of pre- and postsynaptic nerve connectivity and neurotransmitter activity. We will further examine the expression levels of estrogen receptors, namely, ER-α, ER-β and GPER1, in relation to BDNF in the hippocampus.

CHAPTER TWO
INTRODUCTION AND LITERATURE REVIEW

In the last several decades, great strides have been made towards understanding and improving brain health. The scientific community has continually refined search parameters in efforts to minimize and mitigate disease states, while simultaneously directing efforts towards promoting health and well-being. New technological advancements afford us the ability to visualize the brain better than ever. Electroencephalography (EEG) allows us to monitor neural networks with incredible temporal resolution, while neuroimaging, such as functional magnetic resonance imaging (fMRI), helps us spatially define those networks. In the medical field, better technology has evoked a shift in how we approach diagnosis and treatment. Since we can interrogate disease states with much greater precision, a greater emphasis has been placed on identifying biological signs of health and disease. In biomedical sciences, researchers are interested in identifying biomarkers associated with brain disorders so that they may better understand the physiological conditions under which they arise. These efforts are crucial to the development of treatments. However, our understanding of basic brain structure and function is far from comprehensive. The basic constituents of nerve interactions require further attention, and there remains great potential for discovery in this domain.

It is well accepted that brain states are defined by increasingly lower-order systems. Behavioral and cognitive states are derived from interconnected brain regions, which are comprised of neural circuits. Those circuits can be described by neuronal assemblies, or groups of neurons that fire together. And those assemblies can be

described by the information processing that occurs in the synapse between nerve cells. Lower still, intercellular communication is determined by the intracellular goings-on of molecules in motion—the molecular signaling cascades that alter cell excitability and affect gene transcription. To be sure, a complete understanding of any brain state requires an understanding of each underlying system individually, as well as and how they interact to produce the associated phenotype. Animal research has helped reveal the cellular and molecular mechanisms of neuronal communication, which is regarded as the guiding principle of healthy brain function (Snyder & Smith, 2018). An on-going objective in the field of neuroscience is to characterize mechanisms of neuronal communication in order to better understand the role they play, both individually and collectively, in states of health and disease.

Neurotrophins

Healthy brain function relies on neuronal mechanisms of synaptic maintenance and regulation. These mechanisms involve the action of signaling peptides called neurotrophins, which bind to specific transmembrane receptors to govern processes important for neuronal health (Williams & Umemori, 2014). Four distinct members of the neurotrophic family have been identified in the mammalian brain: nerve growth factor (NGF) (Levi-Montalcini, 1966), brain derived neurotrophic factor (BDNF) (Barde, Edgar, & Thoenen, 1982), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5) (Hohn, Leibrock, Bailey, & Barde, 1990). These proteins are homologous in amino acid sequence and structure, share genetic ancestry, and are collectively involved in neuroregulatory actions (Huang & Reichardt, 2001). Like other proteins, neurotrophins arise from precursors

called proneurotrophins, which undergo proteolytic cleavage to produce mature proteins (Seidah et al., 1996). Proneurotrophins and neurotrophins exert distinct effects on target neurons. Their specific action reflects their differential specificity for two classes of neurotrophin receptors: the p75 receptor ($p75^{NTR}$) and the group of tyrosine receptor kinases (Trks) (Chao, 1992). Proneurotrophins are chemically attracted to the $p75^{NTR}$, binding to its site with relatively high affinity (Greene & Kaplan, 1995). $P75^{NTR}$ binding has been associated with neuronal apoptosis and other conspecific mechanisms which oppose the actions of mature neurotrophins (B. Lu, Pang, & Woo, 2005). Mature neurotrophins preferentially bind to their individual high-affinity protein kinase receptors subtypes: NGF selectively binds to Trk-A; NT-3 to Trk-B and Trk-C; NT-4/5 to Trk-B; and BDNF to Trk-B (Bothwell, 1991). The binding of neurotrophins to Trk receptors induces intracellular signaling, which promotes neuronal growth and survival (Chao, 1992).

Neurotrophins play different roles depending on how they are released (Song, Martinowich, & Lee, 2017). Although the traditional trophic model held that neurotrophins are transported in a retrograde fashion, there is now evidence that some neurotrophins, such as BDNF, are also transported in an anterograde fashion (Conner, Lauterborn, Yan, Gall, & Varon, 1997; Fawcett et al., 1998). Generally, the anterograde release of neurotrophins from the presynaptic cell is understood to help organize postsynaptic densities (Vaegter et al., 2011), while the retrograde release of neurotrophins from postsynaptic cells helps situate presynaptic vesicles. Together, these trophic actions help support synaptic connectivity and facilitate neurotransmission, the fundamental constituents of neuronal communication (Choo et al., 2017). The following sections will

focus on BDNF, exploring its putative roles in synaptic signaling, hippocampal plasticity, and neuroprotection.

BDNF and Trk-B signaling

Since its initial purification in 1982, BDNF has been under study for its manifold actions in the brain. Encoded by the *BDNF* gene (K. R. Jones & Reichardt, 1990), BDNF protein exists in two forms. At the cellular level, proBDNF is synthesized in the endoplasmic reticulum (Mowla et al., 2001), stored in presynaptic dense core vesicles (Dieni et al., 2012), and then secreted by neurons in an activity-dependent manner (L. Pozzo-Miller, 2008). In the synaptic cleft, proBDNF can undergo proteolytic processing to produce mature BDNF (Gray & Ellis, 2008). Mature BDNF is a moderately sized (14kDa) polypeptide comprised of 119 amino acids which forms non-covalent dimers (28kDa) and features a basic isoelectric point ($I_p \approx 10.1$) (Barde et al., 1982). Once in the synapse, mature BDNF action is mediated through the binding and dimerization of the Trk receptor type B (Trk-B) located on the membrane of receptive neurons (Kelly-Spratt, Klesse, & Parada, 2002). Following Trk-B activation, tyrosine residues found in their respective cytoplasmic kinase domains become auto-phosphorylated. Activated tyrosine residues allow docking of effector proteins, which in turn activate three main intracellular signaling cascades: the phosphatidylinositol-3 kinase (PI3K), which triggers threonine/serine kinase AKT; the extracellular signal related kinase (ERK, or mitogen-activated protein kinase (MAPK)) pathway, which initiates several downstream effectors; and the phospholipase-C-γ (PLC-γ) pathway, which leads to the activation of calcium-calmodulin dependent kinase (CaMKII) (Figure 2.1) (Zheng & Wang, 2009). The PLC-γ pathway is

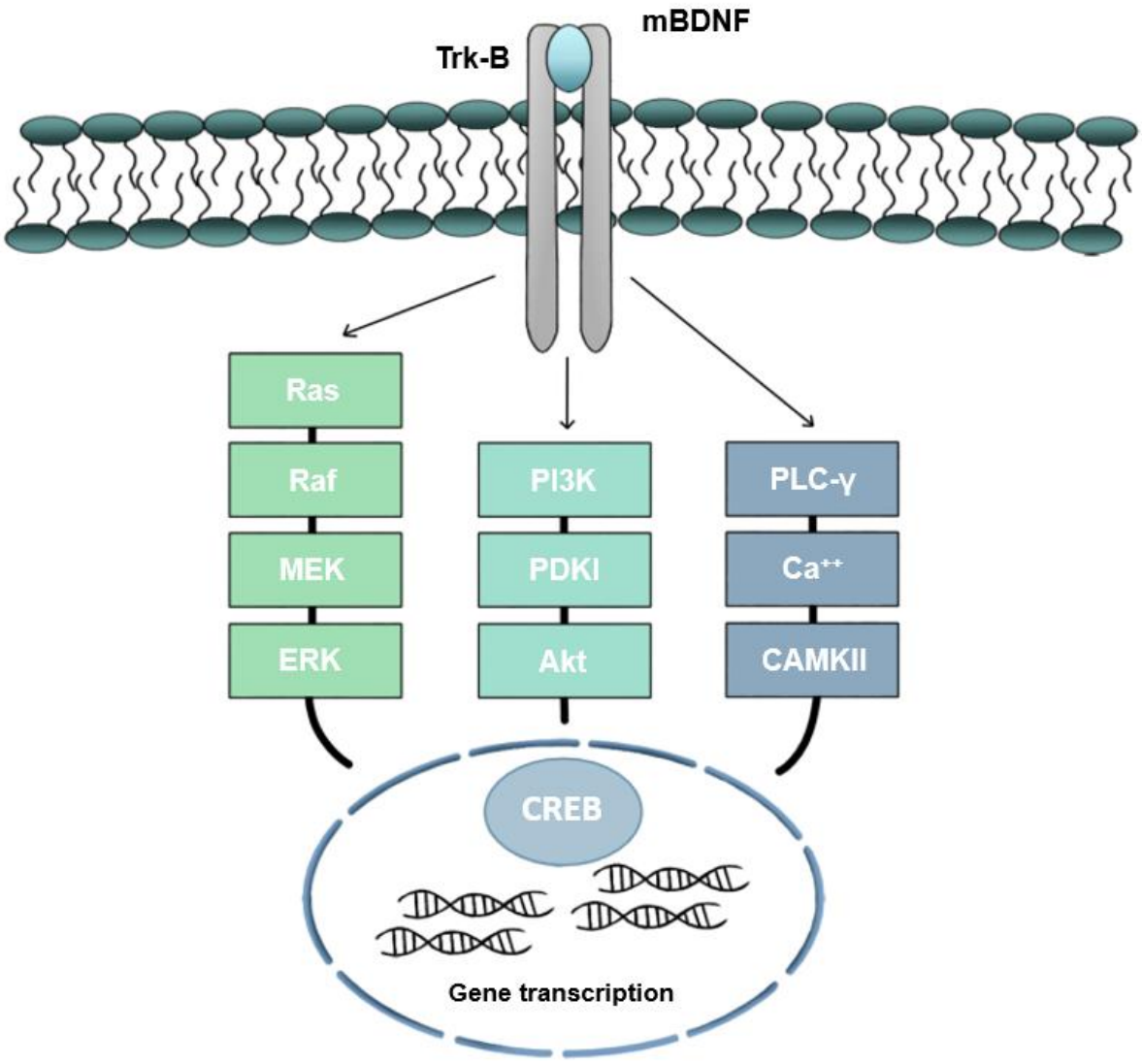

Figure 2.1. BDNF signal transduction. BDNF binds TrkB with high selectivity. TrkB dimerization promotes subsequent autophosphorylation of c-terminal tyrosine residues that serve as docking sites for effector molecules. Effector proteins can bind and trigger one of three signaling pathways: PLC-γ, PI3K, and ERK. These cascades lead to phosphorylation of cAMP response element-binding protein (CREB), mediating gene transcription important for the survival and plasticity of neurons. Activation of PLC-γ also leads to an increase in intracellular calcium and a corresponding increase in CaMKII activity, which is thought to be responsible for the rapid-acting effects of BDNF.

believed to mediate the rapid ion channel effects of BDNF via the release of intracellular calcium stores, while the sustained effects of BDNF\, which involve transcription, are considered downstream of the MAPK and PI3K pathways (Zheng & Wang, 2009). The three major signaling cascades downstream of BDNF-induced Trk-B activation are known to phosphorylate cAMP response element-binding protein (CREB) (Tao, Finkbeiner, Arnold, Shaywitz, & Greenberg, 1998). CREB-mediated gene transcription has a well-established role in neurogenesis, synaptic plasticity, and neuron survival (Yoshii & Constantine-Paton, 2010).

BDNF and the hippocampus

BDNF and other neurotrophins exhibit a similar, but not identical, cellular and regional distribution in the mammalian brain. *In situ* hybridization experiments have shown that BDNF is predominantly expressed by neurons (Zafra, Hengerer, Leibrock, Thoenen, & Lindholm, 1990), and BDNF-expressing neurons are disproportionally concentrated in limbic structures (H. T. Zhang et al., 2007). Immunoreactivity studies further show that BDNF and Trk-B protein expressions are particularly high in various regions of the hippocampus (Conner et al., 1997).

