

Abstract of “*BDNF* Val⁶⁶Met Polymorphism and Gonadal Hormones Modulate Basal and Cognitively-Related Neural Functions in Humans”

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Ovarian steroids have been shown to modulate a wide range of cognitive functions in both animals and humans, but results are inconsistent. The variability of the findings suggests that the response to ovarian hormones may be modulated by a number of yet-to-be identified contextual factors. In this thesis, I demonstrate that brain derived neurotrophic factor (*BDNF*) and ovarian hormones interactively affect human brain function.

In the absence of a direct *in vivo* measure of *BDNF* in human brain, the functional *BDNF* Val⁶⁶Met polymorphism offers a window on how *BDNF* influences human cognition. Using positron emission tomography (PET) to study healthy subjects genotyped for the *BDNF* Val⁶⁶Met SNP, I found that, compared to Val homozygotes, Met carriers had higher resting regional cerebral blood flow (rCBF) in prefrontal and hippocampal/parahippocampal areas. Moreover, there were significant sex-by-genotype interactions in these regions: Val homozygotes had higher rCBF in females than males, whereas Met carriers showed the opposite relationship. Functional correlations of BA25, hippocampus, and parahippocampus with frontal and temporal networks were positive for Val homozygotes, but negative for Met carriers. In addition, sex-by-genotype analysis of functional connectivity revealed that genotype affected the direction of the interregional correlations differently in men than women. These results demonstrate that *BDNF* allelic

variation and sex interactively affect basal prefrontal and hippocampal function as well as functional connectivity between these regions.

Next, to determine whether the observed sex-by-genotype interaction is mediated by ovarian steroids, I used PET in concert with a six-month hormone manipulation protocol to investigate the interaction between *BDNF* polymorphism and the ovarian steroids, estrogen and progesterone, on working memory-related hippocampal function in healthy women. I demonstrated a hormone-by-genotype interaction in working memory-related hippocampal function that reflected abnormal hippocampal recruitment in Met carriers but only in the presence of estradiol, suggesting a *BDNF* genotype-mediated sensitivity to ovarian steroids. Collectively, these data emphasize that interpreting the effects of genotype on brain function in women requires knowledge of the ovarian steroid hormone milieu, and that, conversely, it is crucial to consider *BDNF* genotype when investigating the effects of ovarian steroids on cognitive and behavioral processes.

***BDNF* Val⁶⁶Met Polymorphism and Gonadal Hormones Modulate Basal and Working Memory-Related Neural Functions in Humans**

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Preface

The ovarian steroid hormones, estradiol and progesterone, have been shown to modulate a wide range of cognitive functions in both animals and humans, but results have been inconsistent. The variability of the findings suggests that the differential response to ovarian hormones may be modulated by a number of yet-to-be identified contextual factors. In this thesis, I demonstrate that the brain derived neurotrophic factor (BDNF) is a candidate to mediate the effects of ovarian hormones in humans. Chapter 1 outlines background information that supports this thesis. In this chapter, I describe the localization of ovarian steroids and their receptors in the brain (Section 1.1), summarize the effects of ovarian hormones on neural functioning both preclinically and in humans (Section 1.2), provide an overview of the localization of neurotrophin BDNF and its receptor in the CNS and their influences on neurophysiology and behavior in the brain (Section 1.3), and describe the common molecular mechanisms by which both BDNF and ovarian steroids exert their physiologic effects (Section 1.4). In Chapter 2, I delineate the methods used to acquire the data in this thesis. In Chapter 3, I show that 1) the *BDNF* Val⁶⁶Met functional polymorphism (rs6265) and sex interactively modulate prefrontal and hippocampal basal, resting regional cerebral blood flow (rCBF) as well as the functional connectivity between these regions and other brain regions, and 2) the *BDNF* Val⁶⁶Met functional polymorphism (rs6265) interacts with ovarian steroids to mediate working memory-related hippocampal activity in healthy women. Chapter 4 provides a summary and discussion of the main results of the thesis, and it also includes several proposals for future investigation.

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Chapter 1

INTRODUCTION

Considerable epidemiologic evidence documents the importance of sex differences in the onset, duration, severity and/or course of many neuropsychiatric disorders (WHO, 2008) including Alzheimer's disease (Ventriglia et al. 2002), schizophrenia (Neves-Pereira et al. 2005), (Sklar et al. 2002), anxiety (Soliman et al. 2010), and depression (Gatt et al. 2009). Pre-clinical studies suggest that sex hormones contribute to these observed sex differences by influencing brain organization and function at critical periods of brain development *in-utero* and across puberty, as well as by modulating brain functions during periods of ovarian hormone change in some adults across the life span. There are two predominant ovarian hormones estradiol and progesterone. Pre-clinical studies demonstrate that these ovarian steroids exert a wide range of neuromodulatory effects on both reproductive and non-reproductive behaviors. In addition, there is considerable evidence in humans that ovarian steroid hormones strongly influence the central nervous system (CNS), particularly the prefrontal cortex (PFC) and the hippocampus. Cognitive operations that are dependent on these regions such as executive function, memory, and spatial navigation, are modulated by estradiol and progesterone (Sherwin 1994; Berman, Schmidt et al. 1997; Daniel, Fader et al. 1997; Spencer, Waters et al. 2008). Thus, both basic and clinical studies demonstrate that ovarian steroids have the potential capacity to modulate those brain regions involved with both reproductive and non-reproductive behaviors.

Despite evidence documenting the manifold neuromodulatory effects of ovarian steroids, studies in both humans and animals have shown that the effects of ovarian steroids on brain function and behavior are quite variable. The mechanisms by which alterations in the secretion of ovarian steroids induce changes in behavioral and cognitive states, are as yet undefined. However, several contextual factors have been identified that contribute to the impact of ovarian hormones on brain function, including age, environmental influences (e.g., exposure to early life stress), and variations in ovarian steroid-regulated genes. The diversity of the observed behavioral and cognitive response to the presence or absence of ovarian steroids suggests that further study of the interactions between ovarian steroids and other neuromodulatory systems may help elucidate the mechanisms that mediate the effects of ovarian steroids on neural systems in women.

The effects of ovarian steroids are highly convergent with the actions of several other neuromodulatory systems. For example, brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, has been implicated in mediating the effects of ovarian steroids. Ovarian hormones and BDNF both exert neuromodulatory and neuroprotective effects and are involved in neurogenesis, synaptogenesis, neural growth and differentiation (Pilgrim and Hutchison 1994; Huang and Reichardt 2001). In addition, ovarian steroids and BDNF both play critical roles in PFC-dependent executive functions (Berman, Schmidt et al. 1997; Egan, Kojima et al. 2003) and in hippocampal processes including activity-dependent synaptic plasticity that mediates learning, memory, and spatial navigation (Lu 2003 a, b).

In the brains of rodents, non-human primates, and humans, the *BDNF* gene is most abundantly expressed in the medial temporal lobe, specifically in the hippocampus, as well as in the PFC (Murer, Yan et al. 2001). Moreover, BDNF tyrosine kinase receptors (trkB) and steroid hormone receptors are localized in similar brain regions, particularly the hippocampus and PFC (Miranda, Sohrabji et al. 1993), indicating a potential for the physiologically relevant coupling of their individual functions. It is known that the status of ovarian hormones can greatly affect the expression of *BDNF* and trkB. Estrogen replacement in young ovariectomized female rats increases *BDNF* expression in the medial temporal lobe and the cortex (Gibbs 1999). In addition, the *BDNF* gene contains an estrogen response element (ERE), and the expression of *BDNF* can be directly modulated by the presence of estrogen (Sohrabji et al. 1993). It has also been shown that progesterone increases the expression of *BDNF* (Singh et al. 2012), and metabolites of progesterone such as allopregnanolone may exert their effects through the regulation of BDNF (Nin et al. 2011). While interactions between steroid hormones and BDNF are well documented on the molecular level, there is little behavioral and neural circuitry data in either animal or human.

In animal models, the molecular and neural effects of BDNF can be assessed by direct manipulation and measurement of both *BDNF* gene expression and BDNF protein level, but such experimental control cannot be achieved in the living human brain. However, a functionally relevant single nucleotide polymorphism (SNP) exists in the human *BDNF* gene that leads to naturally occurring alteration in BDNF activity in humans and provides the means to study the effects of variations in BDNF function in the living human brain. The Val⁶⁶Met (rs6265) SNP results in the substitution of methionine

(Met) to valine (Val) in the 5' pro-region of the BDNF protein, and it affects intracellular trafficking and secretion of BDNF. Importantly, neuroimaging studies in humans show that hippocampal recruitment is abnormal in *BDNF* Met carriers during both working (Egan, Kojima et al. 2003) and episodic memory (Hariri, Goldberg et al. 2003).

In this thesis project, I have used positron emission tomography (PET) in healthy volunteers to first study potential sexual dimorphism in the effects of BDNF on basal brain function. Second, in concert with an incisive clinical protocol based on pharmacologically-induced hypogonadism and well-controlled hormone replacement to elucidate the neural mechanisms by which ovarian steroid hormones and *BDNF* genotype affect brain circuitries underlying PFC- and hippocampal-dependent processes. PET studies included resting state and working memory activation paradigms. My results show first that *BDNF* allelic variation affects regional cerebral blood flow (rCBF) and functional connectivity differentially in women versus men (study 1). Further, I demonstrate that in a cohort consisting of healthy women only, the *BDNF* Val⁶⁶Met functional polymorphism interacts with ovarian steroid hormones to affect working memory related hippocampal function (study 2). These findings not only demonstrate important neurogenetic and hormonal mechanisms in the brain, they also provide information of relevance to neuropsychiatric disorders that are differentially expressed in women compared to men, particularly reproductive endocrine-related mood disorders (Schmidt et al. 1998; Bloch et al. 2000). In the next sections, I will describe the localization of ovarian steroids and their receptors in the brain (Section 1.1), summarize the effects of ovarian hormones on neural functioning both preclinically and in humans (Section 1.2), and discuss the localization of neurotrophin BDNF and its receptor in the

CNS and their influence on neurophysiology and behavior in the brain (Section 1.3).

Finally, I will describe the common molecular mechanisms by which both BDNF and ovarian steroids exert their physiologic effects (Section 1.4).

1.1 Ovarian Steroids and Their Receptors in the CNS

The CNS is a major target of steroid hormones and contains specific steroid receptors for peripherally and locally synthesized hormones. There are two major mechanisms by which steroid hormones exert their influences. Steroid hormone actions that are delayed in onset and prolonged in duration usually involve genomic mechanisms, while rapid and short-term steroid hormone effects usually involve nongenomic mechanisms. At the genomic level, steroid hormone effects are mediated through classical receptors belonging to the superfamily of nuclear receptors, and the effects generally have prolonged latency and are long lasting (McEwen et al. 1999). The classical steroid receptors are proteins that act as ligand-activated transcription factors and have multiple functional domains. The DNA-binding domain contains two zinc fingers that are important for receptor binding to specific hormone response elements on the DNA sequence. The ligand-binding domain contains regions that interact with molecules crucial for subsequent transcriptional processes and is involved in determining target specificity. Several chaperone molecules, including heat shock proteins, form complexes with steroid hormone receptors. Upon steroid binding, the receptors undergo several conformation changes, including dissociation of chaperone proteins, dimerization of the receptor-ligand complexes, the binding to specific hormone response elements in the promoters of target genes, and eventually the modulation of transcription of those genes

(Beato et al 1989, Tsai et al. 1994, Brinton et al. 2008). Additionally, the ligand-bound steroid receptor complex leads to the activation of several cellular signaling cascades including mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinases (PI3K), cAMP response element-binding protein (CREB), and phosphoinositide phospholipase C γ (PLC γ) pathways.

In addition to their classical genomic-mediated actions, ovarian steroids have well-documented fast acting, non-genomic actions thought to be mediated by membrane-based steroid receptors (Vasudevan, Kow et al. 2001). These membrane-based receptors are identical in structure to the genomic-acting receptors (with some exceptions, including G protein-coupled membrane estrogen receptor 30 [Revankar et al. 2005] or ER-X [Toran-Allerand CD et al. 2002]), but are located in caveoli-like structures in the membrane. Upon binding of steroid hormone, the activated receptor complex can trigger signaling cascades via a second messenger-regulated DNA-binding protein such as a member of the cAMP response element binding protein (CREB) family (McEwen et al. 1999). The physiologic actions of the steroid membrane receptors serve to facilitate (i.e., speed-up) traditional genomic actions by phosphorylating nuclear-acting steroid receptors and potentially act to alter cellular function independent of traditional steroid actions at the genome level.

Finally, both estradiol and progesterone (or their neurosteroid metabolites) are capable of acting on several classical neurotransmitter receptor systems including gamma-aminobutyric acid (GABA) (Majewska et al. 1986) and glutamate (Wong et al. 1994). The characteristics of ovarian steroids and their receptors, therefore, provide the means by which the response of the CNS to incoming stimuli may be altered. The extent

to which these effects underlie or contribute to behavioral differences is unclear but is of considerable potential relevance for humans.

1.1.1 Estrogen, estrogen receptors, and their distribution in the brain

In women, the most potent circulating form of estrogen is 17 β -estradiol (Gillies et al. 2010), and it is primarily produced by the ovaries, although estradiol can also be synthesized *de novo* in the brain in lower animals (and possibly humans), specifically in the hippocampus (Azcoitia et al. 2011; Fester et al. 2011). The actions of estradiol are mediated through its protein receptors, and the most well-characterized estrogen receptors (ER) are ER α and ER β , which are encoded on different chromosomes (6 and 14, respectively) but share similar structures of their DNA-binding and ligand-binding domains. ER α and ER β belong to the nuclear receptor superfamily and are localized in both the nucleus as well as the cytoplasm of the cell (Gillies et al. 2010). These ERs have different patterns of distribution in the brain, different affinity patterns for certain ligands (e.g., estradiol binds equally well to both ERs, whereas estriol and phytoestrogens have greater affinity for ER β), and a range of different actions (Kuiper et al., 1998; Paech et al., 1997; Shughrue et al., 1997). For example, in rodents, non-human primates, and humans, both ER α and ER β mRNA and/or protein have been identified in many different brain regions, including the hippocampus and frontal cortex (Shughrue, Lane et al. 1997; Laflamme, Nappi et al. 1998; Montague, Weickert et al. 2008). However, within the hippocampus, ER α is localized in proximity to GABAergic interneurons as shown by immunocytochemistry, and studies have demonstrated that estradiol regulates pyramidal cell function by indirect interactions with these GABA interneurons (Weiland, Orikasa et

al. 1997). In contrast, ER β is more commonly seen in direct proximity to hippocampal pyramidal neurons (Li, Schwartz et al. 1997). In non-human primates, both ER α and ER β are expressed in the hippocampus, although ER β appears in higher densities than ER α (Register, Shively et al. 1998). Similarly, in human studies, ER β appears to be the dominant ER within the human hippocampus, with higher densities of ER β identified in the subiculum and entorhinal cortex, whereas ER α has only rarely been observed in these brain areas (Osterlund, Gustafsson et al. 2000). In contrast to the hippocampus, ER α is the predominate receptor type in the PFC. Specifically, immunohistochemistry and *in situ* hybridization have revealed the presence of ER α mRNA/protein positive cells throughout all layers of the PFC, in both pyramidal and nonpyramidal neurons in rats, monkeys, and humans (Montague, Weickert et al. 2008).

1.1.2 Progesterone, progesterone receptors, and their distribution in the brain

Progesterone is the most abundant circulating ovarian steroid in women and is synthesized primarily in the ovaries. In rodents and humans, two structurally distinct isoforms of the classical nuclear progesterone receptor (PR) have been identified, PRA and PRB, the latter of which contains a 164 amino acid N-terminal extension, with different distributions and biological actions (Kastner et al. 1990; Gronemeyer et al. 1991; Chalbos et al., 1994). Progesterone exerts its effects as a ligand-activated nuclear transcription factor by binding to either PRA or PRB. PRA and PRB are both coded by a single gene, with translation being initiated from separate start codons (Conneely et al. 1987, Gronemeyer et al. 1991). PRs are broadly expressed throughout the rodent brain, and although PRA and PRB are co-expressed in different cell types in rodents (Gava et al.

2004), the majority of PR positive cells express both PRA and PRB in humans.

