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Physiological Effects of Estradiol in the Mouse Hippocampal Formation

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PHYSIOLOGICAL EFFECTS OF ESTRADIOL IN THE MOUSE HIPPOCAMPAL FORMATION

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At several points in a woman's life, changes in circulating estradiol are associated with disturbances in mood and cognitive function. To determine the biological basis of these behavioral changes, researchers have concentrated on the hippocampal formation, a medial temporal lobe structure involved in the regulation of mood and cognition in humans. It is now clear that estradiol increases the substrates of hippocampal synaptic plasticity, including dendritic spine density, synapse density, and synaptic protein expression. In some cases, these changes are associated with alterations in mood and hippocampal-dependent learning and memory. The upstream mediators of these estradiol effects remain unknown, but likely candidates may be inferred from known regulators of hippocampal synaptic plasticity and estradiol effects in other tissues.

This thesis explored estradiol activation of two of these candidates, PI3 kinase/Akt and brain-derived neurotrophic factor (BDNF), in the dorsal hippocampus of female mice. In naturally cycling and ovariectomized

female mice, estradiol increased hippocampal Akt and BDNF signaling as determined by immunocytochemistry and in situ hybridization. These effects were associated with changes in synaptic protein expression, and required specific estrogen receptor isoforms, as demonstrated using estrogen receptor knockout mice. Immuno-electron microscopy revealed that estradiol specifically increased signaling through presynaptic TrkB receptors in Schaffer collateral and mossy fiber axons. Estradiol effects on Akt signaling were associated with changes in spatial memory ability, and were profoundly altered in mice expressing the BDNF variant Val66Met.

This study therefore identifies Akt and BDNF signaling as important upstream pathways in the control of hippocampal function by ovarian steroids *in vivo*. Circulating estradiol activates Akt rapidly through a BDNF-dependent mechanism, and increases expression of BDNF more slowly, leading to increased activation of the BDNF receptor TrkB. After activation, Akt and TrkB may participate in estradiol regulation of spine synapse density and hippocampal cell excitability. Future studies should aim to understand the specific role of Akt and BDNF in estradiol modulation of hippocampal function and behavior.

In loving memory of my mother

Dr. Susan Spencer, M.D.

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Chapter 1

Introduction

In females, the sex hormone estradiol is released by the ovaries into the circulation, and passes through the blood-brain-barrier to act directly on the brain to control behavior. Estradiol acts on brain regions controlling both reproductive behaviors, and non-reproductive behaviors, such as mood and cognition. One of the most studied of these regions is the hippocampal formation, a major center for the regulation of cognition and mood in mammals. This brain region has been implicated in the pathology of many human neuropsychiatric disorders, including Alzheimer's disease, age-related cognitive decline, depression, and bipolar disorder (Small et al., 2004; deToledo-Morrell et al., 2007; Frey et al., 2007; Maletic et al., 2007). Estradiol may be an important factor controlling hippocampal function and susceptibility to these disorders.

Estradiol affects hippocampal function in animals and humans

In rodents and nonhuman primates, the function of the dorsal hippocampal formation (known as the posterior hippocampal formation in

humans and hereafter referred to as the hippocampal formation) is often assessed using (among others) tests of spatial learning and memory, which animals with hippocampal lesions are unable to perform successfully (Squire, 1992; Packard and McGaugh, 1996; Bannerman et al., 2004; Good et al., 2007). In humans, the hippocampus is essential for spatial navigation, and declarative and episodic memory, and its function is often assessed using tests of verbal recall (Squire, 1992). Studies of laboratory rodents, nonhuman primates, and humans performing these hippocampal-dependent tasks have shown that estradiol generally enhances hippocampal function.

Suppression of ovarian function causes a decrement in hippocampal-dependent learning and memory, suggesting that endogenous estradiol maintains hippocampal function. For example, in rats, ovariectomy impairs object recognition memory, and estradiol replacement corrects this deficit (Luine et al., 2003; Wallace et al., 2006). Similarly, ovariectomy impairs performance of rats on the hippocampal-dependent active avoidance paradigm, a deficit corrected by 5 weeks of estradiol replacement (Singh et al., 1994). Young adult women given a gonadotropin releasing hormone agonist to suppress ovarian function exhibit verbal memory deficits that can be corrected with estradiol replacement (Sherwin and Tulandi, 1996; Berman et al., 1997).