The hippocampus is important for healthy cognitive function and plays a major role in several learning and memory processes. The neuronal mechanisms underlying long-term potentiation (LTP), a cellular model for learning and memory, involve physiological and structural changes to hippocampal neurons. Importantly, these changes reflect the efficacy of synaptic transmission. Early studies initially demonstrated the effects of BDNF on synaptic transmission in *Xenopus* cultures, where delivery of BDNF increased the

frequency of miniature excitatory postsynaptic currents (EPSCs) (Lohof, Ip, & Poo, 1993). It was later confirmed that BDNF strengthens excitatory synapses and mediates long-lasting enhancements to synaptic transmission in the adult hippocampus (Kang & Schuman, 1995). Since these studies, experimental findings have more directly supported a role of BDNF in LTP. For example, exposure of hippocampal neurons to Trk-B inhibitors prevents LTP from taking place (Figurov, Pozzo-Miller, Olafsson, Wang, & Lu, 1996), and hippocampal tissues from BDNF knockout animals exhibit impaired LTP induction, which is rescued by reintroduction of BDNF (Patterson et al., 1996).

BDNF-induced hippocampal plasticity is also understood to protect against injury or attenuate pathology in the injured brain. For example, Trk-B-induced activation of the MAPK and PI3K pathways promote neuron survival and neurogenesis (Mocchetti & Bachis, 2004), and BDNF signaling in the hippocampus has been shown to ameliorate the effects of ischemic injury (Sheng et al., 2018). Furthermore, BDNF-induced synaptogenesis in the hippocampus improves behavioral outcomes in several models of disease (Xuan, Agrawal, Huang, Gupta, & Hamblin, 2015). Given these reports, there is consensus in the field that hippocampal BDNF is neuroprotective in the injured CNS and may be an effective therapeutic target for several neuropsychiatric and neurodegenerative disorders (Coughlan, Gibson, & Murphy, 2009). Investigations are currently underway to further explore this potential.

BDNF and Neuropathology

Studies of human brain disorders have traditionally relied on the subjective documentation of symptoms linked to impaired brain function. Symptom-based diagnostic

approaches carry several disadvantages, including definitional constraints of symptom duration, the difficulty of identifying individual differences, and the inherent risk of conflating comorbidities. Until recently, human research has involved studies in which higher-order brain mechanisms are dysfunctional, whether due to genetics or the environment. Now, greater emphasis has been placed on determining the fundamental mechanisms involved in diseases of the central nervous system. There has been increasing evidence that neuronal mechanisms of synaptic transmission and connectivity are disrupted in neuropathologic disorders (Kouroupi et al., 2017; Samuel, Alford, Hofstetter, & Hansen, 1997), and this has generated great interest in the idea that dysregulated BDNF is a key player in their development.

Neuropsychiatric disorders

BDNF signaling has been implicated in neuropsychiatric disorders, including the aggregate of affective disorders. Pharmacological studies have linked depressive phenotypes to decreased levels of BDNF mRNA (Altar, 1999), and elevated levels of BDNF mRNA have been associated with antidepressive-like phenotypes (Castren & Rantamaki, 2008). Typical treatments for depression disorders are thought to act primarily through monoaminergic synaptic transmission, and interestingly, BDNF mRNA has been shown to increase alongside the effects of monoamine oxidase inhibitors (MOAIs) and selective serotonin reuptake inhibitors (SSRIs) (Castren & Rantamaki, 2008). In addition, new evidence has linked the antidepressant effects of the NMDA antagonist ketamine to BDNF-dependent signaling in the hippocampus (Song et al., 2017), further supporting the

neurotrophic hypothesis of depression and suggesting a novel mechanism of action for future antidepressant drugs.

BDNF is also postulated to be involved in the mania that characterizes bipolar disorder (Tsai, 2004). Lithium, a traditional drug used for the treatment of depression and bipolar disorder, increases BDNF and Trk-B activation in cerebral cortical neurons (Hashimoto et al., 2002). Furthermore, there is evidence that a DNA variant in the BDNF gene confers susceptibility to bipolar disorder (Neves-Pereira et al., 2002), and the Val66Met polymorphism has been associated with physiological measures in bipolar disorder, as well as schizophrenia (Bonaccorso et al., 2015). In fact, disrupted BDNF appears to play a key role in the development of schizophrenia. First, structural and cognitive changes related to BDNF polymorphisms have been found in patients with schizophrenia (Agartz et al., 2006; Ahmed, Mantini, Fridberg, & Buckley, 2015). Next, the time-dependent effects of antipsychotics on BDNF levels in patients with schizophrenia suggest that the mechanism of their therapeutic action is BDNF-mediated neuroplasticity (Pandya, Kutiyanawalla, & Pillai, 2013). Finally, animal models of schizophrenia show altered BDNF expression in the hippocampus of various species (Angelucci, Brene, & Mathe, 2005), and increased BDNF-mediated hippocampal plasticity has been shown to protect against related cognitive impairments in rats (Faatehi et al., 2019). Collectively, these lines of evidence strongly suggest that BDNF is involved in the pathogenesis of schizophrenia, and that BDNF may be a promising pharmacogenetic target for schizophrenia and related neuropsychiatric disorders (Han & Deng, 2018; Pillai, 2008).

Neurodegenerative disorders

BDNF has also been implicated in the degradation of nerve cells in states of disease. Several reports have reinforced the idea that altered BDNF activity is a hallmark feature of neurodegenerative disorders (Murer, Yan, & Raisman-Vozari, 2001). In Alzheimer's disease (AD), BDNF mRNA is reduced in the hippocampus of humans (Phillips et al., 1991). Moreover, rats with amyloid-beta protein injected into the hippocampus show marked reductions to BDNF activity (Z. Zhang et al., 2005), and BDNF protein is deregulated in early stages of a transgenic animal model of AD (Iulita et al., 2017). These findings support the idea that insufficient supply of trophic activity in the brain corresponds with pathogenic changes to neuronal populations and cellular function (Murer et al., 2001). Interestingly, BDNF upregulation has been observed in plague-related glial cells (Burbach et al., 2004). Since inflammatory and maligned axonal growth processes have been attributed to amyloid-beta protein activity, areas of the brain displaying a high degree of BDNF-mediated plasticity, such as the hippocampus, are particularly vulnerable to plague-associated degeneration (Spires & Hyman, 2004).

BDNF is also involved in the neurodegeneration of the dopaminergic system. Decreased BDNF mRNA has been reported in the substantia nigra of the midbrain in people with Parkinson's disease (Howells et al., 2000), and BDNF/Trk-B expression in dopaminergic grafts was found reduced in a rat model of Parkinson's (Sautter et al., 1998). BDNF may also be used as a treatment in Parkinson's to ameliorate conditions or protect against further degeneration. For instance, transfection of BDNF to dopamine neurons potentiates the effects of a dopamine D3 agonist on striatal innervation,

dendritogenesis, and motor behavior (Razgado-Hernandez et al., 2015). In addition, BDNF receptor blockade has been found to mitigate the neuroprotective effects of exercise in a rat model of Parkinson's disease (Real et al., 2013). In Huntington's disease, the gene Huntingtin, which normally upregulates BDNF transcription, has a mutation that leads to BDNF loss (Cattaneo et al., 2001). These deficits in trophic support to striatal neurons are believed to spell their degeneration and result in the observed pathology (Zuccato et al., 2001). Investigators believe that pharmacological provisions of BDNF may conceivably be therapeutic in Huntington's disease and related degenerative disorders if applied in the appropriate region- and time-specific manner (Spires et al., 2004; Yulug et al., 2018).

BDNF and Sleep

Mammalian sleep is a restorative process of the brain and body during which a number of vital functions take place (Datta, 2010). It is hypothesized that one of these biological functions of sleep is restoring an organism's ability to learn and adapt by facilitating neuroplasticity (Datta, Li, & Auerbach, 2008; Gadea-Ciria, 1976; Walker & Stickgold, 2006). Sleep can be objectively described as a regulated set of behavioral states that are defined by physiological traits. Non-rapid eye movement (NREM) sleep is characterized by synchronized slow wave EEG activity, decreased autonomic activity, and increased growth-hormone release. Rapid eye movement (REM) sleep is characterized by desynchronized fast wave EEG activity, dysregulated autonomic activity, and muscle atonia. The current data suggests that BDNF plays a major role in several processes during both NREM and REM sleep.

BDNF and sleep have been tightly linked. Sleep deprivation significantly alters BDNF levels (K. Schmitt, Holsboer-Trachsler, & Eckert, 2016; Sei, Saitoh, Yamamoto, Morita, & Morita, 2000), and, reflectively, human and animal studies have demonstrated substantial behavioral and cognitive deficits in sleep deprived subjects (Banks & Dinges, 2007; Datta, Mavanji, Ulloor, & Patterson, 2004). Recent studies investigating the link between sleep and BDNF have suggested an important role for BDNF in the regulation of sleep states (Bachmann et al., 2012; Hairston et al., 2004; Kushikata, Fang, & Krueger, 1999). In animal experiments, BDNF injection during wakefulness has been shown to potentiate slow-wave activity (Faraguna, Vyazovskiy, Nelson, Tononi, & Cirelli, 2008), total time spent in NREM sleep, and total time in REM sleep (Kushikata et al., 1999) during the subsequent sleep episode. These data strongly support the neurotrophic hypothesis of sleep regulation, and currently, the field continues to investigate the extent of BDNF involvement in sleep and search for ancillary mechanisms.

Altered BDNF may play a role in sleep disturbances and sleep-related disorders. Poor sleep quality is related to alterations in BDNF concentration (Monteiro et al., 2017), and there is evidence of an interplay between insomnia, stress-induced dysregulation of the HPA axis, and BDNF deficiency (Jiang & Zhu, 2015; K. Schmitt et al., 2016). A recent report has associated decreased peripheral BDNF with the cognitive impairments of insomnia (Fan et al., 2019). Interestingly, this report and related findings correspond with the loss of slow-wave activity and nighttime awakenings in people with Alzheimer's disease (Garcia-Rill, Luster, Mahaffey, Bisagno, & Urbano, 2015; Vitiello & Prinz, 1989). Finally, poor sleep quality is comorbid with several neurodegenerative disorders (Petit,

Gagnon, Fantini, Ferini-Strambi, & Montplaisir, 2004), which have all established a role for BDNF. Moving forward, investigations in the cellular functions of BDNF can provide valuable information for understanding the physiology of sleep pathology and for the diagnosis of related brain disorders.

BDNF and Neuronal Markers of Synaptic Signaling

Neurons are equipped with subcellular mechanisms that govern and maintain synaptic signaling. Healthy synaptic signaling relies on both synaptic connectivity and neurotransmitter release. Neuronal markers of pre- and postsynaptic density can help assess synaptic connectivity, while neuronal markers of transmitter synthesis and mobilization can indicate levels of neurotransmitter release. Regulation of these synaptic proteins directly affect synaptic strength, and in general, neuronal communication and cellular function. Therefore, it is important to understand these markers individually, collectively, and in various contexts.

The relationship between BDNF and neuronal markers of synaptic signaling has been an intense area of research. Several reports have indicated that BDNF, through its dynamic trophic actions, regulates molecular components of synaptic signaling. This has great implications for the study of healthy neuronal and neurologic functions. Moreover, interactions between BDNF and synaptic markers have been implicated in pathological conditions of the central nervous system. The following sections will introduce pre- and postsynaptic elements of synaptic connectivity and neurotransmission, discuss what might occur when these elements are disrupted, and describe their known interactions with BDNF.

Synaptophysin

Synaptophysin is the ubiquitous membrane-bound protein of synaptic vesicles residing in the presynaptic terminal of nerve cells (Wiedenmann & Franke, 1985). It is believed to participate in presynaptic vesicle docking processes that promote transmitter release during neurotransmission (Thiel, 1993). The protein's function has also been associated with that of the synapsins, a family of vesicle proteins responsible for vesicle mobilization and exocytosis (Senda, Ochiai, Nakai, & Fujita, 1991). Due to its ubiquity in presynaptic terminals and its interaction with other vesicle proteins, immunostaining of synaptophysin has been traditionally used for quantification of synapses (Calhoun et al., 1996), and as a neuronal marker of presynaptic density (Masliah, Terry, Alford, & DeTeresa, 1990).