Nonetheless, PR expression varies across brain regions, and PRA as well as PRB are differentially localized in many brain regions in rodents, including the hippocampus and frontal cortex (Hagihara et al. 1992, Guerra-Araiza C. et al. 2000; 2003). Interestingly, some studies show that in the hippocampus, estradiol, and not progesterone, induces PRA isoform expression, suggesting that the balance between PRA and PRB can be altered by estradiol (Guerra-Araiza C. et al. 2003). Information on the localization of progesterone receptors in the human brain and on the specific roles of progesterone in human brain function remains limited.

1.2 Ovarian Steroids Affect Neural Structure and Function in Both Animals and Humans

1.2.1 Estradiol and progesterone affect cell morphology

It has become increasingly evident that dendritic spines are important mediators of neuronal plasticity, and many studies using both quantification of Golgi impregnated tissues and electron microscopy have demonstrated that gonadal steroids regulate the size and density of dendritic spines in the hippocampus and PFC in both rodents and non-human primates. In female rats and mice, the density of both apical and basal dendritic spines in hippocampal CA1 and PFC is reduced after ovariectomy (OVX) by as much as 50% compared to intact rats, and normal densities are restored after estrogen replacement (Gould et al. 1990; Woolley et al. 1990; Woolley and McEwen 1992; Wallace et al. 2006; Velázquez-Zamora et al. 2012). In adult normal cycling rats, the synaptic changes occur rapidly, within 24 hours between proestrous (high estradiol blood levels) and estrous (low

estradiol blood levels and high progesterone blood levels) stages of the estrous cycle, with an approximately 30% decrease in dendritic spine density observed in the estrous stage (Woolley et al. 1992; Smith et al. 2010). The increase in CA1 dendritic spines can also be seen in cultured rat hippocampal neurons (Murphy and Segal 1996). In addition to the increase in spine density, rats in the proestrous stage also have a higher proportion of the stronger, more mature mushroom shaped spines than during the estrous stage (Gonzalez-Burgos et al. 2005).

The administration of progesterone after estrogen treatment results in a biphasic pattern of effects consisting of an initial increase in spine density for 2-6 hours, followed by rapid decreases in dendritic spine density to densities observed post-OVX (Gould et al. 1990; Woolley et al. 1993, 2004). Administration of the progesterone receptor antagonist RU-486 prevents the sharp decrease in spine density. Thus, progesterone acting through PR mediates the decrease in level of dendritic spines.

Similar results have been obtained in non-human primates for CA1 (Hajszan and Leranth, 2010) and the PFC, where estradiol has been shown to increase both dendritic spines and spine synapse density (Tang et al. 2004; MacLusky et al. 2005; Hajszan et al., 2007a, Hajszan et al., 2007b and Khan et al. 2012). OVX results in a significant decrease in dendritic spine density in the dorsal lateral PFC (DLPFC), and the replacement of estradiol and progesterone reverses the decrease (Kritzer and Kohama 1999; Tinkler, Tobin et al. 2004). Similarly, OVX decreases and estrogen replacement increases dendritic spine densities in the CA1 region of the monkey hippocampus (Hao, Janssen et al. 2003). Taken together, these *in-vivo* and *in-vitro* results demonstrate that ovarian

steroids exert both acute and chronic changes in dendritic spine density and are necessary for the maintenance of normal hippocampal and PFC cell structure.

1.2.2 Estradiol and progesterone influence synaptic plasticity

The physiologic relevance of the observed changes in synaptic spine densities is suggested by electrophysiological studies, many of which found that estradiol and progesterone regulate synaptic plasticity in the hippocampus and PFC. Within the hippocampi of female rats, estradiol increases hippocampal excitability and the magnitude of long-term potentiation (LTP), the marker of synaptic plasticity and learning. In an early seminal study, localized seizure threshold in the hippocampus was significantly decreased between the proestrous and estrous stages, and estradiol treatment restored the dampened cyclicity observed in OVX rats (Terasawa et al. 1968). In addition, population spike magnitude significantly increases, and EPSP duration rises after systematic estradiol administration in OVX rats (Teyler, Vardaris et al. 1980; Wong and Moss 1992). *In-vitro* studies using hippocampal slices reveal that estradiol rapidly enhances basal synaptic transmission in hippocampal CA1 regions via a variety of different mechanisms. Through extracellular recordings, significant enhancement of LTP of both the slope and amplitude of N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) mediated excitatory postsynaptic potentials (EPSPs) is observed in the presence of estradiol (Teyler, Vardaris et al. 1980; Wong and Moss 1992; Gu et al. 1998; Foy et al. 1999). Estrogens also can influence synaptic plasticity through modulation of GABA inhibitory input from interneurons onto the pyramidal cells. However, the effects of estradiol on GABAergic function are

complex: by decreasing GABAergic inhibition, estradiol is able to increase excitability of pyramidal cells and, therefore, increase synaptic plasticity (Murphy, Cole et al. 1998); on the other hand, estradiol can increase GABA activity in CA1 by increasing GAD activity in OVX rats (Weiland et al. 1992). Timing may explain these seemingly inconsistent results as estradiol transiently suppresses GABA-mediated inhibition but then subsequently recovers CA1 pyramidal cells inhibition (via NMDA-mediated excitatory inputs) and GAD expression increase (Rudick et al. 2001). In sum, it is evident that estradiol enhances synaptic plasticity through modulating either AMPA- or GABA-mediated excitatory or inhibitory neural functions.

Fewer studies have examined the effects of progesterone on synaptic plasticity and results are mixed. Although some studies show no progesterone effects on LTP (Ito et al. 1999) or on the frequency of EPSPs (Feng et al. 2004), other studies demonstrate a progesterone-related increase in EPSPs and population spike amplitude in hippocampal slices in OVX rats (Edwards et al. 2000). In a recent study, supra-physiologic concentrations (i.e. micromolar) of progesterone decreased hippocampal CA1 baseline synaptic transmission and magnitude of LTP (Foy et al. 2008). The observed progesterone mediated decrease in synaptic plasticity can be explained in part by GABA activity, as progesterone acting through its neurosteroid metabolite allopregnanolone enhances GABA-related tonic inhibition and decreases synaptic plasticity (Brinton, Thompson et al. 2008). These inconsistent findings indicate that the effects of progesterone on synaptic functions are complex and warrant further clarification. Nonetheless, taken together, these results provide evidence for a biologically plausible mechanisms by which estradiol and progesterone influence the regulation of several brain

regions/neurocircuits (i.e., the PFC and hippocampus) involved in affective adaptation and cognitive control.

1.2.3 Behavioral effects of steroid hormones using animal models

Growing evidence indicates that the plasticity of synaptic connections regulated by ovarian hormones is critical in mediating hippocampal and prefrontal dependent cognitive functions including learning, memory and executive functions. Estradiol treatment of both young and old OVX rodents consistently improves learning and memory (Rissanen, Puolivali et al. 1999; Frye, Rhodes et al. 2005). In a delayed matching-to-position T-maze task, estrogen-treated rats acquired the task at a significantly faster rate than the OVX, non-estrogen-treated controls (Gibbs 1999). Hippocampal-dependent spatial working memory was assessed using several different paradigms, including the water-escape radial-arm maze (Bimonte and Denenberg 1999), traditional radial-arm maze (Williams 1996; Daniel et al. 1997; Luine et al. 1998; Fader et al. 1999), delayed matching-to-place version of the water maze (O'Neal et al. 1996; Sandstrom et al. 2004), and T-maze (Dohanich et al. 1994). Results across all of these experimental paradigms are consistent and showed that as working memory load increased, estrogen-treated OVX female rats made fewer errors than non-treated OVX rats. These findings indicate that estrogen replacement can enhance acquisition and reduce performance deficits in spatial memory tasks. In object recognition memory, a task that depends on both hippocampal and prefrontal cortical circuitries, OVX impairs performance (Wallace et al. 2006); and estradiol-treated OVX mice, both young and old, (Vaucher, Reymond et al. 2002), as well as estradiol-treated OVX rats (Luine et al. 2003)

showed significantly greater recall during the task than did non-treated OVX animals. Finally, in avoidance learning, OVX mice treated with estradiol showed faster learning in avoiding footshock in a T-maze than OVX mice who were not treated with estradiol, those with progesterone treatment only, and those with progesterone plus estradiol treatments (Farr, Flood et al. 1995). These findings are remarkably consistent and support estradiol's positive effects on hippocampal dependent cognitive functions and behaviors.

Ovarian steroids regulate several other aspects of behavior in rodents, particularly arousal and response to stress. For example, estradiol increases, whereas progesterone decreases arousal (Morgan and Pfaff 2001). Estradiol increases the HPA axis response to stress through ER α , whereas ER β agonists reduce HPA axis responsively and produce antidepressant-like and anxiolytic effects (Handa, Burgess et al. 1994; Rocha, Fleischer et al. 2005; Walf and Frye 2005). Finally, Shansky and colleagues demonstrated that estradiol amplifies stress-induced reduction in PFC-dependent working memory in rats (Shansky, Glavis-Bloom et al. 2004).

Although many studies suggest that estradiol administration improves performance on a myriad of cognitive functions, some have found the opposite (Frye et al. 1995; Markus et al. 1997; Warren et al. 1997), suggesting that estradiol's effect on behavior may be task- and strategy- specific. To test this idea, OVX female rats after estradiol or control treatments were trained in a plus-shaped maze to perform reference memory tasks engaging either place-learning or response-learning strategies, which have different cognitive requirements. Estradiol treatment significantly improved performance on the place task but not on the reference task, supporting the hypothesis that estradiol

influences cognitive functions in a task-and strategy-specific manner by modulating different aspects of the learning and memory systems (Korol et al. 2002).

Even though studies examining progesterone's influence on cognitive functions are less conclusive, rodent studies also have documented the effects of this ovarian steroid and its neurosteroid metabolite, allopregnanolone on several cognitive processes including spatial memory (i.e., Morris water maze or object placement task), with both progesterone related improvements and declines reported (Johansson, Birzniece et al. 2002; Bimonte-Nelson, Nelson et al. 2004; Frye, Duffy et al. 2007; Frye and Walf 2008). Similarly, effects of estradiol and progesterone on cognition and behavior are also observed in non-human primates. For example, in aged OVX monkeys, impairment in spatial working memory was reversed after administration of cyclic estradiol despite long-term estradiol deprivation (Lacreuse et al. 2002; Rapp, Morrison et al. 2003).

Finally, several contextual factors have been demonstrated to influence the observed effects of ovarian steroids on cognitive performance and behavior including the duration of hypogonadism (Gibbs 2000), the training status (naïve vs. post-training) of the animals (Gresack and Frick 2006), and the dose of steroid administered (i.e., physiologic versus supra-physiologic) (Rissanen, Puolivali et al. 1999; Imwalle, Bateman et al. 2006). Genotypic variations such as *BDNF* Val⁶⁶Met polymorphism (rs6265) also interact with ovarian steroids to affect cognitive and behavioral functions (Spencer et al. 2010; Bath et al. 2012). In summary, animal studies provide strong evidence that ovarian-steroid-related alterations in CNS function can impact behaviors mediated by PFC- and hippocampal-dependent processes. However, these studies also emphasize that important contextual factors can substantially modify the observed behavioral outcomes.

1.2.4 Influence of Ovarian Hormones on CNS Function in Women

1.2.4.1 Neurocircuitry

In women, brain imaging has been used to document the neuromodulatory effects of both estradiol and progesterone on hippocampal and PFC functions. PET and fMRI have been employed to examine the effects of ovarian steroids or the normal menstrual cycle on neural functions under conditions of brain activation. The first neuroimaging study investigating the effects of ovarian steroids on neurocircuitry was performed by Berman et al. using oxygen-15 water ($H_2^{15}O$) PET (Berman, Schmidt et al. 1997). They employed the Wisconsin Card Sort Test (WCST), a measure of executive function and cognitive set shifting, and observed that during conditions of GnRH agonist-induced ovarian suppression, the PFC response was attenuated. Both estradiol and progesterone replacement normalized cortical activity. Similarly, Shaywitz et al. (Shaywitz, Shaywitz et al. 1999) employed fMRI in women who were several years postmenopause, and they showed that estrogen therapy (but not placebo) significantly increased activation in the inferior parietal lobule (IPL) and right superior frontal gyrus during verbal encoding, with significant decreases in the IPL during non-verbal coding. Shaywitz et al.'s findings are supported by a recent report by Craig et al. (Craig, Fletcher et al. 2007), who observed significantly decreased activation in the left PFC, right precentral gyrus, anterior cingulate, and medial frontal gyrus during verbal encoding in a group of women with GnRH agonist-induced "menopause" for the treatment of uterine fibroid tumors.

fMRI studies also have documented menstrual cycle phase-related changes in the function of several brain regions involved in the neurocircuitry of arousal, stress response and reward processing including the amygdala, orbitofrontal cortex, and striatum

(Goldstein, Jerram et al. 2005; Protopopescu, Pan et al. 2005; Dreher, Schmidt et al. 2007). Thus, although the brain regions potentially regulated by estradiol and/or progesterone remain to be fully characterized, the physiological response of the frontal cortex, amygdala and hippocampus, areas subserving executive function, memory, and the regulation of affect, appear to be regulated by ovarian steroids in women.

1.2.4.2 Cognitive Performance

As described above, our understanding of the role of ovarian steroids in cognition has been informed by both *in vitro* and *in vivo* animal studies. Preclinical observations include the following: sexual dimorphisms in performance of cognitive tasks, presumed consequent to differences in gonadal steroid levels or exposure (Frye et al. 2005); changes in cognitive measures across the estrus cycle or following gonadal steroid manipulations (Bimonte-Nelson, Singleton et al. 2004); reversal by gonadal steroids of induced cognitive deficits (Rapp, Morrison et al. 2003); regulation by gonadal steroids of brain regions critical for cognition (Woolley and McEwen 1993); and protection by gonadal steroids of neurons *in vitro* against a variety of insults (Shughrue and Merchenthaler 2003).

Several studies in humans complement these preclinical findings. First, sexual dimorphisms in cognitive abilities such as declarative memory and visuospatial cognition are well documented (Maitland, et al. 2004; Rizk-Jackson, et al. 2006). Second, performance on a number of cognitive measures, including motor dexterity, verbal fluency, and visuospatial ability, varies across the menstrual cycle and correlates with levels of ovarian steroids (Silverman et al. 1993; Hampson 1995). Third, cognitive changes (e.g., in episodic memory, executive function) are observed after manipulations

of ovarian steroids (LeBlanc, Janowsky et al. 2001; Maki, Zonderman et al. 2001; Sherwin 2003), although recent studies in young healthy women showed that short-term changes in ovarian steroids have limited effects on cognitive operations (Owen et al. 2002; Schmidt et al. 2013). Fourth, although estrogen replacement therapy (ERT) has been shown in numerous studies to protect against age- and estrogen deprivation related cognitive decline as well as abnormality in the neurocircuitry underlying these cognitive functions (Resnick et al. 1998; Maki et al. 2000; Hall et al. 2006; Kask et al. 2008), several randomized clinical trials have demonstrated that ERT has no, or even negative, effects on cognitive performance in postmenopausal women (Mulnard et al. 2000; Hogervorst et al. 2002; Rapp et al. 2003; Espeland et al. 2004; Low and Anstey 2006; Yaffe et al. 2006; Lethaby et al. 2008). In fact, the Women's Health Initiative study, a 15-year large scale study involving more than 16,000 postmenopausal women, found that ERT increased the risk for cognitive disorders (Shumaker et al. 2003; 2004). These findings are consistent with several studies showing declining cognitive functions after ERT even in younger perimenopausal women (Gold et al. 2000; Mitchell et al. 2001; Maki et al. 2007). On the other hand, observational studies have consistently shown beneficial effects of estradiol treatment on cognitive functions in perimenopausal women (Yaffe et al. 1998; Bagger et al. 2005; Henderson et al. 2005; MacLennan et al. 2006; Whitmer et al. 2011), suggesting that estradiol's effects in the CNS are intricate, and possibly age-dependent, and may be influenced by contextual factors such as genotypic variation.

In conclusion, although the effects of ovarian hormones on cognitive behaviors are neither uniform nor consistent, in some women the effects of ovarian steroids on the PFC

and hippocampal neural network could mediate the observed changes in cognitive functions across the menstrual cycle or after estrogen therapy. Although both estradiol and progesterone exert important neuroregulatory effects that could have physiologic relevance, the mechanisms underlying the effects of ovarian steroids on brain remain to be clarified. A number of the described effects of ovarian steroids are also observed with changes in BDNF, a member of the neurotrophin family, and its tyrosine kinase receptor (TrkB). Thus, it is possible that alteration in BDNF system function could be an important modulator of the effect of ovarian steroids on brain functions.