Evidence that the estradiol enhancement of these tests indeed represents direct effects on hippocampal function comes from studies showing enhanced performance with estradiol infusion directly into the hippocampus, but not other brain regions (Packard and Teather, 1997b; Zurkovsky et al., 2006). Estradiol's ability to enhance performance on hippocampal-dependent learning and memory tasks in female rats (Daniel et al., 1997; Luine et al., 1998; Korol and Kolo, 2002; Sandstrom and Williams, 2004; Frye et al., 2007), mice (Li et al., 2004; Xu and Zhang, 2006), and rhesus monkeys (Lacreuse et al., 2002; Rapp et al., 2003a) may be due in part to an improvement in memory consolidation, because estradiol treatment immediately after training on spatial memory tasks still enhances performance on subsequent test trials (Packard and Teather, 1997a; Luine et al., 2003; Gresack and Frick, 2006).

Natural fluctuations in circulating estradiol also affect performance on hippocampal-dependent tasks. In female rats, spatial memory fluctuates across the estrous cycle, with the best performance during proestrus when circulating estradiol is high (Korol et al., 2004; Frye et al., 2007). In women, some studies have found that verbal memory and hippocampal activation fluctuate across the menstrual cycle and correlates positively with serum estradiol levels (Rosenberg and Park, 2002; Sherwin, 2003; Dreher et

al., 2007; Protopopescu et al., 2008). This suggests that natural increases in circulating estradiol also enhance hippocampal function in rodents and humans.

In addition to the large literature suggesting that estradiol enhances hippocampal function, some studies have found impaired learning and memory with increased circulating estradiol. In one such study,, rats showed impaired spatial learning in the Morris water maze task during proestrus (Warren and Juraska, 1997). Spatial abilities were also impaired in women during the midluteal phase of the menstrual cycle (high circulating ovarian steroids) relative to the menstrual phase (low circulating steroids) (Hampson, 1990). From estradiol replacement studies it is clear that many factors govern how estradiol will influence learning and memory, including the treatment paradigm and the specific demands of the cognitive task (Frick, 2009).

Estradiol enhances hippocampal synaptic plasticity

The discovery that estradiol enhances hippocampal function pointed to the possibility that it might directly affect hippocampal synaptic plasticity.

Synaptic plasticity includes experience-dependent modifications that are increasingly implicated in learning and memory. These include structural modifications of spine synapses, electrical long-term potentiation, and increased expression of key synaptic proteins (Engert and Bonhoeffer, 1999; Muller et al., 2000; Whitlock et al., 2006; Yashiro and Philpot, 2008; Costa-Mattioli et al., 2009). Indeed, estradiol targets all of these forms of synaptic plasticity.

Spine structure modification. In female rats, ovariectomy decreases the density of dendritic spines in the CA1 stratum radiatum of the hippocampus, an effect rescued by 72 hours of estradiol replacement (Gould et al., 1990; Woolley et al., 1990; Woolley and McEwen, 1992). Estradiol also increases spine density in rat primary hippocampal neurons in culture, where 48 hours of exposure to 0.1 μg/mL estradiol doubles the dendritic spine density (Murphy and Segal, 1996). A similar fluctuation of spine and synapse density also occurs across the estrous cycle: rats in proestrus (high circulating estradiol) show 30% higher spine densities in the CA1 region than rats in estrus (low circulating estradiol) (Woolley et al., 1990). Proestrus rats also have a higher proportion of spines with large heads, or "mushroom" shapes (Gonzalez-Burgos et al., 2005). In mice, estradiol treatment increases the density of these mushroom spines in the CA1 stratum

radiatum (Li et al., 2004). Mushroom spines have a larger head, accommodating larger and more complex post-synaptic densities (Bourne and Harris, 2008). They are widely considered to be functionally stronger and more stable than other spine types, and their density increases with learning (Tada and Sheng, 2006; Hongpaisan and Alkon, 2007; Bourne and Harris, 2008). The effects of estradiol on total and mushroom spine density suggest that it facilitates both the formation of new spine synapses, and the strengthening of existing connections. Indeed, electron microscopy studies showed that estradiol does indeed increase synapse density, measured as an increase in excitatory-type synaptic contacts and multi-synaptic boutons, in female rats (Woolley and McEwen, 1992; Woolley et al., 1996). Estradiol also enhances ultrastructural correlates of synapse strength, including postsynaptic density thickness and synaptic vesicle number, in the CA1 of female mice (Xu and Zhang, 2006).