In disease states, presynaptic mechanisms of vesicle mobilization may be disrupted. For example, research has related reductions of synaptophysin in the striatum to the degeneration of spinous neurons in Parkinson's disease (Goto & Hirano, 1990). Additionally, increased tau phosphorylation has been associated with decreased synaptophysin levels in a mouse model of Alzheimer's disease (Garringer et al., 2013). Disrupted synaptophysin has also been implicated in behavioral and cognitive deficits. For example, total elimination of synaptophysin in mice incurs substantial behavioral changes, including impaired object recognition, deficits in spatial learning, and increased exploratory behavior (U. Schmitt, Tanimoto, Seeliger, Schaeffel, & Leube, 2009). Current research continues to focus on the role synaptophysin plays in neurodegenerative disorders, and how deficits in presynaptic density contributes to neurologic impairments.

It has been established that BDNF potentiates the high frequency neurotransmission of LTP, in part, by inducing physiological changes in the presynaptic terminal (B. Lu & Gottschalk, 2000). Interestingly, BDNF KO mice display impairments to high frequency neurotransmission along with corresponding reductions to synaptophysin expression (L. D. Pozzo-Miller et al., 1999). BDNF has also been shown to modulate presynaptic levels of synaptophysin in the rat hippocampus (B. Lu & Chow, 1999), suggesting that synaptophysin is likely involved in neurotrophin-induced synaptic plasticity (Janz et al., 1999). Synaptophysin may also mediate some of BDNF's neuroprotective effects. For example, recent research has shown that BDNF transfection increases synaptophysin expression to promote neural recovery in rats with cerebral infarction (Y. Zhang et al., 2017). Taken together, the evidence presented here suggests that synaptophysin is an important mediator of BDNF action, and its functional expression is linked to the expression of BDNF.

Postsynaptic density 95

The postsynaptic density (PSD) is an assembly of proteins anchored to the membrane of the postsynaptic nerve cell (Banker, Churchill, & Cotman, 1974). The PSD region faces the presynaptic active zone and ensures tight coordination between neurotransmitter release sites and postsynaptic receptors (Tachibana & Sobue, 2000). Postsynaptic density protein of 95 kDa (PSD-95) is a protein enriched in the PSD, constituting 2.3% of its total mass. PSD-95 is anchored to the NR2 subunit of N-methyl-D-aspartate (NMDA) receptors, the major receptors that bind glutamate (Kornau, Schenker, Kennedy, & Seeburg, 1995). Several reports propose that PSD-95 situates

NMDA receptors in the postsynaptic membrane and serves as a multi-domain anchoring site for several downstream scaffolding and structural proteins (Elias, Elias, Apostolides, Kriegstein, & Nicoll, 2008; Kennedy, 1997; Yoshii & Constantine-Paton, 2014). As such, PSD-95 is widely used as a neuronal marker of postsynaptic density at excitatory synapses (Hunt, Schenker, & Kennedy, 1996; Nagura et al., 2012). When examined alongside neuronal markers of presynaptic density, its expression reflects synaptic connectivity and stability (Elibol-Can, Kilic, Yuruker, & Jakubowska-Dogru, 2014; Kim et al., 2007).

Alterations to postsynaptic density have been implicated in disease states and associated with behavioral impairments. Downregulated PSD-95 expression in the prefrontal cortex is believed to underlie the deficits in glutamatergic signaling found in schizophrenia (Ohnuma et al., 2000), and redistribution of PSD-95 in the striatum has been reported in animal models of Parkinson's disease (Nash, Johnston, Collingridge, Garner, & Brotchie, 2005). In an experimental model of temporal lobe epilepsy, decreased levels of PSD-95 in the hippocampus were correlated with behavioral deficits in rats, including measures of anxiety, locomotor activity, spatial learning and memory (Sun et al., 2009). Additionally, an age-dependent decline in motor neocortex performance in BDNF knockdown mice correlates with a decrease in cortical PSD-95 expression (Carreton et al., 2012). These evidences clearly support the idea that disrupted PSD-95 expression relates to pathologic changes to synaptic connectivity and the glutamatergic system.

There is also abundant evidence that the PSD plays a crucial role in hippocampal plasticity. PSD-95 has known involvement with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor accumulation at the synapse. Previous studies have verified that PSD-95 gene transcription is upregulated by neuronal activity (Beique et al., 2006), and its recruitment to the PSD facilitates AMPA receptor insertion during LTP (Meyer, Bonhoeffer, & Scheuss, 2014). Furthermore, the PSD is found to increase during synaptic plasticity along with a net increase in synaptic size and strength after LTP is induced (Meyer et al., 2014), which suggests a role for neurotrophins in regulating the PSD. BDNF has known interactions with the microstructures of the PSD. A previous study has shown that BDNF is responsible for the transport of PSD-95 to the PSD through PI3K-Akt signaling downstream of NMDA receptor activation (Yoshii & Constantine-Paton, 2007). A more recent study reported an increase in PSD-95 expression in the mouse hippocampus following BDNF treatment (Hu et al., 2011). Together, these evidences suggest that BDNF regulates the expression of PSD-95.

Vesicular glutamate transporter 2

Glutamate is the major excitatory neurotransmitter found throughout the central nervous system. In the presynaptic nerve terminal, glutamate is stored in synaptic vesicles. Vesicular glutamate transporters (VGLUTs) are a family of proteins embedded in vesicle membranes. Unlike excitatory amino acid transporters (EAATs), VGLUTs are not involved in removing glutamate from the synaptic cleft. Instead, they perform the role of vesicle loading inside the presynaptic terminal of excitatory synapses (Vandenberg, 1998). There are three types of VGLUTs known, VGLUT 1-3 (Herzog et al., 2004; Naito

& Ueda, 1983; Takamori, Rhee, Rosenmund, & Jahn, 2001). These transporters display nearly identical uptake characteristics and they each affect quantal release of glutamate (Shigeri, Seal, & Shimamoto, 2004). Immunoreactivity of VGLUT2 has been used to indicate levels of glutamatergic activity, specifically, pre-synaptic glutamate recycling (Takamori et al., 2001). More recently, VGLUT2 has been used as a neuronal marker of presynaptic regulation of glutamate release (Moechars et al., 2006) and spatial-specific activity at excitatory synapses (Wozny et al., 2018).

Excitotoxicity is a well-established outcome of excess glutamate in the synapse. Overexpression of VGLUTs leads to dysregulated glutamate release, and has been shown to cause excitotoxic neurodegeneration (Daniels, Miller, & DiAntonio, 2011). Increased VGLUT expression is also linked to excitotoxic neuropathological phenotypes, such as early lethality, behavioral deficits, and neuronal pathology (Daniels et al., 2011), which have all been observed in human and animal models of schizophrenia (Uezato, Meador-Woodruff, & McCullumsmith, 2009). Conversely, VGLUT expression is decreased in mood disorders, including major depressive disorder (MDD) and bipolar disorder (Uezato et al., 2009). In functional studies, selective global deletion of VGLUT2 in mice has been linked to impaired spatial memory function (Nordenankar et al., 2015) and increased vulnerability to neurotoxin-induced neurotoxicity (Shen et al., 2018). In the hippocampus, VGLUT2 irregularities have been associated with cognitive deficits (King et al., 2014) and age-dependent impairments to learning and memory (Jung et al., 2018).

VGLUT expression is tightly linked to glutamate release and regulation of excitatory synapses. Since BDNF is known to strengthen excitatory synapses and

contribute to LTP, BDNF may be involved in regulating VGLUT expression in the hippocampus. Fluorescence microscopy imaging of cultured hippocampal neurons has shown that BDNF stimulation during embryonic development upregulates the expression and distribution of VGLUT isoforms (Melo et al., 2013). In addition, inhibition of Trk-B prevents BDNF-induced upregulation of VGLUT2 during development (Melo et al., 2013). However, the relationship between BDNF and VGLUT2 in the adult hippocampus has not been thoroughly described.

Choline acetyltransferase

Choline acetyltransferase (ChAT) is a transferase enzyme that is responsible for the synthesis of the transmitter acetylcholine (Ach) (Berman, Wilson, & Nachmansohn, 1953). It is found in high concentrations in cholinergic neurons throughout the central nervous system (Oda, 1999). Produced in the cell body, ChAT is transported down the axon to the presynaptic nerve terminal, where it carries out its catalytic functions. ChAT exists in two forms: a soluble form and a membrane-bound form (Tandon, Bachoo, Weldon, Polosa, & Collier, 1996). The soluble form is the most productive, accounting for a large percentage of total enzyme-activity in the cytoplasm (Pahud et al., 1998). The membrane-bound form is believed to be associated with synaptic vesicles in the hippocampus (Carroll, 1994). The presence of ChAT in brain tissue indicates the presence of acetylcholine, and the expression of this protein is considered a marker of cholinergic activity (Govindasamy et al., 2004).

Cholinergic activity is very important for several neurologic functions. It plays an essential role in activating the forebrain (B. E. Jones, 2005), sustained attention

(Himmelheber, Sarter, & Bruno, 2000), states of sleep (Platt & Riedel, 2011) and learning and memory (Ridley, Bowes, Baker, & Crow, 1984). Pathologic changes to cholinergic system are thought to underlie many neuropathologic diseases. For instance, irregular ChAT levels in the neocortex and hippocampus have been linked to memory loss in Alzheimer's disease (Francis, Palmer, Snape, & Wilcock, 1999), and loss of ChAT immunoreactivity has been related to the motor deficits found in amyotrophic lateral sclerosis (ALS) (Oda, Imai, Nakanishi, Ichikawa, & Deguchi, 1995). Current research is focused on understanding the extent to which cholinergic activity is involved in brain pathology, and whether the molecular components of cholinergic systems are good therapeutic targets. BDNF may be a potential target, as it has known interactions with cholinergic subsystems. *Ex vivo*, BDNF was found to promote the survival and differentiation of rat embryo cholinergic neurons, and this effect was associated with increased ChAT levels in those neurons (Alderson, Alterman, Barde, & Lindsay, 1990). It has also been suggested that BDNF activity in hippocampal neurons is regulated by cholinergic activity, as pilocarpine, a cholinergic receptor agonist, reportedly increased hippocampal BDNF expression *in situ* (da Penha Berzaghi et al., 1993). Finally, it was shown that ethanol exposure caused age-dependent decreases in both ChAT and BDNF in the mouse hippocampus (Jamal et al., 2018). Taken together, these lines of evidence indicate a positive correlation between BDNF and ChAT-mediated cholinergic activity.

Sex Differences in BDNF Expression and the Role of Estrogen

It is well-established that there are sex differences in brain physiology and function. For example, the volume density of mossy fiber synapses in the CA3 area of the

hippocampus is higher in females than in males, and this sex difference has been posited to reflect both pre- and postsynaptic elements (Parducz & Garcia-Segura, 1993). At baseline conditions, it has been reported that males have higher baseline BDNF gene expression in the dentate gyrus and CA1 regions compared with females (Kight & McCarthy, 2017). Spatial-specific sex differences in BDNF expression have also been identified and associated with behavioral phenotypes. In a recent animal study, restraint-stressed female mice, but not males, show decreased BDNF expression in the hippocampus (Yamaura et al., 2013), indicating that BDNF is differentially expressed in the male and female brain in response to stressors. These findings and similar reports have led authors to suggest that neurotrophic activity may be regulated by the sex-specific activity patterns of steroid hormones in the brain (Scharfman & Maclusky, 2005).