1.3 BDNF Affects Neural Functioning in both Animals and Humans

1.3.1 BDNF, BDNF receptors, and their distribution in the CNS

BDNF is the most abundant and broadly distributed neurotrophin in the CNS, playing a key role in neuronal survival and in promoting neuromodulatory and neuroprotective effects. The gene that codes for BDNF has a complex structure with multiple upstream promoters that are individually regulated by diverse stimuli that can alter the gene's expression, including cellular signaling molecules, neurotransmitters, and ovarian hormones. For instance, there is evidence that glutamate receptor agonists induce, whereas GABAA receptor agonists inhibit, the expression of BDNF (Marty, Berzaghi et al. 1997). *BDNF* expression can also be induced/suppressed on an activity-dependent basis, indicating that *BDNF* can act as an immediate-early gene. One of the promoter regions of *BDNF* contains an ERE, and, thus, can be regulated by estradiol (Sohrabji, Miranda et al. 1995), suggesting a close relationship between the physiological functions of BDNF and estradiol. The human BDNF gene spans over 70kb and contains at least

eleven exons and nine functional promoters, in which BDNF transcripts containing exons II, III, IV, V, and VII are mostly brain-specific (Pruunsild et al. 2007). In humans, similar to many studies in rodents (Conner 1997), mRNA expression analysis using reverse transcriptase-polymerase chain reaction (RT-PCR) show that all *BDNF* transcripts are expressed at high levels in many brain regions, including regions where estradiol and progesterone receptors also show high expressions, such as the hippocampus and frontal cortex (Pruunsild et al. 2007). Within the cell, BDNF is present in the mossy fiber system of dentate gyrus granule cells, in excitatory pyramidal neurons in CA1 and CA3 of the hippocampus, and in GABAergic inhibitory interneurons of both PFC and hippocampus (Ernfors et al. 1990; Conner 1997).

The BDNF protein is synthesized as a glycosylated precursor (prepro-BDNF), further processed into pro-BDNF, and then can be converted into mature BDNF either intracellularly or extracellularly (Lu et al., 2005; Matsumoto et al., 2008). BDNF released from cells exerts its effects via binding to the high affinity TrkB or the low affinity p75^{NTR} receptor (Huang et al. 2001; Matsumoto et al., 2008). The binding of BDNF to the extracellular domain of TrkB triggers dimerization and autophosphorylation of the receptor, which induces activation of several intracellular signal transduction pathways, MAPK/ERK, PI3K, CREB and PLC γ pathways (Huang et al. 2001). TrkB is co-localized with BDNF protein in the hippocampus and PFC (Marty, Carroll et al. 1996). Additionally, it has been demonstrated that neurons expressing BDNF mRNA also express TrkB mRNA (Kokaia, Bengzon et al. 1993). Thus, BDNF, similar to estradiol, targets dendrites of pyramidal and GABAergic neurons and exerts both direct and indirect regulation on pyramidal cells through its receptor (Murer, Yan et al. 2001).

Taken together, as discussed below, these data suggest that BDNF and estradiol act through their respective receptors to exert a common influence on PFC and hippocampus.

1.3.2 BDNF affects cell morphology

Many experiments demonstrate that BDNF is essential for the maintenance of dendritic spines in the adult CNS. This neurotrophin induces the production of thin and mushroom-shaped (mature) spines in hippocampal CA1 slice cultures (Tyler et al. 2003), and significant reductions in spine density in cortex and hippocampus are observed in both early- and late-onset BDNF knockout mice, suggesting that BDNF signaling is required for preserving brain circuitry (Vigers et al. 2012). In cerebellar cultures, BDNF increases the spine density of Purkinje cells without affecting dendritic branching complexity. BDNF-TrkB coupled signaling is necessary for the trophic effects on dendritic spine and synapse density in cortical and hippocampal neurons (Tyler et al. 2001; Cohen-Cory et al. 2010). However, a study investigating how postsynaptic TrkB signaling affects cell morphology in the cortex and hippocampus found that, while a reduction in mushroom spine maintenance and synaptic efficacy is seen in adult visual cortical neurons expressing a truncated TrkB receptor, hippocampal CA1 mushroom spine maintenance is unaffected, suggesting that TrkB may play fundamentally different roles in structural plasticity in different brain areas (Chakravarthy et al., 2006). In summary, despite some regional differences in observations on TrkB-induced spine development as well as mixed findings in BDNF's effects on dendritic morphology, it is likely that BDNF-TrkB signaling can facilitate spine morphogenesis which is important for synaptic plasticity and long-term synaptic potentiation.

1.3.3 *BDNF affects synaptic plasticity*

In rodents BDNF and its receptor have been experimentally manipulated to demonstrate the role of BDNF in synaptic plasticity in the hippocampus. Studies have shown that *BDNF* knockout mice have greatly reduced LTP (an indicator of synaptic plasticity), and this effect can be reversed by application of BDNF to hippocampal slices (Patterson, Abel et al. 1996). Similar inhibition of LTP function can be seen following acute application of the BDNF scavenger TrkB-IgG to hippocampal slices (Chen, Kolbeck et al. 1999). Thus, BDNF acting through the TrkB receptor is critical for LTP-dependent synaptic plasticity. In addition, Patterson et al. showed that electrical stimulation capable of inducing LTP also induces *BDNF* expression in the hippocampal formation (Patterson, Grover et al. 1992), and BDNF facilitates LTP in rodent hippocampus by increasing the number of docked (i.e., active) vesicles, but not that of reserved (i.e., inactive) vesicles in CA1 excitatory synapses (Tartaglia, Du et al. 2001). Collectively, these data demonstrate that hippocampal-dependent cognitive functions dependent on augmentation of synaptic plasticity require the presence of BDNF and TrkB activation.

These results demonstrate the importance of BDNF protein and BDNF-TrkB signaling in regulating synaptic plasticity and neuronal survival, migration, differentiation, and dendritic growth (Huang and Reichardt 2001). Together with observations of genotype-related differences between human *BDNF* Val and Met carriers (Egan, Kojima et al. 2003), these data provide a foundation for considering BDNF's involvement in, and the effects of the *BDNF* Val⁶⁶Met polymorphism (rs6265) on hippocampal- and PFC-dependent behavioral functions in humans.

1.3.4 *BDNF's influence on cognitive performance in lower animals*

Based on the molecular evidence, genetic manipulation of BDNF secretion would be expected to lead to deficits in hippocampal-dependent functions such as memory and spatial navigation. In rodents, the Morris water maze task is used to examine spatial memory. Impairment in maze performance is seen in *BDNF* mutant mice (Linnarsson, Bjorklund et al. 1997), mice with hippocampal-specific *BDNF* deletion (Heldt, Stanek et al. 2007), and rats with intra-cerebroventricular infusion of anti-BDNF antibody resulting in depletion of endogenous BDNF (Mu, Li et al. 1999). These data indicate the importance of BDNF in modulating spatial memory. In addition, inhibition of BDNF by continuous intra-cerebroventricular infusion of antisense BDNF oligonucleotide, which acutely impairs BDNF function, results in impairment of both reference and working memory in rats (Mizuno, Yamada et al. 2000). Similarly, mice with forebrain-specific TrkB deletion, specifically adults, also show spatial learning deficits, indicating the importance of TrkB signaling in memory (Minichiello, Korte et al. 1999). Collectively, these data demonstrate that both BDNF and TrkB are necessary in the maintenance of spatial memory. In the absence of a direct *in vivo* measure of BDNF in human brain, the functionally-relevant frequent Val⁶⁶Met polymorphism (rs6265) offers a window into how BDNF influences human cognition.

1.3.5 *The human BDNF Val⁶⁶Met polymorphism*

A SNP in the 5'pro-region of human BDNF in which a valine (Val) is substituted by a methionine (Met) at codon 66 is observed in the population with a frequency ranging from 19% to 25% in Caucasian samples (Pezawas, Verchinski et al. 2004). Cultured

hippocampal neurons transfected with human Met-BDNF-green fluorescent protein (GFP) showed lower depolarization-induced secretion, while constitutive secretion was unchanged. Furthermore, intra-cellular trafficking was attenuated; Met-*BDNF*-GFP failed to localize in secretory granules at synapses (Egan, Kojima et al. 2003). Thus, carrying the Met variant of the *BDNF* SNP could result in neuronal dysfunction via impaired activity-dependent BDNF secretion.

This Met allele-related impairment has also been observed with brain imaging studies in humans. With magnetic resonance spectroscopy imaging, n-acetyl aspartate (NAA) level, an indirect measure of neuronal integrity and synaptic abundance, has been shown to be reduced in Met carriers (Egan, Kojima et al. 2003). The Val⁶⁶Met substitution also affects human cortical morphology. Significant age- and gender-independent reductions in hippocampal volume (Pezawas, Verchinski et al. 2004; Bueller, Aftab et al. 2006) as well as in PFC volume (Pezawas, Verchinski et al. 2004) are associated with the Met allele. These results may provide a context for behavioral and neurofunctional findings related to the Val⁶⁶Met SNP.

Behaviorally, some studies have found that *BDNF* Met carriers perform worse in verbal episodic memory (Egan, Kojima et al. 2003) and in declarative retrieval of novel scenes (Hariri, Goldberg et al. 2003) compared to the Val homozygotes, and fMRI revealed that memory-related hippocampal activity was significantly lower for Met carriers during both encoding and retrieval (Hariri, Goldberg et al. 2003). Egan and colleagues also investigated the effects of the Val⁶⁶Met polymorphism on working memory using an N-back working memory task. The neural circuit underlying performance of this task involves an inverse relationship between the DLPFC and the

hippocampus: during higher working memory load, activity in the DLPFC increases whereas the hippocampus normally shows disengagement and decreases in activity. fMRI results indicated that Met carriers showed a disrupted normal hippocampal disengagement pattern. Specifically, inappropriate over-activation of bilateral hippocampus, instead of the normal decrease in activity, was found in Met carriers (Egan, Kojima et al. 2003). These results provide converging evidence for BDNF's effect on the PFC and hippocampus and on the cognitive processes that depend upon these regions.

1.4 Interaction between ovarian steroid hormones and BDNF

As discussed in the previous sections, the actions of ovarian steroid hormones and BDNF on the CNS are highly congruent. Both *in vivo* and *in vitro* studies document the manifold overlap between ovarian steroids and the BDNF system on molecular, cellular and systems-levels function, and interactions between ovarian steroids and BDNF could provide a mechanism for many of the observed effects of ovarian steroids on brain and behavior in women.

1.4.1 Cellular and molecular mechanisms subserving hormone-BDNF interactions

Table 1 depicts the similarities between the effects of ovarian steroid hormones, particularly those of estrogens, and BDNF on PFC and hippocampal dependent processes. There are two primary mechanisms that could explain these interactions between estrogens and BDNF. First, both estradiol and BDNF activate similar signal transduction pathways within the cell through ERs and trkB, respectively (Table2, Figure 1) (Scharfman and MacLusky 2006). It is possible, therefore, that by activating a common

system of second messengers, estrogens and BDNF can amplify transcriptional activity and thereby influence neural growth, survival, and plasticity in the hippocampus and PFC.

Hippocampal and PFC actions	↑ E ₂ (Ovx & intact females)	↑ BDNF (intact and transgenics)	↑ TrkB (transgenic)
↑ LTP	○ ○ ○ ○ ○	○ ○ ○ ○ ○	○
Memory facilitation	○ ○ ○ ○ ○	○ ○ ○ ○ ○	○
↑ Dendritic spines	○ ○ ○ ○ ○	○ ○ ○ ○ ○	○
Adult neurogenesis	○ ○ ○	○ ○ ○	_____
↑ ↓	○	○ ○	_____
Neuroprotection	○ ○ ○ ○ ○	○ ○ ○ ○ ○	○
↑ ↓	○	○ ○	○

Table 1 Similarities in effects of estradiol (E₂) and BDNF on hippocampal functions. Number of circles indicates strength of evidence for effects in specific neuronal processes (Scharfman and MacLusky 2006).

Effector/Target activated by estrogen and BDNF
MAP/ERK
PI ₃ kinase/Akt
CaMKII
CREB
Src/Fyn

Table 2 Signaling cascades and pathways commonly activated by both estrogen and BDNF (Scharfman and MacLusky 2006).

Alternatively, the effects of estradiol can be mediated directly through estradiol' ability to regulate the activity of BDNF (Figure 2). Thus BDNF can mediate several of the downstream effects of estradiol on neural function.

The BDNF gene contains a functional ERE and estrogen-ligand complexes that are capable of binding this sequence thus inducing BDNF expression (Sohrabji, Miranda et al. 1995). Several studies support this mechanism since

BDNF expression is reduced in gonadectomized female and male rodents, whereas administration of estradiol reverses the reduction in BDNF expression in the hippocampus (Table 3) (Sohrabji and Lewis 2006).

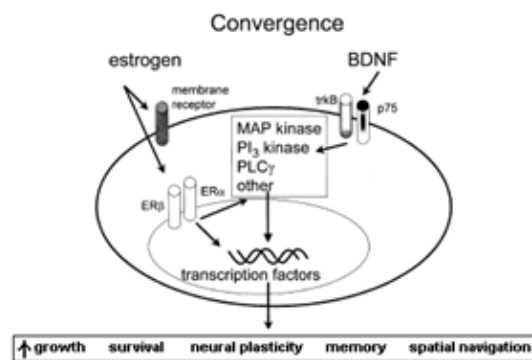


Figure 1 Putative mechanism of converging transcriptional effects of estrogen and BDNF (Scharfman and MacLusky 2006).

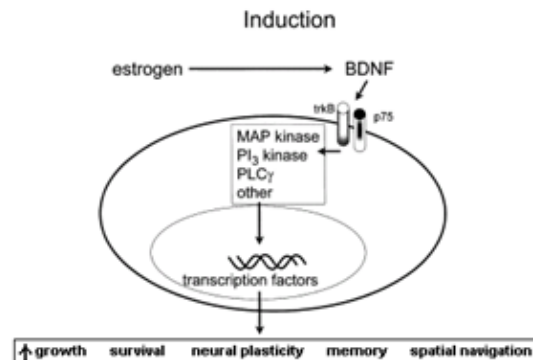


Figure 2 Putative mechanism of estrogen mediated BDNF induced transcription. (Scharfman and MacLusky 2006).

Estrogen modulation of BDNF		
Animal model	Effect on BDNF expression	Brain region
Estrogen-replaced, OVX young adult female rat	↑ BDNF mRNA	CA3, CA4, dentate gyrus
Estrogen-replaced, OVX young adult female rat	No effect on BDNF	Frontal, temporal and parietal cortex
Estrogen-replaced, OVX young adult female rat	↑ BDNF mRNA	Dentate gyrus, CA1, CA3, CA4
Estrogen-replaced, gonadectomized male mice	↑ BDNF mRNA	Pyriform cortex, hippocampus
Estrogen-replaced, gonadectomized male mice	↑ BDNF mRNA and protein	Hippocampus
Estrogen-replaced, OVX young adult female rat	↑ BDNF mRNA	Hippocampus, cortex, spinal cord
OVX young adult female rat	↑ BDNF mRNA and protein	CA1, CA3, medial and basomedial amygdala

Table 3 Evidence of estrogen modulating BDNF using rodent models. Hormonal status influences the expression of BDNF mRNA and/or protein expression in both hippocampus and PFC (Singh et al. 1995; Gibbs 1998; Gibbs 1999; Zhou, et al. 2005).