Excitability and long-term potentiation. The first report on the modulation of hippocampal electrical activity by ovarian steroids was published almost forty years ago, describing electrical changes in the limbic system across the estrous cycle of the female rat (Terasawa and Timiras, 1968). Since then, other groups have demonstrated that estradiol increases hippocampal excitability in the rat (Kim et al., 2006; Scharfman and

MacLusky, 2006; Smith and McMahon, 2006). Estradiol also enhances long-term potentiation (LTP) in awake rats (Cordoba Montoya and Carrer, 1997) and cultured hippocampal slices (Foy et al., 1999; Foy et al., 2008). Across the estrous cycle, hippocampal excitability and LTP at CA3-CA1 synapses are enhanced during proestrus compared to other cycle stages in anesthetized female rats (Warren et al., 1995; Good et al., 1999; Scharfman et al., 2003). Together, these findings suggest that both physiological and pharmacological increases in circulating estradiol increase hippocampal excitability and enhance long-term potentiation in female rats.

Synaptic protein expression. Excitatory synapses on hippocampal pyramidal cells contain a host of specialized synaptic proteins important for synaptic function. At the postsynaptic side, these include the scaffolding protein PSD-95 and actin-binding protein spinophilin, both involved in activity-dependent spine synapse dynamics (El-Husseini et al., 2000; Feng et al., 2000; Ehrlich et al., 2007). Presynaptically, synaptophysin is a synaptic vesicle protein commonly used to localize synapses and implicated in spatial memory ability (Smith et al., 2000; Valtorta et al., 2004), and syntaxins are a family of SNARE proteins involved in synaptic vesicle release. Estradiol has been shown to increase the expression of these proteins in mice, rats, and nonhuman primates. Forty-eight hours of estradiol treatment increases

immunoreactivity for all four synaptic proteins in the rat hippocampus (Brake et al., 2001; Lee et al., 2004b; Waters et al., 2006). In mice, six days of estradiol treatment increases PSD-95, spinophilin, and syntaxin expression in all hippocampal subregions (Li et al., 2004). One month of estradiol treatment increases synaptophysin, syntaxin, and spinophilin expression in the CA1 strata radiatum and oriens of ovariectomized female rhesus monkeys (Choi et al., 2003). Estradiol likely acts directly on neurons to increase the expression of these synaptic proteins, since estradiol treatment increases PSD-95 expression in the NG108-15 neuroblastom cell line *in vitro* (Akama and McEwen, 2003).

Neurotransmitter systems. NMDA receptors are ubiquitously found at excitatory synapses. They are essential for long-term potentiation and learning-induced dendritic spine dynamics (Miyamoto, 2006; Fedulov et al., 2007). Ovariectomy causes a decrease NMDA receptor binding and expression of the NR1 and NR2B receptor subunits in the rat hippocampus (Cyr et al., 2001b; Cyr et al., 2001a), and estradiol replacement increases these measures (Weiland, 1992a; Gazzaley et al., 1996; Woolley et al., 1997; Romeo et al., 2005). Estradiol's effects on NMDA receptors may be important for it to increase dendritic spine density and LTP, as both these effects are blocked by NMDA receptor antagonists (Woolley and McEwen,

1994; Smith and McMahon, 2006). Estradiol also modulates inhibitory inputs in the hippocampus, inducing a transient decrease in GABAergic inhibition followed by a recovery of the inhibition and an increase in GAD expression (Weiland, 1992b; Murphy et al., 1998; Rudick and Woolley, 2001). The effects of estradiol on GABAergic transmission may be specific to different subtypes of GABAergic neurons (Nakamura and McEwen, 2005; Scharfman and Maclusky, 2006; Hart et al., 2007; Nakamura et al., 2007). Finally, estradiol enhances cholinergic transmission by acting directly in the hippocampus and also on hippocampal afferents originating in the basal forebrain (Gibbs et al., 1994; Gibbs, 1997; Daniel and Dohanich, 2001; Marriott and Korol, 2003; Rudick et al., 2003). Cholinergic transmission is essential for estradiol to enhance hippocampal-dependent memory (Daniel and Dohanich, 2001).

In sum, estradiol enhances hippocampal synaptic plasticity in multiple domains. Its effects on dendritic spine dynamics, electribal activity, and protein expression in neurons serve to augment hippocampal cell excitability in general, and to enhance specific activity-dependent modifications. These effects may underly estradiol's ability to enhance hippocampal-dependent learning and memory. They may also underly the estradiol impairment of learning and memory seen in several studies. Experimenter-induced

synaptic potentiation and increased synapse density, similar to to effect of estradiol, can occlude subsequent hippocampal-dependent learning (Madronal et al., 2007; Barbosa et al., 2008). Indeed, the relationship between spine shape and hippocampal function is not always straightforward: mice missing the postsynaptic scaffolding protein Shank have smaller spines and weaker synaptic transmission, but better hippocapal-dependent learning (Hung et al., 2008).