Investigations into the actions of steroid hormones have advanced our understanding of estrogen in the central nervous system. The landmark study conducted by Woolley et al. (1990) demonstrated that 17b-estradiol (E2) modulates spine density in the hippocampus (Woolley, Gould, Frankfurt, & McEwen, 1990), and since then, several studies have examined the extent to which estrogen signaling is involved in neurologic functions. Recently, it was discovered that the brain also produces its own estrogen (Hojo et al., 2004). Brain-derived estrogen has been implicated in modulating cognition (Luine, 2008), regulating synaptic plasticity (Foy, Baudry, & Thompson, 2004; Y. Lu et al., 2019), and promoting neuron survival in both the female and male brain (O'Neill, Chen, & Brinton, 2004). Estrogen has also been shown to be neuroprotective in the injured CNS (Scott, Zhang, Wang, Vadlamudi, & Brann, 2012). Intriguingly, the effects of estrogen and

BDNF in the brain appear to overlap considerably, which has inspired many investigations into their interactions (Scharfman & MacLusky, 2006). Several reports have shown that estrogen regulates BDNF expression in the rat hippocampus (Solum & Handa, 2002), suggesting that estrogen-induced synaptic plasticity may, in part, be mediated by enhanced BDNF. Furthermore, reduced estrogen and BDNF are observed in several neurodegenerative disorders, including Alzheimer's disease and Parkinson's (Sohrabji & Lewis, 2006). The classical estrogen receptors, first characterized in 1958, are understood to transduce the actions of estrogens in the adult brain. Both nuclear receptors, ER-α and ER-β, as well as the extranuclear receptor GPER1, are expressed in the hippocampus. They are known to interact with the neurotrophic system via both genomic and non-genomic mechanisms in an estrogen-dependent manner (Luine, 2008; Yang et al., 2010).

Estrogen receptor alpha

Estrogen receptor alpha (ER-α) is one of two main types of nuclear receptor that is activated by estrogen (Walter et al., 1985). ER-α is a transcription factor localized primarily to the nucleus that is composed of distinct domains important for estrogen binding, DNA binding, and transcription activation (Dahlman-Wright et al., 2006). ER-α has also been identified at extranuclear sites, including the plasma membrane and dendritic spines (Weiland, Orikasa, Hayashi, & McEwen, 1997), which may mediate some of the non-genomic effects of estrogen (Brann & Mahesh, 2008). ER-α is expressed in the male and female adult brain and distributed throughout the cortex and in the hippocampus (Osterlund, Gustafsson, Keller, & Hurd, 2000). Intriguingly, expression in

these regions tends to decline with age (Adams, Furneaux, & White, 2007). In protein expression studies, immunoreactivity of ER-α has been used to demonstrate spatial-specific ER-α expression (Su et al., 2001) and indicate the neuronal actions of estrogen in the brain (Lee, Kim, & Choi, 2012).

In the adult brain, ER-α- appears to be primarily involved in regulating reproductive functions (Lee et al., 2012). However, ER-α may also mediate estrogen's cognitive effects. Evidence that ER-α is involved in cognitive function comes from functional studies, in which ER-α knockout mice display impaired memory performance in hippocampal-dependent tasks (Fugger, Foster, Gustafsson, & Rissman, 2000). These cognitive effects may be explained, in part, by the trophic-like effects of ER-α activation. Previous studies have demonstrated a rapid increase in spine density of hippocampal CA1 neurons following an ER-α selective agonist, and this effect could be blocked by an ER-α selective antagonist (McEwen, Tanapat, & Weiland, 1999). This finding demonstrates a possible nongenomic mechanism by which ER-α mediates estrogen-induced synaptic plasticity, though whether these structural effects are limited to nongenomic mechanisms remains a topic of inquiry (Brann, Dhandapani, Wakade, Mahesh, & Khan, 2007).

ER-α expression rapidly changes in response to injury, suggesting that ER-α may mediate the neuroprotective effects of estrogen in the adult brain. Evidence of ER-α-mediated neuroprotection comes from studies of ovariectomized (OVX) rats subjected to brain injury, in which estrogen-mediated neuroprotection was lost in OVX ER-α-knockout mice (Dubal et al., 2001; Merchenthaler, Dellovade, & Shughrue, 2003). Additionally, ER-

α agonists have been shown to protect hippocampal neurons against glutamate-induced excitotoxicity (L. Zhao, Wu, & Brinton, 2004). However, this result has not always been obtained (Sampei et al., 2000). Currently, work is still underway to determine the extent of ER-α's involvement in the neuroprotective effects of estrogen (Lim et al., 2018).

There is accumulating evidence that estrogen regulates BDNF levels in the healthy and injured CNS (Y. Liu et al., 2001; Solum & Handa, 2002; X. Zhao et al., 2003). An early study identified an estrogen response element (ERE) sequence within the BDNF gene (Klinge, 1999), which can bind to an estrogen receptor complex and regulate BDNF transcription. Moreover, other mechanisms, such as novel estrogen receptor-interacting proteins (Brann, Zhang, Wang, Mahesh, & Vadlamudi, 2008), NMDAR subunit regulation (Gazzaley, Weiland, McEwen, & Morrison, 1996), and non-genomic transsynaptic mechanisms (Blurton-Jones, Kuan, & Tuszynski, 2004) may provide alternative means by which ER-α regulates BDNF expression. Further work is needed to address this issue.

Estrogen receptor beta

Estrogen receptor beta (ER-β) is the other major type of nuclear receptor that is activated by estrogen. ER-β is a transcription factor localized primarily to the nucleus that is composed of an N-terminal DNA binding domain and C-terminal ligand binding domain. Like ER-α, ER-β is also found at various extranuclear sites, including the plasma membrane, dendritic shafts, and dendritic spines. In both males and females, ER-β is expressed throughout various regions of the prefrontal cortex and the hippocampus, with its expression in the hippocampus being higher than that of its related isoform ER-α (Osterlund et al., 2000). Upon estrogen-dependent activation of ER-β at the nucleus, the

encoded protein interacts with DNA sequences to initiate genomic effects. ER-β immunoreactivity has been used to demonstrate spatial-specific ER-β expression (Register, Shively, & Lewis, 1998) and indicate the neuronal actions of estrogen in the brain (Lee et al., 2012).

Like ER-α, ER-β has been implicated in cognition and synaptic plasticity (Li, Cui, & Shen, 2014). For instance, activation of ER-β has shown to regulate hippocampal plasticity and improve memory (F. Liu et al., 2008). In functional studies, ER-β knockout mice display impairments in hippocampal-mediated fear conditioning and retrieved hippocampal slices show significant deficits in LTP amplitudes (Day, Sung, Logue, Bowlby, & Arias, 2005). A more recent study reported increased spine density and newly formed spines in the cortex of female mice after ER-β activation (Wang, Zhu, & Xu, 2018). These data support a role for ER-β in regulating synaptic plasticity in the hippocampus and improving hippocampal-dependent cognitive function.

ER-β has been shown to mediate the neuroprotective effects of estrogen. Studies using selective ER-β agonist have shown to be protective against global ischemia (Carswell, Macrae, Gallagher, Harrop, & Horsburgh, 2004). ER-β subtype agonists have also shown to protect against glutamate-induced excitotoxicity (L. Zhao et al., 2004). Estrogen may exert its neuroprotective effect through its activation of ER-β in astrocytes, as neuroprotective effects of ER-β expressing astrocytes in the cortex have been demonstrated *in vivo* (Dhandapani, Wade, Mahesh, & Brann, 2005). Currently, work is still underway to determine the extent of ER-β's involvement in the neuroprotective effects of estrogen (Lim et al., 2018).

G protein-coupled estrogen receptor 1

G protein-coupled estrogen receptor 1 (GPER1) is a recently discovered protein that binds estradiol. It is a seven-pass transmembrane protein primarily localized to the cytoplasmic face of the endoplasmic reticulum and plasma membrane (Funakoshi, Yanai, Shinoda, Kawano, & Mizukami, 2006). Like the two ER-receptor subtypes, GPER1 is reported to be expressed in the cortex and the hippocampus in various species (Funakoshi et al., 2006). Binding of estradiol to GPER1 results in intracellular calcium release and synthesis of phosphatidylinositol (3,4,5)- triphosphate (PIP_3) in the nucleus (Revankar, Cimino, Sklar, Arterburn, & Prossnitz, 2005). Hence, activation of this receptor plays a role in the rapid nongenomic signaling that is observed following estrogen-induced stimulation (Prossnitz, Arterburn, & Sklar, 2007). In protein expression studies, GPER1 immunoreactivity has been used to demonstrate spatial-specific GPER1 expression (Brailoiu et al., 2007) and indicate the rapid nongenomic effects of estrogen in the brain (Tang et al., 2014).

In the basal forebrain, GPER1 co-localizes with cholinergic neurons (Hammond, Nelson, & Gibbs, 2011) and has been shown to play a role in mediating the effects of Ach release in the hippocampus (Gibbs, Nelson, & Hammond, 2014). In the hippocampus, GPER1 appears to promote hippocampal-dependent memory functions, including spatial memory (Y. Y. Zhang et al., 2019). More directly, GPER1 has been shown to interact with BDNF in the hippocampus. A recent study has found that both estradiol and GPER1-specific agonist rapidly induce BDNF release in field CA3 of the hippocampus, suggesting

a novel mechanism by which BDNF and estrogen regulate hippocampal synaptic plasticity (Briz, Liu, Zhu, Bi, & Baudry, 2015).

Like the previously discussed ER subtypes, GPER1 has been implicated in mediating the neuroprotective effects of estrogen, as well. For instance, the GPER1-agonist, G-1, was shown to protect against apoptotic cell death and enhance motor functional recovery after brain injury (Cheng et al., 2016). Intriguingly, this effect was shown to be downstream of the PI3K/Akt pathway, previously mentioned as one of three main intracellular signaling pathways downstream of BDNF/Trk-B. Other studies have also linked the neuroprotective action of GPER1 to PI3K/Akt signaling, and additionally, to ERK signaling (Deng et al., 2017), suggesting an overlap in neuroprotective pathways between BDNF and E2. Clearly, GPER1 holds great promise as a potential target for therapy. However, more research is required to elucidate the precise mechanisms by which GPER1 mediates neuroprotection, and further, the extent to which BDNF is involved.

Significance of the Study

BDNF signal transduction is linked to neuron survival, synaptic stability, and sustained plastic changes in the hippocampus. Several diseases of the central nervous system involve dysregulation of the BDNF system, which is known to confer disruptions to neuronal function. Currently, efforts are underway to fully realize BDNF as a therapeutic target. However, the basal relationship between BDNF signaling and various components of neuronal function remains a less explored avenue of research. It stands to reason that, in order to understand the role of BDNF in disease, it is important to first characterize the

role of BDNF in healthy, normal function. In addition, sex differences in BDNF signaling and function have been reported. Intriguingly, the differential actions of BDNF have been posited to reflect the sex-dependent actions of sex hormones in the brain. Since sex-specific risk factors have become increasingly salient in epidemiology, it becomes important to characterize BDNF in relation to estrogen signaling in the brain. Therefore, to contribute to the field, this study aims to determine the effects of chronic BDNF loss on synaptic signaling in the hippocampus and explore a potential role of estrogen signaling in modulating this relationship. We hypothesize that synaptic signaling is disrupted in an animal model of BDNF loss, and that this disruption involves sex-dependent alterations to estrogen receptor signaling.

CHAPTER THREE
MATERIALS AND METHODS

Animals

Young adult male (n = 9) and female (n = 9) Sprague Dawley rats were used for this study. The rats were housed individually in a home cage with free access to food and water. Half of each males and females were wild type (WT) rats while the other half were BDNF heterozygous knockout rats ($BDNF^{+/-}$) (SAGE Labs, Boyertown, PA). The $BDNF^{+/-}$ animals had a seven base pair deletion in the BDNF gene. Animals were divided into groups by sex and strain: WT females (n = 5), WT males (n = 5), BDNF KD females (n = 4), and BDNF KD males (n = 4). All the female rats for the study were used as random cycling and the estrous cycle was not monitored. All procedures were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals at the University of Tennessee.