Although the majority of preclinical research suggests that estrogens augment BDNF actions, studies have also identified several factors that can modulate the direction of the effect of estrogens on the neurotrophin system. For example, the ability of estrogens to stimulate the receptor for BDNF is developmental stage specific – estrogens increase BDNF and TrkB during brain development and have no effect during adulthood, but will increase BDNF activity in the adult in the context of CNS injury (Singh, Setalo et al. 1999). Additionally, the effect of estradiol on BDNF expression can be brain-region specific. Some studies demonstrate that estradiol increases *BDNF* expression in hippocampal CA1/CA3 but not the dentate gyrus (Zhou, Zhang et al. 2005), whereas

other studies report that estrogen increases *BDNF* expression in the dentate gyrus only (Scharfman, Mercurio et al. 2003). Moreover, Singh and colleagues demonstrate that estradiol increases *BDNF* mRNA expression in the cerebral cortex of OVX rats (Singh, Meyer et al. 1995). Finally, as described earlier, the effects of the simultaneous administration of both estrogens and progesterone may impact estrogens' effect on *BDNF*. When estrogen treatment was combined with progesterone, *BDNF* expression in the entorhinal cortex decreased (Bimonte-Nelson et al. 2004). Thus, while studies have consistently shown that estradiol regulates the effects of *BDNF* and its receptor, these effects exhibit development-stage and brain-region specific differences, and the effects of estradiol on *BDNF* system function can be modulated by the coexistence of progesterone, similar to estradiol and progesterone's interaction and modulation on synaptic plasticity.

1.4.2 Ovarian hormone-BDNF interaction affects animal behavior

Recent studies in genetically engineered female mice demonstrate that functional variation of the *BDNF* gene influences cellular function and manifests in a differential behavioral responses in anxiety and cognitive function across the estrus cycle compared with wild-type female mice. Specifically, compared to wild-type Val female mice, females with the human Met allele exhibited impaired hippocampal dependent memory. In addition, a significant estrous cycle stage and *BDNF* genotype interaction revealed that memory performance was best in proestrous (high estradiol level) in Met mice, indicating that the effect of the Met allele on object memory is dependent on estrous cycle stage (Spencer et al. 2010). In another study, anxiety-related behavior was elevated in Met knock-in female mice during estrous relative to the metestrous phase, whereas anxiety-

like behavior is not affected by estrous status for wild-type Val mice (Bath et al. 2012). Collectively, these results suggest that the Met allele of the *BDNF* gene introduces an ovarian steroid-dependent behavioral sensitivity in mice.

To date, no studies in humans have been performed to examine the interaction between ovarian steroids and the BDNF system, despite the fact the presence of the Val⁶⁶Met polymorphism offers an opportunity for such investigation. This thesis investigates gonadal steroid hormone-BDNF interaction in the living human brain. First, I tested for a sexual dimorphism in the effects of *BDNF* allelic variation in basal resting state rCBF. I hypothesized that *BDNF* and sex interactively affect resting rCBF in key regions including the hippocampus and PFC, and that, at the circuit level, functional connectivity between these regions and other brain regions is modulated by both *BDNF* Val⁶⁶Met polymorphism and sex. Second, I used a hormone manipulation paradigm analogous to those employed in animal studies to examine the effects of the presence or absence of ovarian steroids and the effects of estradiol and progesterone separately, since both potentially affect BDNF function. I hypothesized that an ovarian hormones-by-*BDNF* interaction would be observed in regions subserving the working memory network specifically the hippocampus and PFC. In addition to elucidating the effects of ovarian steroids on the BDNF system for the first time in humans, the results of these studies may identify a substrate of sensitivity to the CNS effects of ovarian steroids, a phenomenon that may clarify the diversity of effects of ovarian steroids on brain functions in women, and may offer a framework within which to investigate the neurobiological substrate of sexually dimorphic neuropsychiatric disorders.

Chapter 2

GENERAL METHODS FOR STUDIES 1 AND 2

2.1 Subject Selection

Healthy volunteers were recruited through local advertisements or self-referred through the National Institutes of Health (NIH) Normal Volunteer Office. Participants were free of significant medical illness (currently and in the past two years), were taking no medications, and had normal physical exams and laboratory results. Specifically, complete blood counts, blood chemistry, and thyroid function tests (thyroid-stimulating hormone) were all within normal limits. The absence of current or past psychiatric illness was confirmed by the Structured Clinical Interview for DSM-IV. Protocols were reviewed and approved by the National Institutes of Health Combined Neuroscience Institutional Review Board, and oral and written informed consent was obtained from all subjects. All participants were paid for their participation according to the guidelines of the NIH Normal Volunteer Office.

We restricted subject selection to Caucasians of European ancestry (Study 1) or covaried to control for race (Study 2) because *BDNF* allele frequencies vary across different populations. Notably, Met allele frequency is increased from 0.55 to 19.9 and 43.6% in Sub-Saharan Africans, Europeans, and Asians, respectively (Petryshen et al. 2010).

2.2 *BDNF* Genotyping

Blood was collected from all subjects and DNA was extracted with standard methods. Genotyping and allelic discrimination were determined for the *BDNF* Val⁶⁶Met (rs6265) SNP by TaqMan 5' exonuclease assay using a custom-designed assay (Applied Biosystems, Foster City, CA). To test for occult genetic stratification, participants were also genotyped for a common functional polymorphism in *COMT* Val¹⁵⁸Met (rs4680), and no significant variation in allele frequency was found in the study populations. As is commonly done in studies of genotype-phenotype associations with the *BDNF* Val⁶⁶Met SNP, *BDNF* Val/Met heterozygotes and Met/Met homozygotes were combined into a “Met carrier” group because of the rarity of Met homozygotes (<5% in Caucasian samples [Shimizu et al. 2004]).

2.3 Positron Emission Tomography (PET)

rCBF, a measure of general neural activity that is closely coupled to cerebral metabolic rate (Fox PT et al. 1988), was determined with the oxygen-15 water (H₂¹⁵O) PET technique (Herscovitch P et al. 1983). PET is a nuclear imaging technique that produces three-dimensional images of the distribution and quantity of a radioactive tracer in the brain, . To create PET images, a radiopharmaceutical containing a cyclotron-produced positron-emitting radionuclide is injected into a peripheral vein. The radiopharmaceutical decays by the emission of positrons, antiparticles of electrons with opposite charge. The positrons are emitted from the radiopharmaceutical because it has too few neutrons to stabilize the positrons in the nucleus. When the positron encounters

an electron, they annihilate one another and give rise to two annihilation photons traveling at the speed of light approximately 180 degrees to each other, each with an energy of 511 keV. The photon pairs are detected when they reach the scintillation detectors in the PET scanner, and coincidence detection is used to localize the annihilation events in the brain. An annihilation event is recorded only when two photons simultaneously reach the detectors. The annihilation event is assumed to have occurred somewhere on a line between the two detectors that recorded the event, and an activity map is constructed from many such intersecting lines. Several cyclotron-produced radionuclides, including oxygen-15, nitrogen-13, carbon-11, and fluorine-18, are used to investigate *in vivo* metabolism and biologically relevant physiological functions in the brain. These short-lived radionuclides are isotopes of atoms that constitute common organic molecules, with fluorine typically substituting for hydrogen, and these positron-emitters can, therefore be incorporated into substances included in metabolic processes. The half-lives of the radioisotopes are different, with oxygen-15 having the shortest half-life ($^{15}\text{O} = 2.05$ minutes; $^{13}\text{N} = 9.96$ minutes; $^{11}\text{C} = 20.34$ minutes; $^{18}\text{F} = 110$ minutes). Because of the particularly short half-life of oxygen-15, the H_2^{15}O method for measuring rCBF is useful for studying cognitive functions because it permits rapid, repeated testing in a single subject (scans are collected every six minutes).

The PET technique has several advantages for the present studies. First, the oxygen-15 water PET technique is a gold standard method that has not changed at the NIH or at other imaging centers for well over a decade; this methodological consistency is in contrast to fMRI, where changes in important technical features such as field strength, magnet, and pulse sequence are quite frequent. This technical stability is crucial for

studies requiring a large sample size, such as for the genotype-by-sex analysis in Study 1, or for those that are clinically difficult and span significant periods of time, such as the Lupron-based, six-month hormone manipulation protocol in Study 2. While clearly an optimal approach for controlling hormonal state (as discussed in Section 3.3.2), this protocol is clinically demanding for our participants, and, as a result, this study has required a number of years to complete.

Second, unlike fMRI signals, which are highly susceptible to magnetic field inhomogeneity and movement, PET data are not affected by air-fluid-tissue interfaces. Therefore, PET has no signal dropout in regions such as the hippocampus and PFC, which are particularly important for the studies in this thesis. Finally, unlike fMRI, which typically depends on detecting changes in signal between different task conditions performed in the same imaging run, the PET method maps rCBF during each cognitive condition separately with entirely independent scans. This capability is particularly important in both Study 1, which focuses on the basal resting state alone, and Study 2, an investigation of pharmacologically controlled hormonal changes on task-related activation, which could reflect rCBF alterations during the task of interest, the sensorimotor control task, or both, thus complicating interpretation of the data. In addition to allowing assignment of neural changes to specific task-related neural circuits, analyses of the control task alone can uncover or rule-out non-specific changes such as those related to vascular function *per se*, which may be particularly relevant in studies of hormone manipulation.

2.3.1 PET data acquisition

Subjects were instructed to refrain from alcohol, nicotine, and caffeine for four hours prior to the scan, and were told not to ingest over-the-counter medications that could affect rCBF for the preceding 24 hours. PET data were collected with a GE Advance (Milwaukee) three-dimensional PET scanner (septa retracted, 4.25 mm slice separation, 35 slices, axial field of view 15.3 cm). A thermoplastic mask was individually molded to each participant's face and attached to the scanner bed to limit head movement. Each scan session began with an eight-minute transmission scan to directly measure the attenuation of counts by the skull, scalp, and intracranial tissues. For this procedure, a 511 kilo-electron volt photon-emitting source of $^{68}\text{Ga}/^{68}\text{Ge}$ is placed in the tomograph and rotated around the subject's head. The data from the transmission scan were used to correct the emission scan data collected during the cognitive activation tasks or resting condition. During a single PET session, several 60-second emission scans, six minutes apart, were collected. The beginning of each emission scan followed the collection of 60 seconds of background counts and the subsequent injection by IV bolus of 10 mCi of H_2^{15}O . Data acquisition began when a steep rise in the count rate of 511 keV photons indicated the arrival of the radioactive tracer in the head.

2.3.2 PET data preprocessing

Emission scans were corrected for background and attenuation, and were reconstructed into 32 axial planes (6.5 mm full width at half maximum [FWHM]). The reconstructed PET data were preprocessed and analyzed with SPM5 (Wellcome Department of Cognitive Neurology, <http://www.fil.ion.ucl.ac.uk/spm/>). Images were

co-registered, corrected for background activity, anatomically normalized to an average template, scaled proportionally to remove variations in counts and global blood flow, and smoothed using a 10mm Gaussian kernel.

Chapter 3

SPECIFIC METHODS AND RESULTS FOR STUDIES 1&2

3.1 Specific Methods for Study 1: *BDNF* Val⁶⁶Met Polymorphism affects Resting rCBF and Functional Connectivity Differentially in Women versus Men (Wei et al., Journal of Neuroscience, 2012)

3.1.1 *Subject information*

Ninety-four healthy, right-handed Caucasians, aged 18 to 50, were recruited for this study. We matched genotype groups for age, handedness, and sex ratio and we excluded participants with a history of psychiatric illness because all of these factors have been shown to influence *BDNF*'s effects on cognition, behavior, and brain function. Each of the 94 participants underwent two resting PET scans and was genotyped for the *BDNF* Val⁶⁶Met functional polymorphism. There were 66 Val homozygotes (mean age=31.1±[S.D.]8.5) consisting of 33 males and 33 females (mean age = 30.1±8.1 and 30.0±8.8, respectively), as well as 28 Met carriers (mean age = 32.1±9.1), consisting of 14 males and 14 females (mean age = 31.1±8.6 and 33.0±9.8). *BDNF* genotype frequencies were in Hardy-Weinberg equilibrium ($X^2=0.09$).

3.1.2 *PET data acquisition during rest*

Participants were instructed to lie still in the scanner and rest with their eyes closed. An intravenous bolus of 10 mCi of oxygen-15 water was administered prior to each of the two 60-second scans performed six minutes apart.

3.1.3 PET data analyses

After preprocessing, the two resting rCBF scans of each subject were averaged to increase signal and entered into voxel-based group-level analyses. These analyses were designed to test the hypotheses that both *BDNF* genotype and sex affect resting rCBF and functional connectivity, and that their actions are interactive. Because both *BDNF* and ovarian steroid hormones exert strong influences on hippocampal/parahippocampal regions and the PFC, these brain areas were chosen as *á priori* regions of interest in addition to performing voxel-wise whole brain analyses.

3.1.3.1 rCBF analyses

To test for rCBF differences between genotype groups (Figure 3 and Table 4) and for main effects of sex (Figure 4 and Table 5) across the entire brain, a voxel-wise random effects analysis was performed. In light of our *á priori*, region-specific hypotheses, small volume correction (SVC) for family-wise error (FWE) was additionally applied to results within the hippocampal/parahippocampal complex and the PFC; anatomical regions of interest were created with the Wake Forest Pick-Atlas tool in SPM for left and right hippocampi, parahippocampal gyri, and PFC (composed of Brodmann area [BA] 9, BA10, BA11, BA25, and BA46, combined). To test for male-female differences in the effect of the Val⁶⁶Met polymorphism on resting rCBF, a voxelwise sex-by-genotype interaction analysis was performed (Figure 5 and Table 6).

3.1.3.2 Functional connectivity analyses

To test for between-genotype differences in functional connectivity, rCBF was extracted from spheres with 5mm radii drawn around seed voxels within regions of interest hypothesized *á priori* (hippocampus/parahippocampal gyrus and PFC) that best distinguished the Val homozygotes from the Met carriers as delineated by the between-genotype analysis (shown in Table 4). First, to determine a search area for the between-genotype and the sex-by-genotype correlational analyses, averaged rCBF from these spheres was entered into a series of voxel-wise regression analyses that were run on the entire cohort, regardless of sex or genotype. Next, the averaged rCBF values for each of these spheres were entered into voxel-wise regression analyses for each genotype group separately to determine group-specific correlational patterns within the search area. Finally, between-genotype analyses were carried out on the resulting correlational maps for each seed region (Table 7 and Figure 6).

To test for sex-by-genotype effects on functional connectivity, we used the same search areas as defined above: connectivity maps for the hippocampus/parahippocampal gyrus and PFC seed regions that best distinguished rCBF in the Val homozygotes from rCBF in the Met carriers for the entire group, regardless of genotype or sex. Within these whole-group connectivity maps, sex-by-genotype interaction analyses were carried out on the correlational data (Table 8 and Figure 7).

Results significant at the $p < 0.001$ level with cluster size greater than 10 voxels are reported. Peak voxel p-values are given, with FWE correction for multiple comparisons indicated when SVC was applied.

3.2 Results for Study 1

3.2.1 *BDNF* genotype and rCBF

Whole-brain voxel-wise analysis examining the main effect of genotype revealed no regions in which rCBF of Val homozygotes exceeded that of Met carriers. In contrast, compared to Val homozygotes, Met carriers demonstrated increased resting rCBF in regions hypothesized *á priori* (Figure 3), specifically in ventromedial PFC (BA25 extending into BA10; $p=0.00006$, uncorrected; $p=0.05$, SVC for FWE), left parahippocampal gyrus ($p=0.0003$, uncorrected; $p=0.05$, SVC for FWE), and right hippocampus ($p=0.0007$, uncorrected). Met carriers also showed greater rCBF in left insula and right lateral temporal regions not hypothesized. Details of these findings are reported in Table 4.

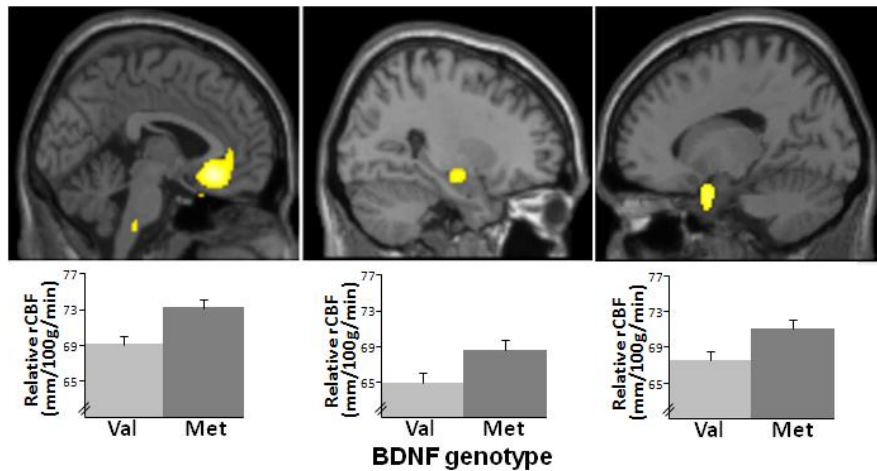


Figure 3. Areas of significant between-genotype group differences. Met carriers showed greater rCBF values than Val homozygotes. Sagittal views (top) and rCBF values (bottom) for (a) BA25, $p=0.00006$, $p<0.05$, SVC for FWE; (b) right hippocampus, $p=0.001$, uncorrected; and (c) left parahippocampal gyrus, $p=0.0003$, $p<0.05$, SVC for FWE. Error bars are ± 1 standard error of the mean (SEM). There were no regions where rCBF in Val homozygotes was greater.