Although most of the studies of estradiol effects on hippocampal synaptic plasticity were conducted in female rats, conservation of estradiol effects on synaptic protein expression in mice, rats, and nonhuman primates raises the possibility that they are also conserved in humans. This possible human relevance increases the urgency of determining how estradiol signaling leads to its enhancement of synaptic plasticity.

Estradiol acts on estrogen receptors via nuclear- and membrane-intiated signaling

Numerous studies have provided evidence that estradiol effects in the hippocampal formation depend on estrogen receptors (ERs). For example, pharmacological ER antagonism blocks estradiol-mediated pyramidal cell

disinhibition and spinogenesis in the rat hippocampus (McEwen et al., 1999; Rudick et al., 2003). There are two types of classical ER, alpha and beta, which are expressed in the mouse, rat, and monkey hippocampal formation (Shughrue et al., 1997; Weiland et al., 1997; Register et al., 1998; Mitra et al., 2003). Knockout of either receptor in female mice impairs hippocampal-dependent learning (Fugger et al., 2000; Rissman et al., 2002; Day et al., 2005), and both have been implicated in hippocampal spinogenesis and synaptic protein expression (Szymczak et al., 2006; Mukai et al., 2007; Spencer et al., 2008). Nevertheless, these receptors may activate distinct mechanisms. Knockout of ER beta impairs LTP (Day et al., 2005), while knockout of ER alpha but not ER beta eliminates estradiol's ability to increase spinophilin expression (Spencer et al., 2008). Much work remains to discover the distinct actions of each estrogen receptor in the hippocampal formation, and which actions are necessary for estradiol's effects on hippocampal function. The distinction between the roles of ER alpha and ER beta will be especially important as laboratory work is translated to clinical studies. ER alpha and beta have different expression profiles throughout the body and in the brain that will be important in considering the efficacy and side effects of estrogenic agents (Shughrue et al., 1997; Shang and Brown, 2002).

Estrogen receptors engage in two modes of signaling: nucleus- or membrane-initiated steroid signaling (Levin, 2005; Hammes and Levin, 2007; Vasudevan and Pfaff, 2007). In nucleus-initiated signaling, cytoplasmic ERs bound to estradiol translocate to the nucleus and bind estrogen response elements (EREs) in the DNA to influence gene transcription (Hammes and Levin, 2007). In membrane-initiated signaling, extranuclear estrogen receptors affiliated with the plasma membrane or membranous organelles couple to G proteins, growth factor receptors, and intracellular kinases to activate signaling events (Pedram et al., 2006b; Hammes and Levin, 2007; Pedram et al., 2007). The resulting signaling cascades can act on transcription factors to affect gene transcription, or effect more rapid changes independent of new gene transcription (Levin, 2005; Mannella and Brinton, 2006; Pedram et al., 2006a).

Very few nuclear ERs are found in the hippocampal formation. Work in the McEwen laboratory has shown that nuclear estradiol binding and ER alpha-immunoreactivity is found in scattered inhibitory GABAergic interneurons in the rat hippocampal formation (Loy et al., 1988; McEwen and Milner, 2007). In contrast, neurons in the rat hippocampal formation contain no detectable nuclear ER beta, although some can be found in astrocytes (Azcoitia et al., 1999; Milner et al., 2005). This suggests that

estradiol may modulate GABAergic neurotransmission and astrocyte function through nucleus-initiated signaling.

The near-absence of nuclear ER alpha and beta in pyramidal cells, the principal excitatory neurons in the hippocampal formation, suggests that estradiol may signal through membrane-associated receptors in these cells. Indeed, estradiol binding and ultrastructural studies from the McEwen lab and others have identified extranuclear estrogen receptors in neurons of the rat hippocampal formation (Shughrue and Merchenthaler, 2000; Milner et al., 2001; Mitra et al., 2003; Kalita et al., 2005; Milner et al., 2005; Milner et al., 2008). Electron microscopy studies have revealed distinct distributions of the two receptor subtypes: ER alpha is found mostly in axons and axon terminals and to a lesser degree in dendritic spines, while extranuclear ER beta is mainly affiliated with the membrane of somata and dendrites of principle cells (McEwen and Milner, 2007). These different distributions indicate that ER subtypes may play different roles in the maintenance of hippocampal function.