Tissue Harvest

Animals were euthanized using isoflurane overdose. Animals were decapitated and the brains were removed. Individual brains were cut at the midsagittal plane and the hippocampus from each hemisphere was dissected out. Hippocampal tissues were stored at -20°C and homogenized for protein detection via Western blot analysis.

Tissue Lysis

Hippocampal tissue was homogenized using RIPA buffer (Teknova). Tissue suspended in RIPA buffer was subjected to sonication (power level: 2) until the tissue

was visibly solubilized in the buffer. The homogenized tissue was then allowed to rest on ice for 1 min. The homogenate was centrifuged at 12,000 rpm for 10 minutes. Supernatant was collected in fresh tubes and used for further protein analyses.

Protein Estimation

The bicinchoninic acid method (BCA Protein Assay Kit, Thermo Fisher Scientific) was used for quantification of total protein in each sample. Protein estimation was performed using the 96-well plate method, as per the instructions of the kit. Bovine Serum Albumin (BSA) was used as the standard. Absorbance was measured at 562 nm using a Biotek plate reader and the protein concentration was calculated using Gene5 software.

Western Blotting

The samples for western blotting were prepared by mixing 1X Laemmli buffer (BioRad) with tissue homogenate and incubating in a water bath at 100°C for 10 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by loading the samples on 4-20% precast gels (BioRad). Protein samples were then transferred on 0.45 µm nitrocellulose membrane (80V for 90 min) and blocked using 5% BSA for 1 hour at room temperature on shaker. The blots were then incubated with primary antibody (Table 3.1) overnight at 4°C on shaker. After the primary antibody incubation, blots were washed with 1X TBST (10 min X 3) and incubated with the respective HRP-conjugated secondary antibody. Following this, blots were washed, developed using ECL (Clarity Western ECL substrate, BioRad), and scanned using chemiluminescence on Chemidoc Touch Imaging System (BioRad).

Table 3.1. Primary Antibodies used for Western Blotting

Name	Company	Catalog #
BDNF	Thermo Fisher Scientific	OSB00017W
Synaptophysin	Abcam	ab8049
PSD-95	Abcam	ab18258
VGlut2	Sigma	V2514
ChAT	Abcam	ab181023
ER-α	Abcam	ab32063
ER-β	Thermo Fisher Scientific	PA1-313
GPER1	Lifespan Biosciences	LS-C403194
GAPDH	Abcam	ab8245
Beta-actin	R&D Systems	MAB8929
Vinculin	BioRad	MCA465GA

Quantification of Proteins

Blots were quantified using ImageJ software downloaded from NIH.gov. The band intensity was measured and corrected for the corresponding housekeeping values. Data are presented as average fold change of each group. Error bars indicate positive standard error of the mean.

Statistical Analysis

Multiple t tests were used to compare significance between sex matched groups; WT males compared to BDNF KD males; WT females compared to BDNF KD females. Statistical significance was determined using the Bonferroni-Dunn method, with alpha set to 0.05. Analyses were performed using PRISM software (version 7).

CHAPTER FOUR
RESULTS

Western Blot Confirms BDNF Heterozygous Knockout

The animals used in our study had a 7-base pair deletion in the BDNF gene, resulting in a genetic model of BDNF loss. To verify that our BDNF KD animals had functional deficits in BDNF protein expression, we first examined both forms of functional BDNF in the mutant animals. As seen in Figure 4.1, protein levels of pro- and mature BDNF were detected from hippocampal tissue harvested shortly after euthanasia. Western blot results are shown in Figure 4.1A-B while quantification of the results from all samples is shown in Figure 4.1C. The data are represented in terms of normalized protein expression as compared to Wild Type (WT) animals. As shown in Figure 4.1, the results demonstrate that protein levels of BDNF are decreased in the hippocampus in both male and female BDNF KD rats. Statistical analysis, as shown in Table 4.1, revealed that the reduction of proBDNF in the males was significant ($p < .05$). While the reduction in the KD females was not statistically significant, the p value, 0.055, is right at the brink of significance. For mature BDNF, the statistical analysis (Table 4.2) revealed that there were significant reductions in both males and females following KD of BDNF.

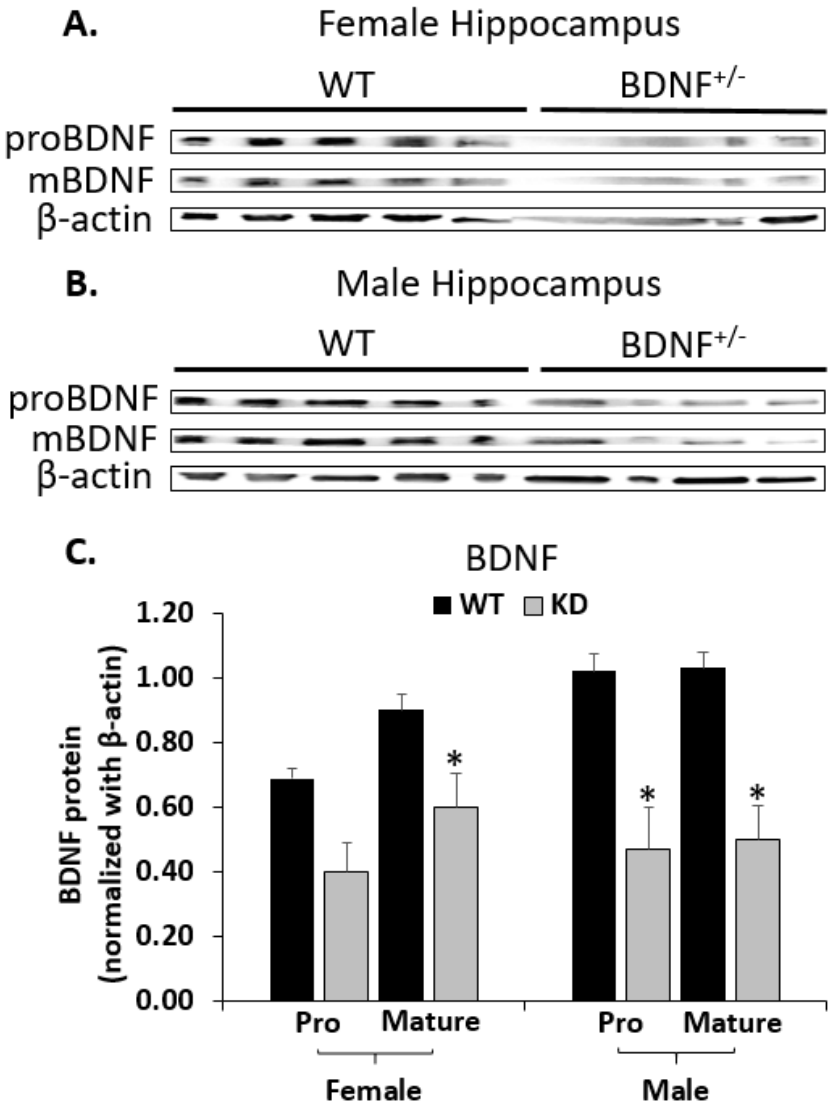

Figure 4.1. Western blot analysis confirmed BDNF heterozygosity. Tissue samples were collected from the hippocampus of adult BDNF KD and WT rats, both males and females. (A, B) Western blot images of BDNF expression in the female and male hippocampus show visual reductions in BDNF in the mutant (KD) rats, as compared to WT controls. (C) Quantification of blots indicates reductions in BDNF in both male and female KD rats. (*, $p < 0.05$).

Table 4.1. Statistical Analysis for proBDNF

Gender	Significant?	P-value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	No	0.027786	0.9020	0.6025	2.768	7	0.055572
Male	Yes	0.001533	1.034	0.4950	5.019	7	0.003065

Note: ***** = Adjusted using Bonferroni-Dunn method.

Table 4.2. Statistical Analysis for Mature BDNF.

Gender	Significant?	P-value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	Yes	0.013222	0.6880	0.4000	3.294	7	0.026443
Male	Yes	0.003192	1.024	0.4725	4.391	7	0.006384

Note: ***** = Adjusted using Bonferroni-Dunn method.

BDNF Knockdown and Altered Synaptic Densities

Synaptophysin

To understand the effects of BDNF loss on synaptic connectivity, we examined the effects of BDNF KD on synaptophysin expression in the hippocampus of both male and female rats. As shown in Figure 4.2, protein levels of synaptophysin were detected using western blot analysis. Representative immunoblots are shown in Figure 4.2A-B. Quantification of blots is shown in Figure 4.2C. As shown in Figure 4.2, the results demonstrated that protein levels of synaptophysin are significantly increased in BDNF KD males. Although BDNF KD females showed decreased levels of synaptophysin relative to WT females, the difference is not statistically significant (Table 4.3). The increase in synaptophysin in the BDNF KD males is suggestive of an altered presynaptic component of synaptic connectivity.

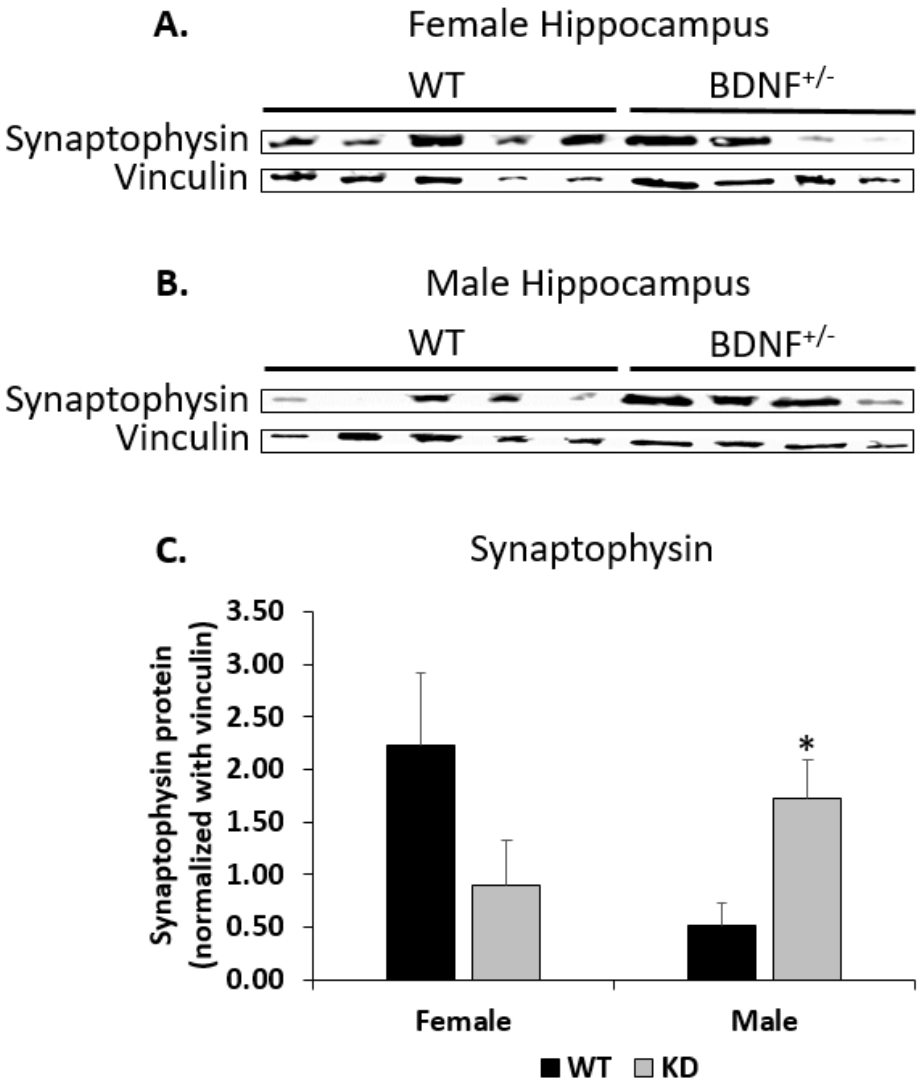

Figure 4.2. Synaptophysin was increased in BDNF KD males. Tissue samples were collected from the hippocampus of adult BDNF KD and WT rats, both males and females. (A) Western blot images of synaptophysin protein expression in female hippocampus samples show a visual decrease in the KD rats, as compared to WT controls. (B) Western blot images of synaptophysin protein expression in male hippocampus show a visual increase in the KD rats, as compared to WT controls. (C) Quantification of immunoblots showed a significant increase in synaptophysin protein in the mutant males, relative to WT males. The difference between female groups is not statistically significant. (*, $p < 0.05$).