Brain Region	MNI coordinates			cluster size	T values	P values
	x	y	z			
L subgenual cingulate (BA25)	-4	28	-12	176	4.04	0.00006*
L insula	-42	-10	-2	168	3.97	0.00007
R inferior temporal gyrus	50	-60	-2	43	3.71	0.0002
L parahippocampal gyrus	-14	6	-28	41	3.58	0.0003*
R middle temporal gyrus	60	4	-18	60	3.53	0.0003
R hippocampus	32	-10	-12	13	3.30	0.0007

Table 4. Regions showing increased rCBF for BDNF Met carriers compared to Val homozygotes. No regions showed the opposite effect. * - FWE corrected at $p < 0.05$ for SVC. Cluster size reported at $p < 0.001$.

3.2.2 Sex and rCBF

Whole-brain voxel-wise analysis examining the main effect of sex in this cohort revealed an extensive group of regions in which resting rCBF was higher in women than in men. These included regions hypothesized *a priori* (medial PFC/ BA10 [$p \leq 0.002$, FDR corrected], right hippocampus [$p = 0.0001$, FDR corrected], and bilateral parahippocampal gyri [$p \leq 0.002$, FDR corrected]). There were no regions in which rCBF values for men were greater than women in either genotype group. Details of these findings are reported in Table 5, and representative data are shown in Figure 4.

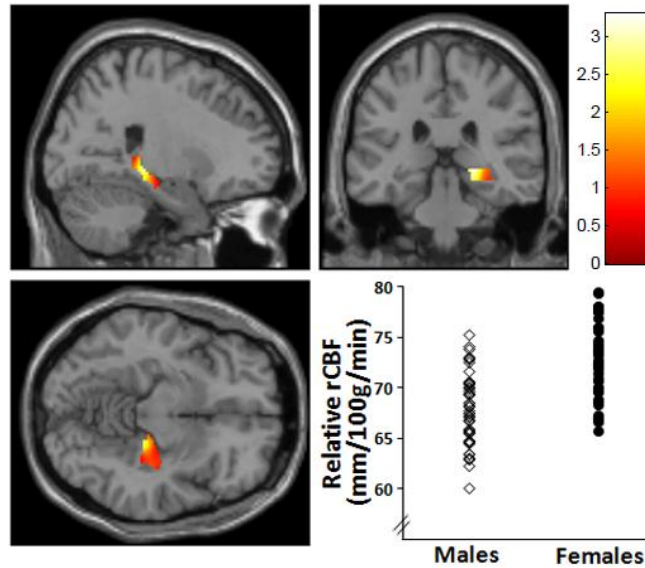


Figure 4. Statistical parametric map and graph (MNI $x,y,z = 22,-30,-2$) showing sex differences in right hippocampus where rCBF was higher in females than males. $p < 0.05$, FWE corrected. Additional regions where this effect was seen are given in Table 2.

Brain Region	MNI coordinates			cluster size	Z values	P values
	x	y	z			
R hippocampus	22	-30	-2	16822	5.66	0.000000007*
L parahippocampal gyrus (BA36)	-38	4	-32	209	4.56	0.000003*
R parahippocampal gyrus (BA36)	32	8	-38	254	4.08	0.00002
L superior frontal gyrus (BA10)	-16	52	-4	453	4.47	0.000004*
L superior frontal gyrus (BA8)	-38	26	46	123	4.24	0.00001
R superior frontal gyrus (BA10)	16	58	-6	2724	4.06	0.00002
R middle frontal gyrus (BA10)	50	12	50	216	4.33	0.000008
R middle frontal gyrus (BA10)	40	52	22	2724	3.98	0.00003
L middle frontal gyrus (BA10)	-34	60	6	453	3.89	0.00005
L middle frontal gyrus (BA6)	-36	4	58	74	3.53	0.0002
R inferior prefrontal gyrus (BA47)	34	46	-2	2724	4.20	0.00001
L inferior prefrontal gyrus (BA47)	-52	24	-8	10	3.33	0.0004
R orbital frontal gyrus (BA11)	40	58	-10	2724	4.05	0.00003
R DLPFC (BA46)	52	30	30	77	4.04	0.00003
L DLPFC (BA9)	-4	58	34	2724	3.77	0.00008
Dorsal anterior cingulate (BA32)	0	26	32	2724	3.90	0.00005

Additional regions including thalamus, right putamen, right postcentral gyrus, left cerebellum, bilateral superior temporal gyrus, angular gyrus, supramarginal gyrus and visual association cortex also showed higher rCBF for women than men at $p \leq 0.0006$, uncorrected.

Table 5. Locales within regions hypothesized *a priori* (PFC and medial temporal cortex) in which resting rCBF was higher in women than in men. No regions showed the opposite effect.

* = FWE corrected at $p < 0.05$. Cluster size reported at $p < 0.001$.

3.2.3 Sex-by-genotype interaction on rCBF

Whole-brain voxel-wise analysis evaluating the relationship between BDNF genotype and sex revealed interactions in several brain regions as detailed in Table 6. Specifically, genotype effects differed in men versus women in the right parahippocampal gyrus ($p=0.0006$, uncorrected) and the right dorsolateral PFC (DLPFC, BA9, $p=0.0009$, uncorrected), as well as in the right caudate ($p=0.0003$, uncorrected), left fusiform gyrus (BA37, $p=0.0005$, uncorrected), and left inferior temporal gyrus (BA20, $p=0.0009$, uncorrected). For all of these regions, rCBF was higher in females than males for the Val homozygotes, whereas the opposite relationship was seen for Met carriers. This consistent pattern is demonstrated in Figure 5 for right parahippocampal gyrus. Conversely, this pattern was reversed in only a single locus in the left precuneus, where rCBF was higher in males compared to females for Val homozygotes and the opposite was seen for Met carriers (BA7, $p=0.0001$, uncorrected).

Brain Region	MNI coordinates			cluster size	T values	P values
	x	y	z			
<u>Val>Met (female>male)</u>						
R caudate	18	4	18	29	3.52	0.0003
L fusiform gyrus	-28	-48	-14	63	3.39	0.0005
R parahippocampal gyrus	18	-2	-30	69	3.33	0.0006
L inferior temporal gyrus (BA20)	-30	-8	-42	227	3.23	0.0009
R DLPFC (BA9)	16	60	32	21	3.16	0.0009
<u>Val>Met (male>female)</u>						
L precuneus (BA7)	-10	-54	62	358	3.83	0.0001

Table 6. Regions showing significant interaction between BDNF allelic variations and sex differences on rCBF. Cluster size reported at $p<0.001$.

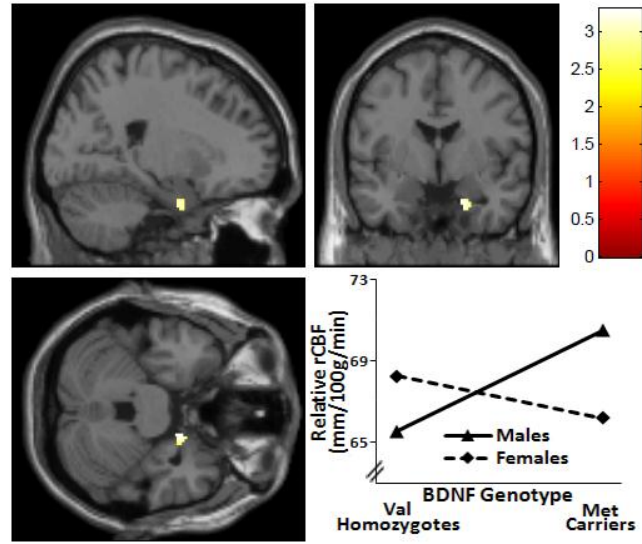


Figure 5. Statistical parametric map and graph (MNI $x,y,z = 18,-2,-30$) showing sex-by-genotype interaction in right parahippocampus where rCBF was higher in females than males for the Val homozygotes, whereas the opposite relationship was seen for Met carriers. $p=0.0006$, uncorrected.

3.2.4 BDNF genotype and functional connectivity

Three separate between-genotype analyses of functional connectivity were carried out using seed regions from (1) left BA 25 in the PFC, (2) right hippocampus, and (3) left parahippocampus as defined by the between-genotypes analysis shown in Table 1. Detailed results of these analyses are given in table 3. Between-genotype differences in inter-regional correlations of these three regions showed a predominant pattern such that the correlations were positive in Val homozygotes, but robustly negative in Met carriers. This predominant pattern is demonstrated in Figure 6 for the relationship between the BA 25 seed region and inferomedial BA 11. Findings in the opposite direction (i.e., inter-regional functional connectivity positive in Met carriers, but negative in Val homozygotes) were sparse (Table 7).

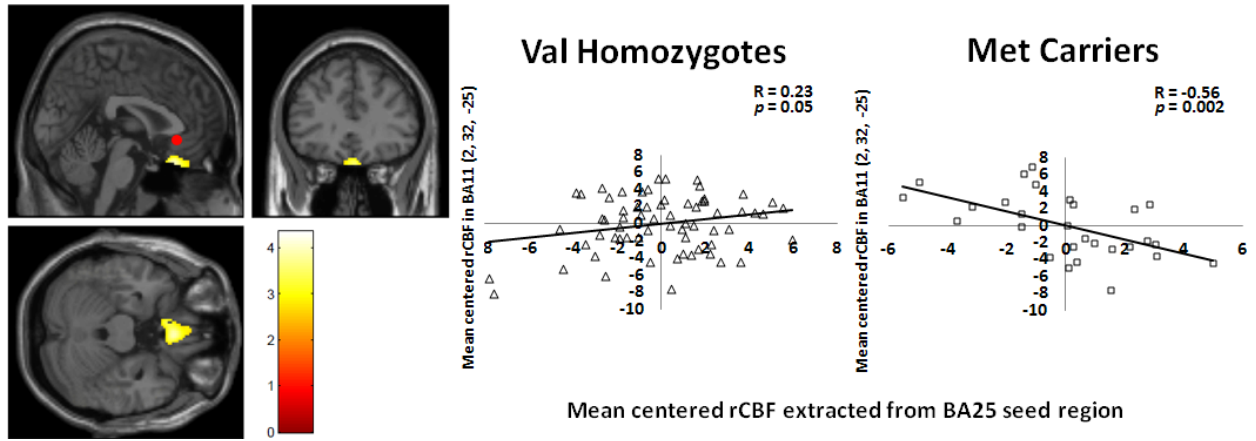


Figure 6. Statistical parametric map and graphs showing differential functional connectivity between genotype groups. Between-group difference in functional connectivity between the BA25 seed region (red circle) and BA11; between-group difference in the slopes of the correlations was significant at the $p=0.0001$ level. Post hoc analysis of extracted rCBF values (at MNI $x,y,z=2,26,-24$) demonstrated a positive correlation for the Val homozygotes ($r=0.23$, $p=0.05$) and a negative correlation for the Met carriers ($r=-0.56$, $p=0.002$).

Seed/Brain Region	MNI coordinates			cluster size	T values	P values
	x	y	z			
Connectivity with BA25						
Val>Met						
R fusiform gyrus (BA37)	48	-64	-20	119	4.33	0.00002
R precuneus (BA7)	10	-84	44	122	4.11	0.00005
L fusiform gyrus (BA37)	-46	-68	-18	248	4.00	0.00007
Orbital frontal gyrus (BA11)	2	26	-24	70	3.88	0.0001
R inferior temporal gyrus (BA20)	52	-30	-22	46	3.80	0.0001
R inferior temporal gyrus (BA20)	50	14	-30	23	3.67	0.0002
R precuneus (BA7)	24	-64	60	44	3.59	0.0002
L inferior temporal gyrus (BA20)	-50	-32	-22	16	3.47	0.0004
Met>Val						
R PFC (BA10)	14	56	22	12	3.56	0.0003
L precentral gyrus (BA4)	-30	-26	52	19	3.48	0.0004
Connectivity with Right Hippocampus						
Val>Met						
R middle temporal gyrus (BA21)	50	-36	-12	251	4.11	0.00004
R inferior frontal gyrus (BA45)	46	28	6	70	3.98	0.00007
R parahippocampal gyrus (BA28)	22	-12	-24	47	3.79	0.0001
R inferior frontal gyrus (BA47)	24	26	-20	19	3.45	0.0004
Connectivity with Left Parahippocampus						
Val>Met						
L inferior frontal gyrus (BA44)	-52	8	8	88	3.78	0.0001
L fusiform gyrus (BA37)	-44	-48	-20	25	3.61	0.0003
L middle frontal gyrus (BA8)	-22	26	48	13	3.49	0.0004
R inferior frontal gyrus (BA45)	48	22	8	12	3.45	0.0004
Met>Val						
L precentral gyrus (BA4)	14	-30	58	23	3.63	0.0002

Table 7. Regions showing differential functional connectivity of rCBF for BDNF Val homozygotes and Met carriers. Cluster size reported at $p < 0.001$.

3.2.5 Sex-by-genotype interaction on functional connectivity

To investigate sex-by-genotype interactions on functional connectivity, we used the same approach as in the genotype analysis of functional connectivity mentioned above: connectivity maps for three seed regions from left BA 25, right hippocampus, and left parahippocampus as defined in Table 1 were derived for the entire group regardless of genotype or sex, and these three connectivity maps were used as search areas for our analysis of sex-by-genotype effects on functional connectivity. A consistent pattern of interactions was seen for functional connectivity with all three seed regions such that for Val homozygotes, interregional correlations were more robustly positive for females than males, whereas for Met carriers, this pattern was reversed in that correlations for males were more robustly positive than for females. An example of this pattern is shown in Figure 7 for the relationship between the left parahippocampal seed region (red circle indicated on the right rather than left sagittal view for illustration) and the right parahippocampal gyrus. No interactions in the opposite directions were observed. Complete details are given in Table 8.

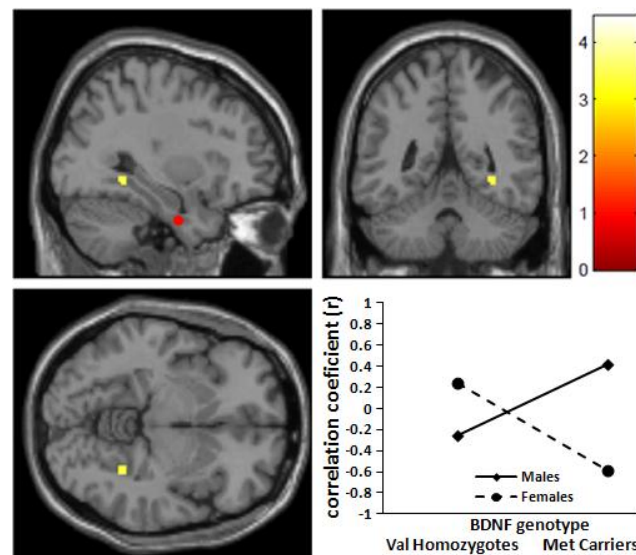


Figure 7. Statistical parametric map and graph (MNI $x,y,z=32,-48,-6$) showing genotype-by-sex interaction ($p=0.0004$, uncorrected) in functional connectivity between the left parahippocampal seed region (red circle indicated on the right rather than left sagittal view for illustration) and the right parahippocampal gyrus. The correlation was robustly positive for female Val homozygotes, whereas in male Val homozygotes the correlation was negative; conversely, for Met carriers, the inter-regional correlation was robustly positive for males, but negative for females.