Table 4.3. Statistical Analysis for Synaptophysin.

Gender	Significant?	P-value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	No	0.169570	2.226	0.8975	1.531	7	0.339140
Male	Yes	0.020749	0.5140	1.728	2.972	7	0.041498

Note: ***** = Adjusted using Bonferroni-Dunn method.

Postsynaptic density 95 (PSD-95)

Synaptic connectivity was further examined using PSD-95 as a neuronal marker of postsynaptic density. We examined the effects of BDNF KD on PSD-95 expression in the hippocampus of both male and female rats. As shown in Figure 4.3, protein levels of PSD-95 were detected using western blot analysis. Representative immunoblots are shown in Figure 4.3A-B. Quantification of blots is shown in Figure 4.3C. As shown in Figure 4.3, the results demonstrated that protein levels of PSD-95 were significantly decreased in BDNF KD males. PSD-95 expression levels did not differ significantly between the female groups (Table 4.4). The decrease in PSD-95 in BDNF KD males is suggestive of an altered postsynaptic component of synaptic connectivity.

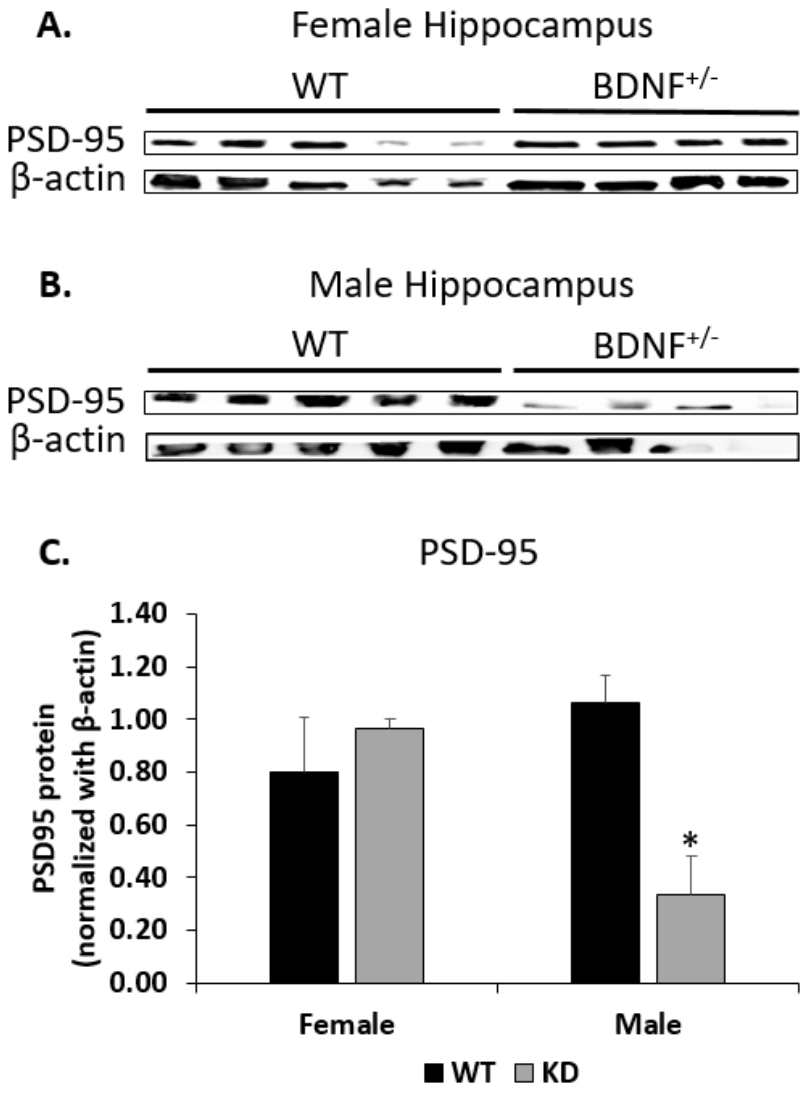

Figure 4.3. PSD-95 was decreased in BDNF KD males. Tissue samples were collected from the hippocampus of adult BDNF KD and WT rats, both males and females. (A) Western blot images of PSD-95 protein expression in female hippocampus samples are comparable between groups. (B) Western blot images of PSD-95 protein expression in male hippocampus show a visual decrease in the KD rats, as compared to WT controls. (C) Quantification of immunoblots showed a significant decrease in PSD-95 protein in the mutant males. (*, $p < 0.05$).

Table 4.4. Statistical Analysis for PSD-95.

Gender	Significant?	P-value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	No	0.515249	0.7980	0.9650	0.685	7	>0.99999
Male	Yes	0.006444	1.064	0.3333	4.088	7	0.012887

Note: * = Adjusted using Bonferroni-Dunn method.

Vesicular glutamate transporter 2 (VGLUT2)

VGLUT2 is a neuronal marker of presynaptic glutamatergic activity. It is widely used as a marker for glutamatergic neurons. We examined the effects of BDNF KD on VGLUT2 expression in the hippocampus of both male and female rats. As shown in Figure 4.4, protein levels of VGLUT2 were detected using western blot analysis. Representative immunoblots are shown in Figure 4.4A-B. Quantification of blots is shown in Figure 4.4C. As shown in Figure 4.4, the results demonstrated that protein levels of VGLUT2 were comparable to those of WT controls, in both males and females. No statistically significant changes were observed (Table 4.5), suggesting that VGLUT2 expression is unaltered in BDNF KD animals relative to WT controls.

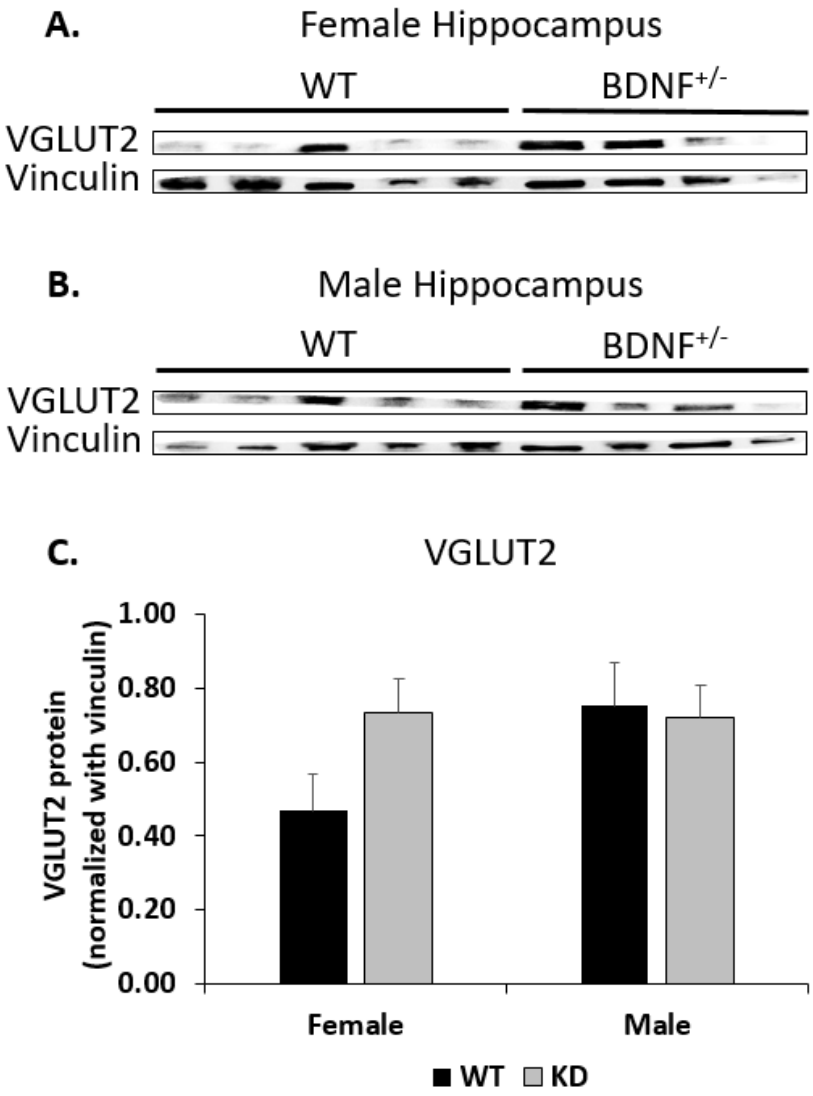

Figure 4.4. VGLUT2 did not differ across sex-matched groups. Tissue samples were collected from the hippocampus of adult BDNF KD and WT rats, both males and females. (A) Western blot images of VGLUT2 protein expression in female hippocampus samples show a visual increase in the KD rats, as compared to the WT controls. (B) Western blot images of VGLUT2 protein expression in male hippocampus are visually comparable between groups. (C) Quantification of blots did not indicate any significant differences between sex-matched groups. (*, $p < 0.05$).

Table 4.5. Statistical Analysis for VGLUT2.

Gender	Significant?	P-value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	No	0.093050	0.4680	0.7350	1.944	7	0.186101
Male	No	0.854349	0.7520	0.7225	0.191	7	>0.99999

Note: ***** = Adjusted using Bonferroni-Dunn method.

Choline acetyltransferase (ChAT)

ChAT is a neuronal marker of presynaptic cholinergic activity as well as an accepted marker of cholinergic neurons. We examined the effects of BDNF KD on ChAT expression in the hippocampus of both male and female rats. As shown in Figure 4.5, protein levels of ChAT were detected using western blot analysis. Representative immunoblots are shown in Figure 4.5A-B. Quantification of blots is shown in Figure 4.5C. As shown in Figure 4.5, the results demonstrated that protein levels of ChAT were comparable to those of WT controls, in both males and females. There was a minor elevation in protein expression in the BDNF KD animals, however, this change was not statistically significant (Table 4.6). These results indicate that ChAT expression is unaltered in BDNF KD animals relative to WT controls.

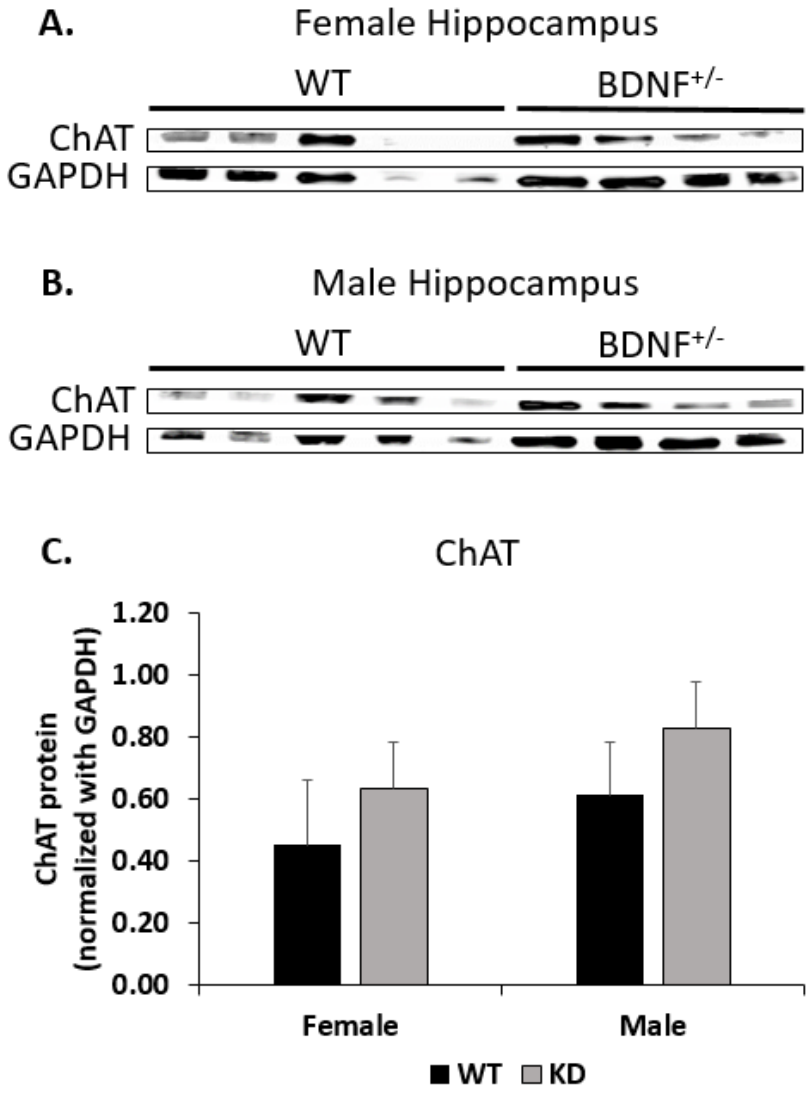

Figure 4.5. ChAT did not differ across sex-matched groups. Tissue samples were collected from the hippocampus of adult BDNF KD and WT rats, both males and females. (A) Western blot images of ChAT protein expression in female hippocampus samples show a visual increase in the KD males, as compared to the WT controls. (B) Western blot images of ChAT protein expression in male hippocampus are comparable between groups. (C) Quantification of blots did not indicate any significant differences between sex-matched groups. (*, $p < 0.05$).

Table 4.6. Statistical Analysis for ChAT.

Gender	Significant?	P-value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	No	0.524007	0.4520	0.6350	0.671	7	>0.99999
Male	No	0.389807	0.6140	0.8300	0.917	7	0.779614

Note: * = Adjusted using Bonferroni-Dunn method.

BDNF Knockdown and Altered Estrogen Receptor Expression

Estrogen receptor alpha (ER-α)

Analysis of ER-α was used to aid to the analysis of underlying mechanisms of sex-specific differences in BDNF action. We examined the effects of BDNF KD on ER-α expression in the hippocampus of both male and female rats. As shown in Figure 4.6, protein levels of ER-α were detected using western blot analysis. Representative immunoblots are shown in Figure 4.6A-B. Quantification of blots is shown in Figure 4.6C. The protein expression of ER- β was visually lower in WT males relative to WT females (Figure 4.6A-B). As shown in Figure 4.6, the results are indicative of a trend in which female mutant rats displayed a minor decrease in ER-α, while male mutants show an increase in ER-α, relative to their respective WT controls. Statistical analysis, shown in Table 4.7, revealed that the changes in both males and females were not significant. These results convey that ER-α mediated estrogen signaling is unaltered in BDNF KD animals. However, the p value for males, 0.060, was right at the brink of significance. Appropriate power analysis and increasing the n number for future studies should yield more stringent statistical evidence and show sex-dependent changes in ER-α expression following chronic BDNF loss.

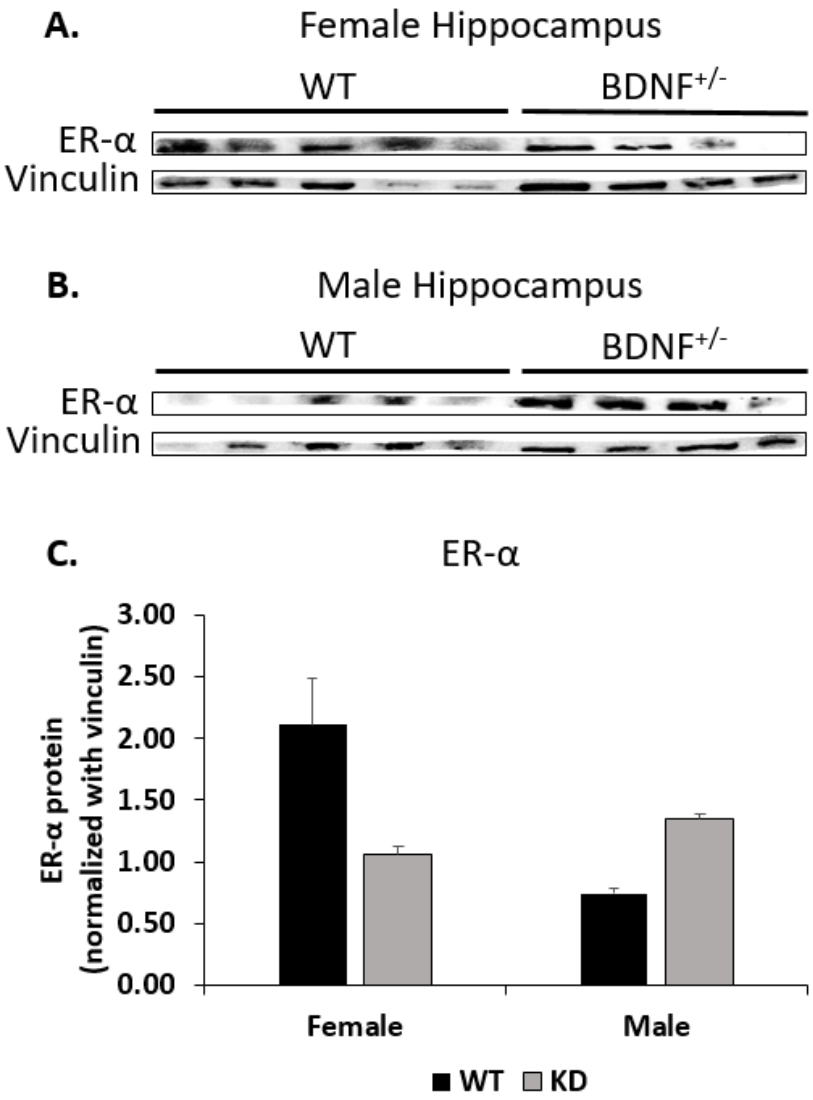

Figure 4.6. ER-α did not differ across sex-matched groups. Tissue samples were collected from the hippocampus of adult BDNF KD and WT rats, both males and females. (A) Western blot images of ER-α protein expression in female hippocampus samples show a visual decrease in the KD rats, as compared to WT controls. (B) Western blot images of ER-α protein expression in male hippocampus show a visual increase in the KD rats, as compared to WT controls. (C) Quantification of immunoblots did not indicate significant differences between sex-matched groups. (*, $p < 0.05$).

Table 4.7. Statistical Analysis for ER-α.

Gender	Significant?	P-value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	No	0.030178	2.112	0.8475	2.710	7	0.060356
Male	No	0.113734	0.7460	1.123	1.807	7	0.227467

Note: ***** = Adjusted using Bonferroni-Dunn method.

Estrogen receptor beta (ER-β)

Besides ER- α, ER-β is another important mediator of genomic estrogen signaling in the brain. We examined the effects of BDNF KD on ER-β expression in the hippocampus of both male and female rats. As shown in Figure 4.7, protein levels of ER-β were detected using western blot analysis. Representative immunoblots are shown in Figure 4.7A-B. Quantification of blots is shown in Figure 4.7C. The protein expression of ER- β was visually lower in WT males as compared to WT females (Figure 4.7A-B). As shown in Figure 4.7, the results are indicative of a trend in which female mutant rats displayed a minor decrease in ER-β, while male mutants show an increase in ER-β, relative to their respective WT controls. Statistical analysis, shown in Table 4.8, revealed that protein levels of ER-β are significantly increased in male BDNF KD animals as compared to WT males. There was no statistically significant difference in ER-β between the female groups (Table 4.8). These results are suggestive of a sex-dependent alteration in ER-β mediated estrogen signaling after BDNF loss.

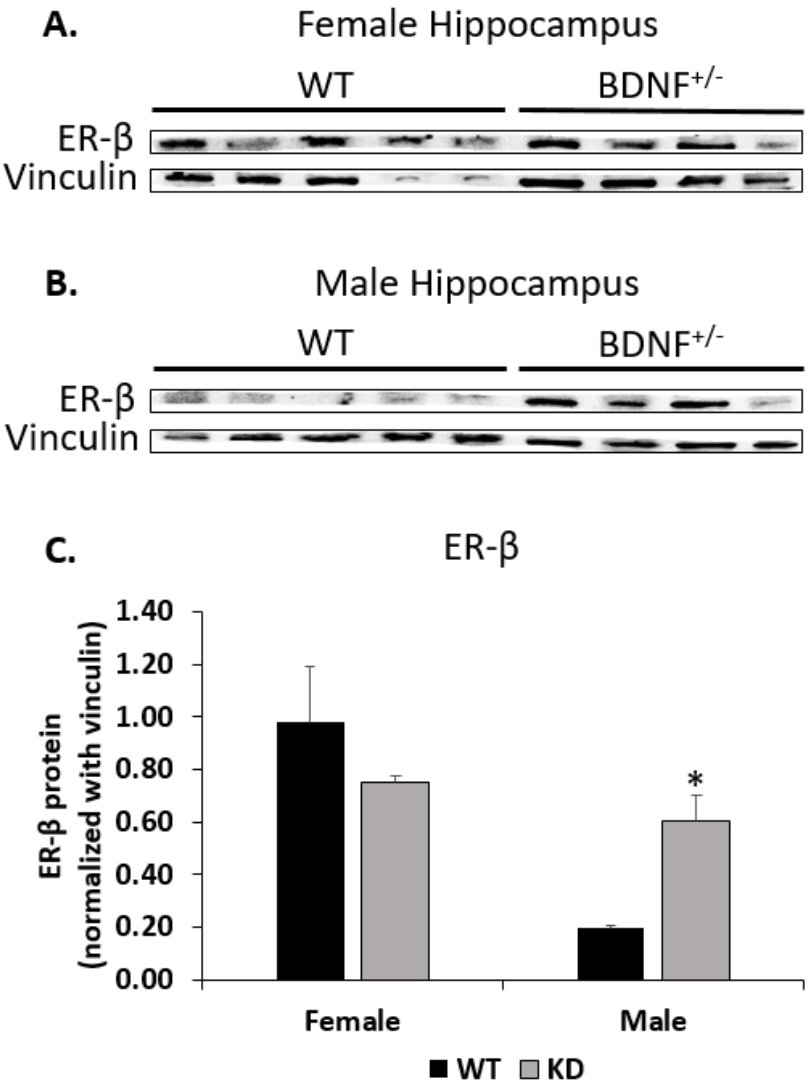

Figure 4.7. ER-β was increased in BDNF KD males. Tissue samples were collected from the hippocampus of adult BDNF KD and WT rats, both males and females. (A) Western blot images of ER-β protein expression in female hippocampus samples show a visual decrease in the KD rats, as compared to WT controls. (B) Western blot images of ER-β protein expression in male hippocampus show a visual increase in the KD rats, as compared to WT controls. (C) Quantification of immunoblots showed a significant increase in ER-β protein in the mutant males. The difference between female groups is not statistically significant. (*, $p < 0.05$).

Table 4.8. Statistical Analysis for ER-β.

Gender	Significant?	P-value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	No	0.379082	0.9780	0.7500	0.939	7	0.758164
Male	Yes	0.002148	0.1940	0.6075	4.724	7	0.004296

Note: ***** = Adjusted using Bonferroni-Dunn method.