Seed/Brain Region	MNI coordinates			cluster size	T values	P values
	x	y	z			
Connectivity with BA25						
Val>Met (female>male)						
R inferior frontal gyrus (BA45)	36	22	8	66	3.80	0.0001
R hippocampus	34	-42	-2	21	3.70	0.0002
L middle frontal gyrus (BA8)	-40	28	46	44	3.64	0.0002
Ventral anterior cingulated (BA24)	4	-4	46	27	3.47	0.0004
Connectivity with Right Hippocampus						
Val>Met (female>male)						
L DLPFC (BA9)	-54	14	34	470	4.69	0.000005*
L middle frontal gyrus (BA6)	-38	2	50	166	4.61	0.000007*
L middle frontal gyrus (BA10)	-22	56	22	212	4.59	0.000008*
L middle temporal gyrus (BA39)	-50	-72	28	471	4.52	0.00001*
R DLPFC (BA9)	56	12	38	184	4.29	0.00002
R Cingulate gyrus (BA29)	8	-48	8	98	4.23	0.00003
R hippocampus	30	-16	-20	29	4.19	0.00003
R postcentral gyrus (BA4)	18	-36	74	300	4.18	0.00003
R DLPFC (BA9)	28	44	38	148	3.97	0.00007
R precentral gyrus (BA4)	24	20	58	161	3.97	0.00007
R superior frontal gyrus (BA8)	10	38	54	42	3.80	0.0001
R cerebellum	44	-68	-26	250	3.77	0.0002
R postcentral gyrus (BA4)	28	-44	64	45	3.76	0.0002
L middle frontal gyrus (BA10)	-26	64	-4	87	3.72	0.0002
R precentral gyrus (BA4)	30	-16	64	47	3.59	0.0003
R parahippocampal gyrus (BA35)	20	-34	-12	61	3.59	0.0003
Dorsal anterior cingulate (BA32)	-4	32	-10	45	3.58	0.0003
L medial frontal gyrus (BA6)	-18	-2	64	28	3.52	0.0003
L postcentral gyrus (BA4)	-22	-32	72	36	3.42	0.0005
Connectivity with Left Parahippocampus						
Val>Met (female>male)						
L precentral gyrus (BA6)	-60	-18	44	120	4.44	0.00001
R fusiform gyrus (BA18)	26	-78	-20	159	4.17	0.00004
R precentral gyrus (BA8)	8	-26	74	96	3.99	0.00007
L fusiform gyrus (BA18)	-28	-86	-22	111	3.77	0.0002
R postcentral gyrus (BA4)	58	-20	54	17	3.72	0.0002
R middle frontal gyrus (BA8)	34	32	52	16	3.61	0.0003
R ventral anterior cingulated (BA24)	14	-6	46	33	3.44	0.0003
R middle temporal gyrus	38	6	-24	24	3.54	0.0003
R parahippocampal gyrus	32	-48	-6	18	3.51	0.0004
L parahippocampal gyrus (BA28)	-24	4	-22	13	3.36	0.0004

Table 8. Regions showing interaction between sex and BDNF Val⁶⁶Met polymorphism on rCBF functional connectivity. * - FWE corrected at $p < 0.05$. Cluster size reported at $p < 0.001$.

3.3 Specific Methods for Study 2: Interaction between *BDNF* Val⁶⁶Met Polymorphism and Ovarian Steroid Hormones Affects Working Memory-Related Hippocampal Function

3.3.1 Subject information

Thirty-six healthy women between the ages of 18 and 50 years were recruited. In addition to the medical and neuropsychiatric work-up detailed in Section 2.1, criteria for study entry included a normal gynecologic exam within the past six months, normal menstrual cycles, and confirmation of the absence of menstrual-related mood or behavioral symptoms by daily symptom self-ratings for two menstrual cycles. Twenty-six participants were Val homozygotes and 10 were Met carriers. Genotype frequencies were in Hardy-Weinberg equilibrium ($X^2=0.94$, d.f.=1, $p=0.33$, n.s.), and there were no significant age, racial distribution, or handedness differences between women who were *BDNF* Val homozygotes and those who were Met carriers (Table 9).

Genotype Demographics	Val/Val Homozygotes	Met Carriers	Statistical Significance
	Mean± SD	Mean± SD	
Age (years)	34.0±8.2	37.6±8.3	$P=0.8$, N.S.
Race	19C/6AA/1A	7C/2AA/1A	$P=0.4$, N.S.
Handedness	21R/5L (80.8%R)	9R/1L (90%R)	$P=0.5$, N.S.

Table 9. Study 2 subject demographics

3.3.2 Hormone manipulation protocol

In Study 2, we induced a reversible state of ovarian suppression through the administration of a gonadotropin-releasing hormone (GnRH) agonist for six months. Additionally, we added-back physiologic doses of both 17 β -estradiol (the predominant type of estrogen secreted by the ovary) and progesterone during separate months during

the last three months of the six-month protocol. This experimental design has several advantages over naturalistic studies performed across the menstrual cycle. First, it permits us to examine the effects of the absence of ovarian steroid hormones compared with those after the replacement of ovarian steroids in a manner similar to studies performed in ovariectomized lower animals. Second, with this protocol the effects of estradiol can be observed separately from those of progesterone, whereas these two ovarian steroids are simultaneously present at varying levels during the normal menstrual cycle. Finally, all women received the same, physiological doses of both estradiol and progesterone, thereby removing a potential confound related to inter-individual variations in the quantities of these hormones secreted across the natural menstrual cycle.

GnRH, a decapeptide produced by the hypothalamus, stimulates the anterior pituitary to release the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), which in turn, stimulate the production of estradiol and progesterone by the ovary (Reid, Van Vugt et al. 2007). The GnRH agonist employed in this study, depot leuprolide acetate (Lupron), is a synthetic nonapeptide that is 80 to 100 times more potent than native GnRH. After an initial stimulation of the pituitary-ovarian axis, Lupron results in a downregulation of pituitary GnRH receptors, and ultimately in a reversible cessation of pituitary ovarian axis

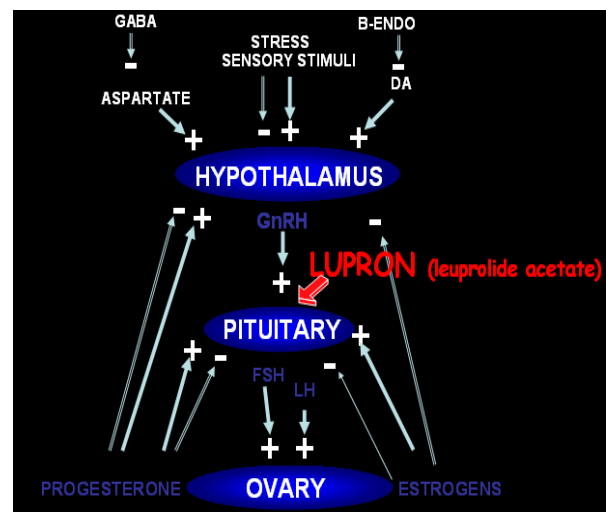


Figure 8. Hypothalamic-pituitary-gonadal axis. The administration of Lupron results in a reversible suppression of hypothalamic-pituitary-gonadal axis and, thus, the suppression of the ovarian steroids, estradiol and progesterone.

function, i.e., both gonadotropin and ovarian steroid secretion are suppressed (Figure 8). Ovarian secretion of estradiol and progesterone remains suppressed (comparable to levels in post-menopausal women) as long as Lupron is administered on a regular monthly basis, but resumes in pre-menopausal women after cessation of Lupron treatment.

Prior to starting the hormone manipulation protocol, all women had a two month baseline period during which mood and behavioral ratings were obtained to confirm the absence of menstrual-cycle-related mood disorders. Following the baseline period, subjects were administered 3.75 mg of Lupron via intramuscular injection on a monthly basis for 24 weeks. After week 12 of Lupron treatment, participants were randomized to either Group A or B in a double-blind, counterbalanced manner and received, in a sequential fashion, estradiol or progesterone as follows (Figure 9).

Group A: While continuing on the GnRH agonist, subjects were administered 0.1 mg/day of 17 β -estradiol via skin patch (estraderm) for a period of five weeks. After the fourth week, progesterone suppositories (200 mg BID) were added to provide progesterone withdrawal-induced shedding of the endometrium and menses in order to prevent any effects on the endometrium of prolonged exposure to unopposed estrogen. After the four weeks of estradiol and the one week of combined estradiol and progesterone administration, there was a two week washout period when neither estradiol nor progesterone was given. Subjects were then administered progesterone suppositories (200 mg BID) twice daily for a period of five weeks, after which progesterone was discontinued.

Group B: Hormone addback was identical to Group A except that the order of estradiol and progesterone treatments was reversed: progesterone suppositories (200 mg)

were administered twice daily for the first five weeks, and after a two week washout period, estradiol was administered alone for four weeks and, then, in combination with progesterone during the fifth week.

After the administration of the GnRH agonist, in addition to the completion of daily mood and behavioral rating scales, blood samples were drawn biweekly for the duration of the study (24 weeks), and participants were seen by physicians every 2 weeks in the NIMH outpatient clinic. Neuroimaging procedures (described below) were performed during each of three separate hormonal conditions as follows: (1) after at least six weeks of Lupron alone (hypogonadism), (2) after at least two weeks of estradiol addback, and (3) after at least two weeks of progesterone addback.

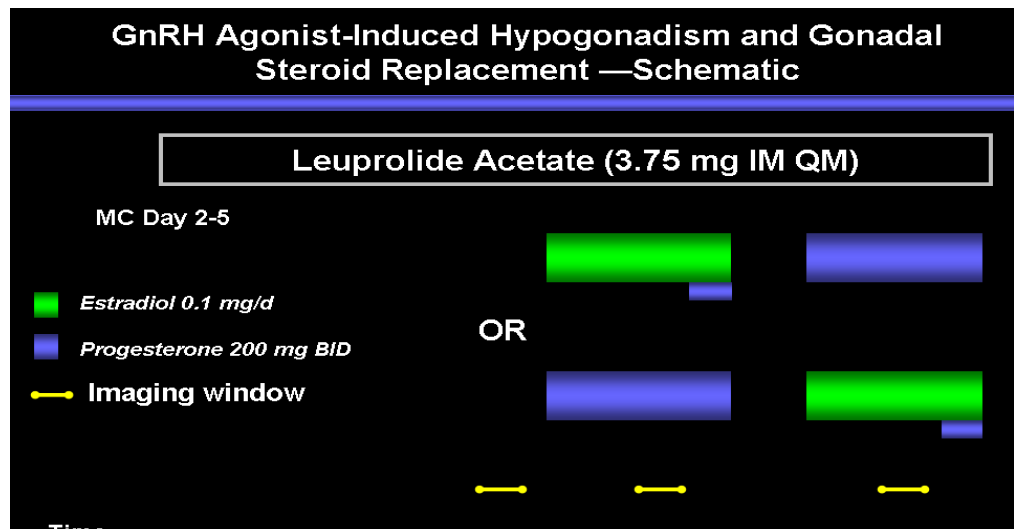


Figure 9. Schematic diagram of GnRH Agonist-Induced Hypogonadism & Gonadal Steroid Replacement. Following a two month baseline period, women received 3.75 mg of Lupron (leuprolide acetate, purchased from TAP Pharmaceuticals, Chicago, IL) by intramuscular injection every four weeks for six months. Lupron alone was administered for the first 12 weeks. Women received, in addition to Lupron, 17 β estradiol (0.1 mg/day) by skin patch or progesterone suppositories (200 mg BID) for five weeks each. They then switched to the alternative treatment (in counterbalanced design). The two replacement regimens were separated by a two week washout period. Four neuroimaging sessions were acquired during the baseline period, Lupron alone, estradiol addback, and progesterone addback periods.

3.3.3 *Hormone assays*

Blood samples were centrifuged, aliquoted and stored at -70°C until time of assay. Plasma levels of progesterone were analyzed by radioimmunoassay (Diagnostic Systems Laboratory, Webster, TX), and plasma levels of estradiol were measured by a competitive chemiluminescent analyzer (Immulite 2000, Siemens Healthcare Inc., Deerfield Ill, and NIH Department of Laboratory Medicine). Intra- and inter-assay coefficients of variation for progesterone were 7.0-7.3% and 8.0-9.2%, respectively and for estradiol, 7.0% and 4.4-4.5%, respectively.

3.3.4 *N-back working memory paradigm*

Because the work in this thesis is focused on identifying modulatory effects of both *BDNF* and ovarian steroids and their interactions in PFC and hippocampus (regions shown to be affected by both modulators, as discussed in Chapter 1), during neuroimaging subjects performed a cognitive task that reliably affects both regions and is commonly used in neuroimaging, the n-back working memory task. A number of studies have demonstrated that during this task, DLPFC is activated (Owen et al. 2005), whereas hippocampal regions are “deactivated” (i.e., have less neuronal recruitment during working memory than at baseline, possibly reflecting the need to depend on short-term memory, rather than hippocampal episodic memory, mechanisms for efficacious performance of the task; Meyer-Lindenberg et al. 2001; Stretton et al. 2012). This imaging task paradigm thus provides a well-documented and reliable tool to investigate the potential interactive effects of *BDNF* and ovarian hormones in these regions.

The version of the n-back task employed in this work requires the constant on-line monitoring and updating of a series of stimuli. Subjects were shown a series of diamond-shaped number arrays, with one of four numbers highlighted in random sequences with a 2 second ITI (Figure 10). For the 0-back sensorimotor control task, participants were asked to push a button corresponding to the number shown at the time of the trial. For the 2-back working memory task, participants pushed a button corresponding to the number displayed two trials previously. Because the level of task performance *per se* (e.g., percent correct) may relate to activity levels in PFC (Callicott et al. 1999) and hippocampus (Stretton et al. 2012), we sought to minimize this potential confound by minimizing between- and within-subject performance differences. To achieve this goal, participants were overtrained on the n-back working memory paradigm, and were retrained before each of the PET scanning sessions to achieve at least 80% accuracy on the 2-back working memory task before performing the task in the scanner.

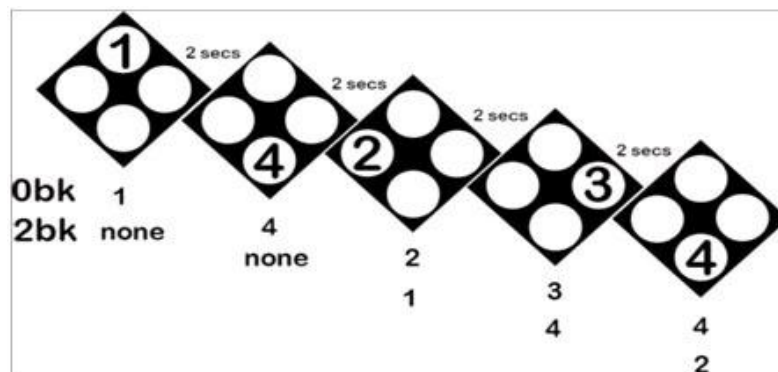


Figure 10. N-back working memory paradigm. The numbers 1-4 were presented on a computer monitor at set locations at the points of a diamond shape and were shown in random order at a one every two seconds interval. Subjects were instructed to respond to each trial by pressing one of four buttons on a response box with the same configuration as the diamond shape on the screen. For the 0-back sensorimotor control task, subjects were told to press the button that corresponds to the number shown on the monitor at the time of the trial. During the 2-back working memory block, the instruction was to press the button corresponding to the number shown two trials previously. Working memory is emphasized and no recognition is involved. Because a response is required for each trial, continuous attention to the task can be documented.

3.3.5 PET data acquisition

PET sessions were performed during each of three separate hormonal conditions, (1) after at least six weeks of Lupron alone (hypogonadism), (2) after at least two weeks of estradiol addback (0.1mg/day), and (3) after at least two weeks of progesterone addback (200mg BID). Due to the design of the hormone manipulation protocol and the clinical difficulty of it for participants, hypogonadism must be induced and tolerability of this treatment must be ensured for each participant prior to estradiol or progesterone addback. Therefore, the Lupron session always preceded the addback conditions and was not counterbalanced (as the subsequent estradiol and progesterone addback PET sessions were). During each hormone-specific scan session, two 60-second rest scans and fourteen 60-second task scans (alternating between seven 0-back and seven 2-back scans) were collected six minutes apart, each following an intravenous bolus of 10 mCi of oxygen-15 water, as described above. Each task began approximately 10 seconds prior to the injection of tracer and continued throughout the entire 60-second scan and until 90 seconds of behavioral data were collected.

3.3.6 PET data analyses

3.3.6.1 rCBF Analyses

Because *BDNF* and the ovarian steroids estradiol and progesterone have been shown in animal studies to interactively affect PFC and hippocampal function (as discussed in Chapter 1) these brain areas were chosen *á priori* as regions of interest for our analyses. Voxel-wise analyses within these regions were carried out to test the hypothesis that *BDNF* Val⁶⁶Met genotype and hormone status interactively effect cognitively-related brain function in women.

For the rest analysis, the two resting rCBF scans of each subject were averaged and entered into voxel-based group-level analyses similar to that described below. For the 2-back versus 0-back activation/deactivation analysis, first, for each woman, one first-level 2-back versus 0-back activation/deactivation map per hormone condition was entered as a repeated measure, and genotype was entered as a between-groups measure in a flexible factorial model in SPM5. Next, to restrict the findings to *á priori* regions of interest (i.e. hippocampus and PFC, specifically DLPFC), a bilateral hippocampal mask (manually segmented in standard stereotaxic space based on an averaged T1 structural MRI template from 250 healthy volunteers independent of the present study cohort) and an independently derived DLPFC mask (as cytoarchitectonically defined in standard stereotaxic space in postmortem human brain by Rajkowska and Goldman-Rakic et al. 1995) was applied. Finally, within these region-specific masks, genotype-by-ovarian hormone interactions in working-memory activation/deactivation (2-back versus 0-back) were evaluated with a voxel-wise statistical threshold of $p < 0.001$, uncorrected. Additionally, SVC for FWE was applied to results within these *á priori* regions of interest. Extracted average activation/deactivation values from a 5mm diameter sphere surrounding the most robust voxel in the hippocampus or DLPFC were used for the post-hoc between-genotype, between-hormone condition, and gene-by-hormone interaction analyses (Figure 13).

Because altered activation (2-back versus 0-back) could reflect rCBF changes in either the 0-back or 2-back conditions or in both, the 2-back and 0-back rCBF maps were also analyzed separately to disambiguate the activation/deactivation genotype-by-hormone findings. The procedures were identical to the 2-back versus 0-back activation/deactivation analysis except that for each women, one first-level 2-back or 0-back activation/deactivation

map per hormone condition was entered before performing the second-level full factorial analysis (Figure 13).

3.4 Results for Study 2

3.4.1 Estradiol and progesterone measurements and n-back performance (Table 10)

As expected, plasma measurements of serum estradiol and progesterone confirmed hormone suppression during administration of Lupron alone, as well as replacement of the appropriate ovarian steroid to physiological levels during each addback condition. Specifically, during hypogonadism, plasma levels of estradiol and progesterone were suppressed to < 20 pg/ml and < 0.6 ng/ml, respectively, whereas during estradiol replacement, plasma levels of estradiol were in the mid-follicular phase range (80-120 pg/ml), and during progesterone replacement, plasma levels of progesterone were comparable to those in the mid-luteal phase (8-15 ng/ml). There were no significant hormone level differences between Val homozygotes and Met carriers (Figure 11) and no genotype-by-hormone interactions.

During the PET sessions, all participants performed the n-back working memory task well above chance (25%) on all runs in all hormone conditions. There were no performance differences between genotype groups or across hormone conditions, and no genotype-by-hormone interaction (Figure 12).

	Lupron		Progesterone		Estradiol		ANOVA-R Main Effect of Hormone Condition F (P value)	ANOVA-R Main Effect of Genotype F (P value)	ANOVA-R Interaction of Hormone by Genotype F (P value)
	Val	Met	Val	Met	Val	Met			
Plasma Estradiol (pg/ml)	29.80 (14.56)	25.71 (9.54)	31.29 (16.22)	29.12 (7.02)	116.70 (51.89)	130.56 (71.75)	43.48 (P<0.001)	0.34 (P=0.56)	0.71 (P=0.50)
Plasma Progesterone (ng/ml)	0.37 (0.12)	0.39 (0.16)	13.44 (5.99)	12.69 (5.81)	0.38 (0.22)	0.47 (0.22)	64.32 (P<0.001)	0.13 (P=0.72)	0.28 (P=0.76)
0-back	98.80 (1.70)	99.11 (1.39)	98.05 (3.34)	99.54 (0.77)	99.07 (1.05)	99.24 (1.17)	0.34 (P=0.71)	1.84 (P=0.18)	0.75 (P=0.48)
2-back	84.51 (12.80)	79.05 (16.06)	87.11 (12.52)	81.31 (15.47)	85.84 (12.57)	83.75 (10.23)	1.47 (P=0.24)	1.03 (P=0.32)	1.28 (P=0.29)

Table 10. Hormone levels and n-back performance

Post-hoc Bonferroni t-tests:

Post-hoc testing compared average estradiol levels of both Val and Met during each hormone condition

Estradiol Levels: Est vs Lup, $p<0.01$; Est vs Prog, $p<0.01$; Lup vs Prog, $p=N.S.$

Post-hoc testing compared average progesterone levels of both Val and Met during each hormone condition

Progesterone Levels: Est vs Lup, $p=N.S.$; Est vs Prog, $p<0.01$; Lup vs Prog, $p<0.01$

Post-hoc testing compared average 2-back accuracy of both Val and Met during each hormone condition

Nback Accuracy: Est vs Lup, $p=N.S.$; Lup vs Prog, $p=N.S.$; Est vs Prog, $p=N.S.$

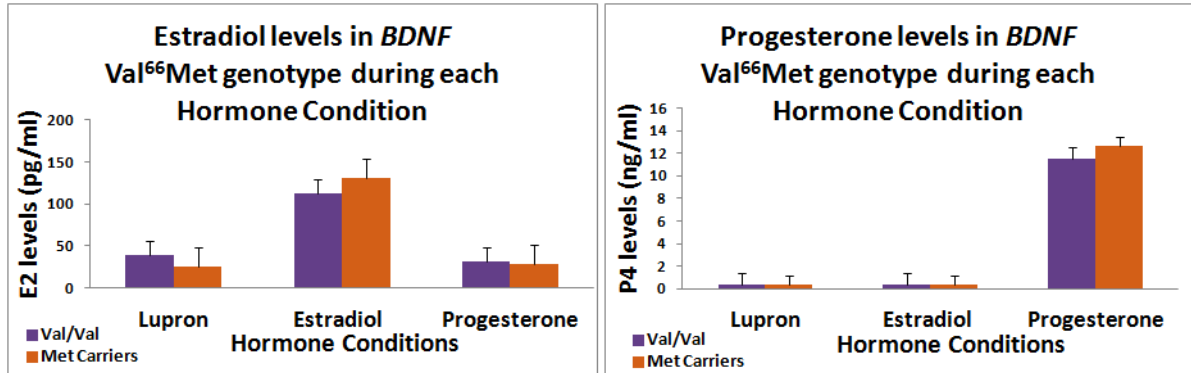


Figure 11. Serum estradiol and progesterone levels during hormone suppression (Lupron alone), estradiol- and progesterone-replacement conditions. During hypogonadism, plasma levels of estradiol and progesterone were suppressed (< 20 pg/ml and < 0.6 ng/ml, respectively), whereas during estradiol replacement, plasma levels of estradiol were in the mid-follicular phase range, and during progesterone replacement plasma levels of progesterone were comparable to those in the mid-luteal phase. Hormone levels did not differ significantly between genotype during any of the three hormone conditions.

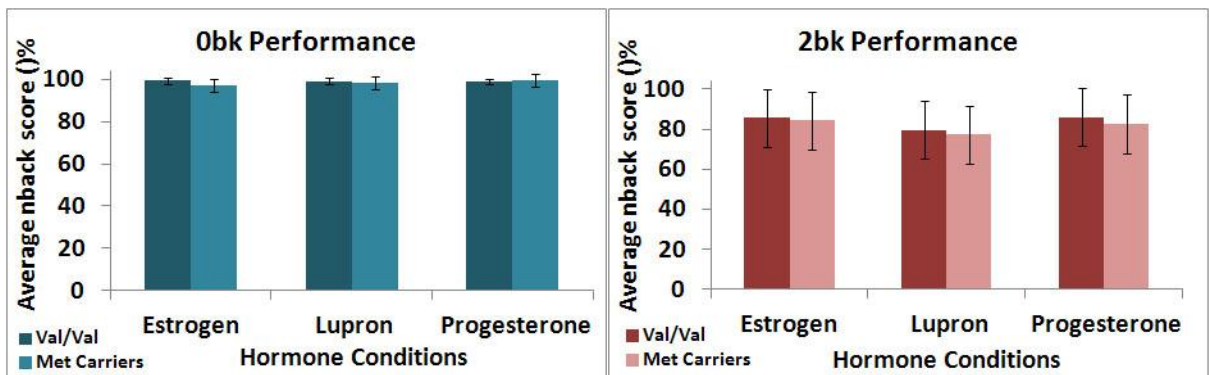


Figure 12. N-back working memory task performance during all hormone conditions. There were no significant accuracy differences between genotypes or across hormone conditions for either the 0-back sensorimotor control or the 2-back working memory tasks.

3.4.2 Rest and Working memory related hippocampal activation/deactivation

No significant interactions between *BDNF* genotype and gonadal steroid condition were found during rest. In addition, there were no significant *BDNF*-by-ovarian hormones interactions in the PFC. However, significant interactions between *BDNF* genotype and ovarian hormones were observed within the hippocampus, a region typically deactivated in the n-back working memory paradigm. Specifically, an interaction was observed in the right hippocampus for 2-back versus 0-back activation/deactivation ($F=9.11$, $p=0.02$, FWE corrected; voxel-level, MNI x,y,z coordinates: 24, -36, -2). A similar trend was also observed in the left hippocampus ($F=5.83$, $p=0.004$, uncorrected; voxel-level, MNI x,y,z coordinates: -34, -14, -18). Post-hoc analyses of a 5mm sphere surrounding the most significant right hippocampal voxel in the interaction analyses (Table 11) revealed that for Val homozygous women there was no significant change in activation across hormone conditions ($F=2.47$, $p=0.09$, uncorrected), whereas Met carriers showed robust hormone-specific changes ($F=3.55$, $p=0.04$, uncorrected): the hippocampus was abnormally activated (not deactivated) in Met carriers during estradiol but not during the hypogonadal state or during progesterone replacement; for Met carriers the abnormal hippocampal activation was significantly different compared to Lupron alone ($T=2.29$, $p=0.03$, uncorrected) and to progesterone ($T=2.42$, $p=0.03$, uncorrected), but there was no difference between Lupron alone and progesterone addback. There was a significant effect of genotype during estradiol replacement with Met carriers having abnormally elevated activation compared to Val homozygotes ($T=3.52$, $p=0.001$, uncorrected), but there was no genotype effect during Lupron alone or progesterone addback.

These findings suggest a convergence in the actions of *BDNF* and ovarian steroids on the modulation of working memory-related hippocampal activation/deactivation. However, changes in activation (2-back versus 0-back) could reflect rCBF changes in either the 0-back or 2-back conditions or in both. To disambiguate the activation findings, and determine whether hormone-related changes occurred specifically during the working memory task (2-back) or the sensorimotor control task (0-back), I took advantage of the PET approach which maps rCBF during each cognitive condition separately with entirely independent scans and analyzed rCBF during 0-back and that during 2-back separately. These analyses (Figure 13) clearly indicated that the activation findings were due to neural activity during the working memory condition and not the sensorimotor control task. Specifically, the pattern of hippocampal rCBF changes in the 2-back working memory condition analyzed alone showed a significant genotype-by-hormone interaction effect with a pattern very similar to that seen in the 2-back versus 0-back activation/deactivation analysis. In contrast, no *BDNF*-by-hormone interaction was observed when the 0-back control condition was analyzed alone. In addition to clearly assigning the findings to neural activity during recruitment for working memory, these analyses rule out non-specific effects such as those that could be due to vascular changes *per se*.

	Right hippocampal activation/deactivation ANOVA or 2-sample t-test F or T (p value)
post-hoc T tests:	
Met hormone effect	F=3.55 ($p=0.04$)
Val hormone effect	F=2.47 ($p=0.09$)
Met est>lup	T=2.29 ($p=0.03$)
Met est>prog	T=2.42 ($p=0.03$)
Met>Val est	T=3.52 ($p=0.001$)

Table 11. Post-hoc analyses: Working memory-related right hippocampal activation/deactivation for 2-back versus 0-back.

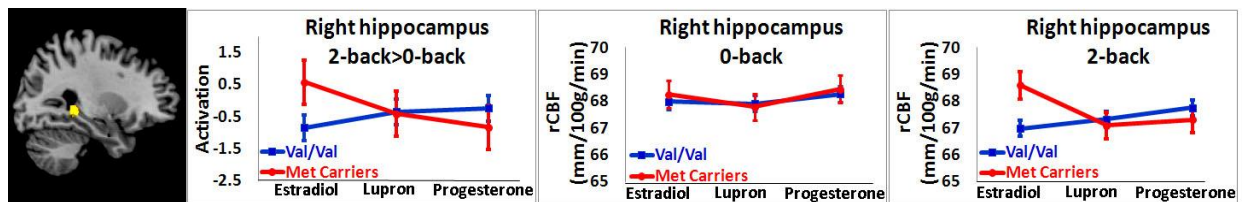


Figure 13. Working memory-related right hippocampal activation/deactivation displaying differential BDNF-by-hormone interaction on rCBF changes in the 2-back>0-back activation/deactivation (left). Analyses of 2-back working memory and 0-back rCBF maps (right and middle, respectively) demonstrated that the activation/deactivation results were specific to neural activity during 2-back working memory and not to the 0-back control condition.

Chapter 4

DISCUSSION

4.1 STUDY 1: BDNF Val⁶⁶Met Polymorphism Affects Resting rCBF and Functional Connectivity Differentially in Women versus Men (Wei et al., 2012)

4.1.1 *BDNF affects resting rCBF*

Study 1 tested for effects of the *BDNF* Val⁶⁶Met polymorphism and for sex-by-genotype interactions on resting rCBF, a measure tightly linked to regional cerebral metabolism. I further examined whether rCBF in genotype-associated regions covaried with rCBF in other nodes throughout the brain in a genotype- and/or sex-dependent fashion. The results showed that resting rCBF in hippocampal/parahippocampal and PFC regions, as well as functional connectivity between these regions and other parts of the brain were modulated by both the *BDNF* Val⁶⁶Met polymorphism and sex. These results are of particular interest in light of both clinical and preclinical literature.

BDNF is a key regulator of long-term potentiation and promotes synaptic plasticity and efficacy via excitatory glutamatergic neurotransmission in the hippocampus (Messaoudi et al. 1998; Drake et al. 1999; Tartaglia et al. 2001; Bramham et al. 2005). The *BDNF* Val⁶⁶Met polymorphism attenuates activity-dependent BDNF signaling in cultured hippocampal neurons (Egan et al. 2003; Lu 2003) and has been linked to both hippocampal and prefrontal abnormalities by functional neuroimaging. Previous fMRI experiments, where measurements inherently depend on computing differences between two brain states and thus provide only relative measures of activation over baseline (Deyoe et al. 1994), have demonstrated *BDNF* genotype-dependent hippocampal recruitment during memory-related

tasks compared to control tasks (Egan et al. 2003; Hariri et al. 2003; Hashimoto et al. 2008). Also using fMRI, Soliman and colleagues (2010) found that *BDNF* Met carriers showed reduced ventromedial PFC activation during memory extinction. However, it has remained unclear until the present work whether this polymorphism has implications for the cerebral metabolic landscape during the so-called basal or “resting” state. I used a gold-standard PET method that allows task-independent brain function, specifically rCBF, a parameter tightly coupled to regional cerebral glucose metabolic rate, to be measured and mapped without reference to any other brain state.

Our data demonstrate that even at rest, rCBF in bilateral hippocampal/parahippocampal and medial frontal regions of healthy individuals is affected by the *BDNF* Val⁶⁶Met polymorphism. In addition to providing an important perspective on fMRI activation studies, these data suggest that there exists a potent, genetically mediated bias in the basal activity of frontotemporal circuitry. Because BDNF generally facilitates neural activity in hippocampus and cortex (Henderson 1996; Benraiss et al. 2001), this bias in Met carriers – increased regional activity at baseline – could reflect a neural systems level accommodation for the relatively inefficient, activity-dependent BDNF signaling at the cellular level associated with Met alleles (Egan et al. 2003). Whether this is additionally related to previously reported compensatory increases in peripheral serum concentration of BDNF in healthy Met carriers (Lang et al. 2009) is unclear, and given BDNF’s broad impact on neuronal development, activity, and plasticity, delineating the precise roots of such speculated accommodation necessarily requires further research. Nonetheless, it is noteworthy that even subtle differences in this molecule result in measurable neurofunctional alterations, particularly in

key components of the default network (Raichle et al. 2001), in a cognitively and affectively unchallenged state.