G protein-coupled estrogen receptor 1 (GPER1)

A third, most recently discovered estrogen receptor is the GPER1, which primarily moderates non-genomic estrogen signaling in the brain. GPER1 was used to complete a thorough analyses of ERs after BDNF loss. We examined the effects of BDNF KD on GPER1 expression in the hippocampus of both male and female rats. As shown in Figure 4.8, protein levels of GPER1 were detected using western blot analysis. Representative immunoblots are shown in Figure 4.8A-B. Quantification of blots is shown in Figure 4.8C. The protein expression of GPER1 was visually lower in WT males as compared to WT females (Figure 4.8A-B). As shown in Figure 4.8, the results are indicative of a trend in which female mutant rats displayed a minor decrease in GPER1, while male mutants show an increase in GPER1, relative to their respective WT controls. Statistical analysis, shown in Table 4.9, revealed that the changes in both males and females were not significant. These results convey that GPER1 mediated estrogen signaling is unaltered in BDNF KD animals. However, the p value for males, 0.076, was close to being significant. Appropriate power analysis and increasing the n number for future studies should yield more stringent statistical evidence for sex-dependent changes in GPER1 expression following chronic BDNF loss.

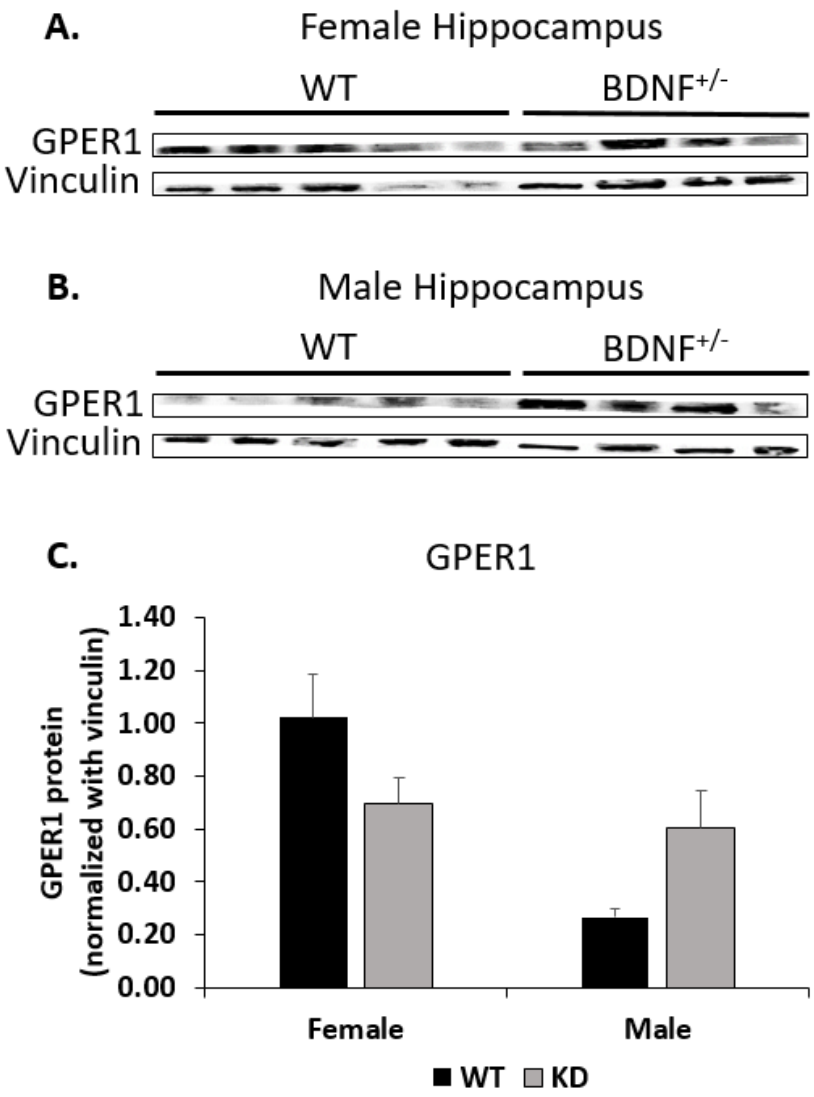

Figure 4.8. GPER1 did not differ across sex-matched groups. Tissue samples were collected from the hippocampus of adult BDNF KD and WT rats, both males and females. (A) Western blot images of GPER1 protein expression in female hippocampus samples show a visual decrease in the KD rats, as compared to WT controls. (B) Western blot images of GPER1 protein expression in male hippocampus show a visual increase in the KD rats, as compared to WT controls. (C) Quantification of immunoblots did not indicate significant differences between sex-matched groups. (*, $p < 0.05$).

Table 4.9. Statistical Analysis for GPER1.

Gender	Significant?	P-value	Mean of WT	Mean of KD	T ratio	df	Adjusted P value*
Female	No	0.159127	1.024	0.6975	1.576	7	0.318253
Male	No	0.037972	0.268	0.6050	2.552	7	0.075944

Note: ***** = Adjusted using Bonferroni-Dunn method.

CHAPTER FIVE
DISCUSSION AND CONCLUSIONS

Discussion

As discussed earlier, we hypothesized that synaptic signaling is disrupted in a condition of BDNF loss, and that this disruption involves alterations to estrogen receptor signaling in a sex-specific manner. We tested this hypothesis using a $BDNF^{+/-}$ KD rodent model. In agreement with our hypothesis, findings from our study reveal that: (1) $BDNF^{+/-}$ KD led to sex-specific alterations in levels of protein expression of synaptophysin and PSD-95, (2) BDNF loss did not alter protein levels of VGLUT2 or ChAT in either male or female hippocampus, and (3) sex-specific changes in the hippocampus were observed in protein expression of ER-β following $BDNF^{+/-}$ KD.

From our initial findings, we observed that there are specific pre- and postsynaptic molecular changes in the male hippocampus, but not in the female, after $BDNF^{+/-}$ KD. Presynaptic marker, synaptophysin (Figure 4.2), was significantly increased in the male KD as compared to WT. But, the postsynaptic marker, PSD95 (Figure 4.3), was decreased in the KD male as compared to WT. These results are suggestive of a disrupted neuronal communication/connectivity in the hippocampus, which may be the underlying cause of several other reported behavioral deficits in relation to BDNF loss (Linnarsson, Bjorklund, & Ernfors, 1997; Mizuno, Yamada, Olariu, Nawa, & Nabeshima, 2000). No changes in any of the pre- or postsynaptic markers were reported in the female hippocampus, suggesting a pre-existing rescue mechanism. Other studies have also confirmed that disrupted BDNF signaling has altered mechanisms of synaptic connectivity

both *in vivo* and *in vitro* (L. D. Pozzo-Miller et al., 1999) (Yoshii & Constantine-Paton, 2014).

Examination of changes in synaptic markers was followed by analyses of neuronal-type specific markers; VGLUT2 and ChAT. These are markers of glutamatergic and cholinergic neuronal activity, respectively. Recently, others have documented that disruptions in BDNF signaling lead to deficits in VGLUT2 levels in cultured hippocampal neurons (Melo et al., 2013), and that BDNF loss corresponds to reductions in ChAT levels in the mouse hippocampus (Jamal et al., 2018). Our analyses showed that there were no significant changes in the protein levels of either VGLUT2 or ChAT in both $BDNF^{+/-}$ KD males as well as females as compared to WT (Figures 4.4 & 4.5). These results tempt us to reason that BDNF loss may affect neuronal communication but not glutamatergic or cholinergic neuronal activity. There can be several other underlying mechanisms that may show a similar effect, and therefore, require further deciphering. Here, we propose a few possibilities as to why no change in VGLUT2 and ChAT was observed in our model of chronic BDNF loss. One of the reasons could be that effects of BDNF loss may be restricted to specific neuronal pathways other than VGLUT2 or ChAT. It is also possible that there are compensatory mechanisms in the brain to rescue VGLUT2 and ChAT. Time may also be a factor in our results. For example, BDNF loss and the resulting disruption of VGLUT2 and ChAT could be occurring in aged animals only. Finally, ChAT and VGLUT2 expression in $BDNF^{+/-}$ KD animals may be differentially altered by neuronal insult. Therefore, a next step would be to examine these markers under pathologic conditions, in relation to $BDNF^{+/-}$ KD.

Next, the sex-specific significant differences in pre- and postsynaptic markers were investigated in the context of steroid hormone-specific signaling pathways. For this study, we looked at the differences in estrogen signaling molecules, the estrogen receptors (ERs), namely, ER-alpha, ER-beta, and GPER1. Protein expression of all three receptors showed an expected pattern where WT males had lower levels of ERs as compared to WT females. Analysis of female groups showed no change in ER expression in KD females relative WT females (Figures 4.6, 4.7, 4.8). However, our findings reported that $BDNF^{+/-}$ KD males show a significant increase in ER-β, along with a similar upward trend in ER-α and GPER1, when compared to respective protein levels in WT males. Importantly, the increases were not as high as the basal levels in WT females. Given these results, we believe that $BDNF^{+/-}$ KD in the males led to an activation of the neuroprotective pathways that may be necessary to rescue the effects of BDNF loss. We posit that estrogen receptor signaling may achieve these neuroprotective effects. In support of our interpretation, previous studies have shown that BDNF and estrogen both serve neuroprotective roles in the male and female brain (Cho et al., 2003; Pandya et al., 2013), and that estrogen neuroprotection can be mediated by estrogen receptor signaling (Carswell et al., 2004; Merchenthaler et al., 2003). We propose that, since males have indigenously low levels of ERs (as shown in Figures 4.6, 4.7, 4.8), they require an upregulation in ER expression to combat the negative effects of BDNF loss. Further support of this view comes from the fact that males express higher levels of BDNF mRNA in the developing hippocampus as compared to females (Kight & McCarthy, 2017), suggesting a superior functional role of and dependency on BDNF in this region of the

adult male brain. Hence, BDNF loss in males could be more detrimental to signaling processes, thereby requiring an alternative neuroprotective response in the form of estrogen signaling. We believe that the naturally higher levels of ERs in females may suffice for protective levels of ERs, and, thus, do not require an upregulation of estrogen receptors after BDNF loss.

Taken together, our study adds a piece in the puzzle of understanding BDNF action in the brain, more specifically, in the region important for memory and cognition – the hippocampus. We have examined the effects of BDNF loss under basal conditions, without any brain insult or other interfering variables. We showed specific neuronal signaling pathways and estrogen receptor signaling pathways that may play a crucial role in BDNF action. We also accounted for sex differences, as understanding sexual dimorphism in the brain is pivotal for the understanding of human physiology. Additionally, this study adds to the development of therapeutic targets, as BDNF is neuroprotective in several neuropathologic disorders. Finally, our findings can be implicated in mechanisms of aging and in post-menopausal health, where reductions in both BDNF and E2 signaling have been very well documented.

Limitations of the Study

We acknowledge that there are additional investigations that need to be performed as part of future directions for this project. An important future objective that must be met is to investigate the temporal and spatial effects of BDNF loss in the brain. To that end, parallel studies in the lab are investigating the effects of BDNF loss in other limbic structures, cortex, and the brainstem. As the facts have it, the brain is formed of 90% glial

cells and 10% neurons. Therefore, it is also imperative to study the effects of BDNF loss on other cells types and on neuroinflammation. Other studies from our lab have reported effects of BDNF loss on inflammation in the brain.

Since our results are suggestive of a pattern that involves sex-dependent changes in estrogen receptor expression, it is important to take into consideration known sexual dimorphic components of neurophysiology and of the periphery, as well. For example, the observed changes in our study may be explained, in part, by the sexual dimorphic actions of select neurosteroid receptors in the brain, including, but not limited to, progesterone, androgen, and insulin growth factor 1 (IGF1) receptors. While beyond the scope of this study, future objectives would do well to include such parameters. Furthermore, estrous activity is known to modulate estrogen receptor expression. Though our female specimens were on random cycles, controlling for estrous cycle may prove valuable in determining the causal factors involved in estrogen receptor changes following BDNF loss.

Conclusions

Our study features an important pre-clinical model with immense translational value. We conclude that BDNF remains a crucial neurotrophic factor in neuronal signaling and healthy brain function. Moreover, the effects of BDNF in the brain are different in males and females and the steroid hormone, estrogen, might be an important mediator of BDNF action.

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VITA

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