4.1.2 *BDNF and sex affect resting rCBF*

Our finding of increased resting rCBF in women compared to men is consistent with several previous studies (Devous et al. 1986; Yoshii et al, 1988; Slosman et al. 2001) and has been hypothesized to reflect physiologic compensation for the smaller brain size found in women (Ho et al. 1980a,b; Ankney et al. 1992). The observation that sex effects in several regions within frontotemporal structures are interactively modulated by *BDNF* allelic variation is in line with a substantial body of animal literature demonstrating parallel and interacting actions of estradiol and *BDNF* in the brain. Not only do estrogen receptors, *BDNF*, and *trkB* show regional coexpression in the hippocampus and frontal cortex (Miranda et al. 1993; Sohrabji et al. 2006), both *BDNF* and estradiol increase adult neurogenesis (Ormerod et al. 2004; Sairanen et al. 2005), provide neuroprotection (Kiprianova et al. 1999; Shughrue et al. 2003), and facilitate memory formation (Mizuno et al. 2000; Frick et al. 2002). Additionally, *BDNF* mRNA levels are reduced in ovariectomized female rats and are rescued by estradiol replacement in the hippocampus and cortex (Singh et al. 1995; Sohrabji et al. 1995; Liu et al. 2001). Despite this ample preclinical evidence for hormone-by-genotype interaction, there is a paucity of human data demonstrating true statistical interactions between genotype and sex, though several authors have reported genotype effects occurring only in one sex and not the other (Henningsson et al. 2009; van Wingen et al. 2010;). Our observation of *BDNF* genotype effects that were generally greater in magnitude in men than women in these select regions agrees with studies finding genotype

effects on frontotemporal measures selectively in men, including those examining serotonin transporter availability (Henningsson et al. 2009) and activation during memory tasks (van Wingen et al. 2010). However, our findings of reversed sex effects on resting rCBF in Val homozygotes versus Met carriers indicate that the combined effects of sex and BDNF signaling on neurophysiological function are likely to be complex.

4.1.3 *BDNF and sex affect functional connectivity*

Because both BDNF and estradiol play important roles in axonal guidance and dendritic arborization (Dominguez et al. 2004) as well as in synaptogenesis (Tanapat et al. 1999; Scharfman et al. 2005), I postulated that BDNF function, as predicted by genotype, both alone and in conjunction with sex, may modulate the functional connectivity of areas impacted by *BDNF* genotype. In keeping with this hypothesis, significantly greater positive correlations between rCBF in BA25 and rCBF in mPFC, and between hippocampus and parahippocampus, were seen in Val homozygotes compared to Met carriers during rest, consistent with prior documentation of greater resting network connectivity in these areas in Val homozygotic children (Thomason et al. 2009). These findings are complimented by the observed sex-by-genotype interactions, which demonstrated consistent genotype-determined differences in sexually dimorphic patterns of interregional cooperativity within BA25 and hippocampal/parahippocampal regions. The correlations of rCBF between these regions were modulated differentially by sex, with correlations more robustly positive for Val homozygotic females than males, whereas for Met carriers, this pattern was reversed. These results together suggest that when investigating genotypic modulation of neurophysiology, sex difference is an important factor that should be considered in explaining the findings.

4.1.4 Abnormal resting rCBF in BDNF Met carriers may be related to the pathophysiology of depression

Our results are also relevant in considering the neural substrate of neuropsychiatric disorders. For example, the ‘neurotrophin hypothesis’ of depression speculates that incompetent BDNF function is directly related to the pathophysiology of depression. Although previous genetic association studies of the *BDNF* Val⁶⁶Met polymorphism in depression have generated inconsistent results (Verhagen et al. 2008; Surtees et al. 2007; Chen et al. 2008), these data suggest that several brain regions known to display depression-related abnormalities could also be modulated by the *BDNF* Val⁶⁶Met polymorphism. The hippocampus, BA25, and mPFC have been shown to exhibit abnormal depression-related variations, including hyperperfusion in BA25 in chronic/treatment resistant patients (Mayberg 1994; Drevets et al. 1997; Mayberg 2005), hyperactivation of hippocampus to emotional stimuli (Lau et al. 2010), and hyperrecruitment of PFC when down-regulating amygdala responses to negative stimuli (Johnstone et al. 2007). Our finding of increased resting rCBF in these same regions in healthy Met carriers without clinical depression mirrors this systems-level pathological phenotype and suggests that inefficient BDNF protein processing may translate to limbic functional changes that could have importance in illness. On the other hand, the abnormally increased subgenual cingulate and default network functional connectivity that has been observed in depressed patients (Greicius et al. 2007) was manifested differently in Met carriers in the current study, suggesting that the confluence of *BDNF* genotype effects and depression pathophysiology is intricate and requires further investigation.

4.1.5 Strengths and limitations of the present study

This work benefited from several methodological strengths, including the use of the gold standard H_2^{15}O rCBF PET method, and the acquisition of a relatively large study sample. However, there are several caveats that deserve mention. First, menstrual cycle phase could not be documented for all women in the study, which prevented investigation of the effect of this important variable in the current data. Future research including such hormonally-related parameters will provide crucial information. Second, even though this study controlled for age, race, sex and past psychiatric illness, genes interact with many factors including early life stress, hormones, and the environment to influence the underlying neurobiology of the intermediate phenotype. Although the sample size is adequate, these factors could contribute variance to findings in the present study. In addition, “resting state” by definition implies unrestricted thought process, and variation in the content of the thought process or in the response to the scanning session may be affected by genotype; these effects also may interact with sex and other personality-related features. Such experiential variability could be reflected in the rCBF results.

4.1.6 Implications and future directions

Study 1 identified in healthy individuals *BDNF* genotype-determined differences in basal resting activity and interregional activity relationships within medial frontotemporal nodes important in neuropsychiatric illnesses such as depression. The fact that sex differences in these regions are modulated by *BDNF* genotype serves as an important impetus to further examine *BDNF*-gonadal hormone interactions. Since both *BDNF* and estradiol exert important influences on hippocampal-dependent processes, Study 2 examined the interaction

between these two neuromodulators using a cognitive task with a strong hippocampal signal (in the case of the n-back working memory task, deactivation), in conjunction with a pharmacological hormone manipulation protocol.

4.2 Study 2: Interaction Between the BDNF Val⁶⁶met Polymorphism and Ovarian Steroid Hormones Affects Working Memory-Related Hippocampal Function

4.2.1 *BDNF and ovarian steroid hormones interactively affect working memory-related hippocampal rCBF*

Study 2 is the first demonstration in humans that *BDNF* genotype and ovarian hormones interactively affect brain function. The n-back working memory paradigm, which has consistently shown PFC activation and hippocampal “deactivation” (i.e. less neuronal recruitment during working memory than at baseline), was utilized to examine neurofunctional changes mediated by the interaction between *BDNF* and ovarian steroids. While a *BDNF*-by-ovarian hormone interaction was not observed in the PFC, the results revealed that atypical hippocampal activation (not deactivation) in 2-back versus 0-back working memory was associated with the presence of the functionally less efficient Met allele in women, consistent with a previous fMRI study (Egan et al. 2003). Moreover, the abnormal effects of the Met allele on rCBF were only demonstrated in the presence of estradiol. Post-hoc analyses showed that there were no hormone-related activation differences in Val homozygotes, and that there were no genotype-related differences during either the Lupron alone (hypogonadal) or progesterone add-back conditions. Recent knock-in mouse studies (Spencer et al. 2010; Bath et al. 2012) shed light on the finding in women of hormone-sensitive change in hippocampal activation that is particularly pronounced in *BDNF* Met carriers.

4.2.2 Findings of gene-by-ovarian hormone interactions in women converge with pre-clinical studies

Interestingly, estrous cycle sensitivity has been demonstrated in knock-in mice carrying the human Met allele (Spencer et al, 2010; Bath et al, 2012). Specifically, compared with wild-type mice (i.e., Val homozygotes), *BDNF* Met knock-ins displayed enhanced mnemonic function (i.e., object placement) during proestrus (when estradiol levels are high), whereas anxiety-like behaviors (i.e., elevated-plus maze, open field) were increased during estrus compared with wild-type mice. Our findings in women also demonstrate an estrogen sensitivity in the context of the Met allele, and thus provide an important translational step from these studies in mice by demonstrating that the *BDNF* Met allele-ovarian steroid interaction could have a differential impact on neural circuitry. Harboring a *BDNF* Met allele conveys a robust estradiol-related sensitivity in the hippocampus that may have clinical implications. For example, the Met allele could increase vulnerability to ovarian steroid-related cognitive and mood disorders (Epperson et al. 2012). Together these clinical and preclinical findings underscore the physiological relevance of the convergence of estrogen receptor signaling and BDNF system function.

4.2.3 Mechanisms underlying BDNF-estradiol interactions in the hippocampus

The majority of preclinical studies indicate that both BDNF and estradiol facilitate, rather than inhibit, neural activity in the hippocampus (Huang et al. 2001; Spencer et al. 2008). Estradiol and BDNF activate similar signaling cascades and pathways through estrogen receptors and trkB, respectively (Scharfman et al. 2006), and conjointly influence neural growth, survival, and plasticity in the hippocampus (Henderson et al. 1996; Benraiss et al.

2001). There are several potential mechanisms that could mediate an interaction between the effects of estradiol and BDNF on hippocampal-dependent processes. For example, estradiol increases BDNF function by binding to the ERE on the *BDNF* gene and/or by inducing the BDNF receptor, TrkB (Sorabji et al. 1995). This interaction could have special relevance in the context of the altered function of the BDNF system associated with the Met allele, as demonstrated in transfected hippocampal cultured hippocampal neurons where the *BDNF* Met protein impairs intracellular processing and activity-dependent modulation of BDNF (Egan et al. 2003, Chen et al. 2004). Using gene-targeting strategies, it might be possible to disrupt the ERE within the *BDNF* gene to examine the specific effects of estradiol on *BDNF* gene function and clarify this interaction.

4.2.4 *BDNF and progesterone in the CNS*

Similar to estradiol, there is pre-clinical evidence showing that progesterone regulates BDNF function (Singh et al. 1995; Gonzalez et al. 2004, 2007; Kaur et al. 2007). However, in contrast to what was observed with estradiol in the present study, there were no genotype-related differences in hippocampal function during progesterone addback. Currently, there are no data demonstrating an interaction between *BDNF* Val⁶⁶Met allelic variation and progesterone, and it is possible that the Met carriers (i.e. less efficient activity-dependent BDNF function) are less sensitive to the effects of this ovarian steroid. Preclinical studies have documented the physiologic importance of estradiol's induction of progesterone receptors in brain regions involved in reproductive behavior as well as the hippocampus; this induction increases the effects of progesterone in these regions (Mani et al. 2012). For example, the effects of progesterone on lordosis behavior in the rodent is not observed unless

the animal is first exposed to estradiol (i.e., estradiol priming) to induce progesterone receptors in hypothalamic regions mediating lordosis. Thus, it is possible that if an estrogen priming phase had been used to increase CNS progesterone receptors in the present study, an effect of progesterone on hippocampal function might have been observed. In addition, a larger sample size might also have uncovered BDNF-by-progesterone interactions in the hippocampus.

4.2.5 *Strengths and limitations of the present study*

There are several strengths in the present work. First, the gold standard PET method used in the present experiment remains stable over the extended period required to collect these data and is not susceptible to signal dropout in regions important in this study (e.g., hippocampus). Moreover, the PET method maps rCBF during each cognitive condition separately with entirely independent scans. This feature of the PET rCBF method was important to the present work because the genotype-by-hormone interactions found could reflect rCBF changes in either the 0-back (sensorimotor control task) or 2-back (working memory) conditions, or in both. It was determined that during the 2-back working memory condition alone, the pattern paralleled that seen in the 2-back versus 0-back activation/deactivation analysis, whereas no *BDNF*-by-hormone interaction was observed when the 0-back control condition was analyzed alone. These analyses confirm that the findings of *BDNF*-by-hormone interaction in the hippocampus were specifically related to the cognitive circuit recruited by the n-back working memory paradigm and were not due to non-specific effects such as rCBF changes resulting from vascular changes *per se*.

Second, the six-month hormone manipulation protocol has several advantages over naturalistic studies performed across the menstrual cycle in women. This incisive but clinically difficult protocol allows the investigation of the effects of the absence of ovarian steroid hormones compared with those after the replacement of ovarian steroids in a manner similar to studies performed in lower animals that employ ovariectomy and ovarian steroid replacement. Moreover, the effects of estradiol can be observed separately from those of progesterone which is not possible during the course of the normal menstrual cycle. Additionally, the hormone addback phases were carefully controlled; all women received comparable doses of both estradiol and progesterone, thereby removing a potential confound related to inter-individual variations in the quantities of these hormones secreted across the natural menstrual cycle. Finally, because the level of task performance, itself, could affect brain activity during cognitive activation, between- and within-subject performance differences were minimized by over-training participants on the n-back working memory paradigm and by retraining them before each of the PET scanning sessions.

There are also several limitations that warrant consideration. First, the relatively small sample size, while sufficient to demonstrate genotype-by-hormone effects in the hippocampus that are supported by pre-clinical studies, may have limited the ability to further elucidate the role of ovarian steroids in the PFC findings in Study 1. Second, a larger sample size would have allowed testing for gene network interactions with hormone state and for race-specific genotype-by-hormone effects.

4.2.6 *Implications and future directions*

This work extends previous imaging studies, some (Egan et al. 2003; Hariri et al. 2003), but not all (Cerasa et al. 2010; Dennis et al., 2011), of which have reported a relationship between *BDNF* allelic variation and hippocampal engagement, by demonstrating that effects of *BDNF* genotype on hippocampal function can be modulated by gonadal steroids. Since the present findings showed that the abnormal hippocampal activation associated with the Met allele was only observed in the presence of estradiol, it is possible that genotype effects could be obscured by the inclusion of women if they were in differing reproductive states or phases of the menstrual cycle with different levels of estradiol. Thus, these data emphasize that interpreting the effects of genotype on brain function in women requires knowledge of the ovarian steroid hormone milieu, and that, conversely, it is crucial to consider *BDNF* genotype when investigating the effects of ovarian steroids on cognitive and behavioral processes.

These findings have relevance both to women's health and to the long-standing effort to understand individual differences in the effects of ovarian steroids on behavior and brain function. Data such as these may also inform the search for substrates of risk for endocrine-related mood and cognitive disorders and for mechanisms of sex-specific expressions of neuropsychiatric illness. Future research should focus on exploring the relevance of the interaction between *BDNF* genotype and ovarian steroids on the pathophysiology of these disorders, and on investigating potential gene-gene interactions, such as those between *BDNF* genotype and other ovarian steroid-regulated genes (e.g., *catechol-o-methyl transferase*). In addition, the interactive effects of *BDNF* and gonadal steroids on hippocampal functional connectivity remain to be defined. Finally, since the

hippocampal signal in the present study consisted of a working memory-related deactivation, a cognitive neuroimaging probe that actively recruits the hippocampus, such as a spatial navigation task, may further elucidate the interactive relationship between these two crucial neuromodulators.

4.3 Overall Thesis Summary

Numerous studies have demonstrated modulatory effects of *BDNF* functional polymorphism and ovarian steroids both pre-clinically and in humans. The data in this thesis demonstrate that these influences on cellular and circuit-level functions are interactive. Study 1 showed that *BDNF* Met carriers have abnormally increased basal, resting activity in PFC and hippocampal regions. Additionally, there were sex-by-genotype interactions on rCBF as well as on functional connectivity in these regions. This was the first study in humans to demonstrate a *BDNF* genotype-sex interaction on resting brain activity, but the specific contributions of ovarian hormones could not be determined. Study 2 further explored these interactions with the use of a cognitive activation neuroimaging probe in conjunction with a pharmacological hormone manipulation protocol in women, and found an interaction of ovarian hormones and *BDNF* genotype on hippocampal function that reflected estradiol sensitivity in Met carriers. These studies pave the way for future basic and clinical investigation to clarify the mechanisms of these effects and their implications for cognitive and other neuropsychiatric disorders. Additionally, future studies should address the possibility that genotype modulates the effects of sex steroids on brain development during puberty.

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