Several pieces of evidence suggest that membrane-initiated signaling mediates estradiol effects on hippocampal function. First, many estradiol effects on signal transduction and synaptic potentiation in hippocampal neurons occur too quickly to be mediated by nucleus-initiated signaling.

The effects of estradiol on gene transcription are visible 12-24 hours after hormone exposure (Vasudevan et al., 2001; Gottfried-Blackmore et al., 2007), while signaling pathway activation can be measured from 15 minutes through six hours of continuous estradiol exposure (Akama and McEwen, 2003; Abraham et al., 2004; Lee et al., 2004a; Yuen, 2006; Fernandez et al., 2008). In keeping with these rapid effects, estradiol enhancement of spatial memory can be achieved by administration immediately, but not two hours, after training, suggesting a rapid effect of the hormone on memory consolidation during this narrow two-hour time window following training (Packard and Teather, 1997a; Luine et al., 2003; Gresack and Frick, 2006). In addition, estradiol increases PSD-95 and spinophilin expression through a post-transcriptional mechanism, suggesting the involvement of membrane-initiated signaling (Piva et al., 1995; Quinones-Jenab et al., 1997; Akama and McEwen, 2003; Lee et al., 2004b). Finally, compounds designed as antagonists at nuclear ERs have in fact shown some estrogenic properties in the brain. For example, the estrogen receptor antagonist ICI 182,780 acts as an agonist to enhance spatial learning when infused directly into the hippocampus of female rats (Zurkovsky et al., 2006). Low doses of 17-alpha estradiol, an isomer of the natural 17-beta estradiol, have no effect on lipid metabolism or uterine weight but rapidly enhance spatial memory and

increase CA1 spine synapse density in ovariectomized rats within four hours of subcutaneous injection (Lundeen et al., 1997; MacLusky et al., 2005). This evidence suggests that these compounds act through mechanisms other than the classical nucleus-initiated signaling to achieve these effects in the brain.

The evidence gathered therefore points to both rapid and delayed effects of estradiol in different hippocampal cell types, with an emphasis on the importance of extranuclear ERs in hippocampal pyramidal cells. In addition to the classical ERs alpha and beta, novel membrane ERs such as the ER-X, (Toran-Allerand et al., 2002) and the G-protein receptor, GPR30 (Bologa et al., 2006), may participate. Thus it is clear that many questions still remain in the investigation of how estradiol affects hippocampal function.

Mechanism of estradiol enhancement of hippocampal synaptic plasticity

Because of the numerous types of ER and their multiple modes of signaling, the mechanism of estradiol enhancement of hippocampal function is undoubtedly complex. Elucidation of the key upstream mediators will be an important step toward understanding this process. Studies conducted

mostly in cultured hippocampal neurons have identified several candidate mediators, including the extracellular signal-related kinase (ERK) pathway, phosphoinositol-3-kinase (PI3K) pathway, and brain-derived neurotrophic factor (BDNF).

ERK and CREB. Estradiol treatment leads to activation of the cyclic AMP response element binding protein in the dorsal hippocampus of female rats and mice (Bi et al., 2001; Abraham et al., 2004; Zhou et al., 2005). In cultured primary and immortalized neurons, estradiol activates CREB via the ERK pathway (Wade and Dorsa, 2003; Lee et al., 2004a; Mannella and Brinton, 2006; Szego et al., 2006). This mechanism may also operate *in vivo*, as estradiol rapidly activates ERK in the mouse hippocampus, and ERK2 activation fluctuates across the estrous cycle in female rats (Bi et al., 2001; Fernandez et al., 2008). The importance of the ERK pathway in synaptic plasticity and memory (Sweatt, 2004) suggest that estradiol activation of MAPK/CREB may be responsible for some of its effects on hippocampal synaptic plasticity and behavior. CREB phosphorylation has been implicated in estrogen-induced spine formation and upregulation of synaptic protein expression in primary cultured hippocampal neurons (Murphy and Segal, 1997; Zhao et al., 2005). Moreover, hippocampal

infusion of an ERK inhibitor blocks estradiol enhancement of an object recognition task in female mice (Fernandez et al., 2008).

PI3K/Akt. The PI3Ks are a family of protein kinases implicated in a wide array of neuronal functions, including cell survival and long-term potentiation (Sanna et al., 2002; Sui et al., 2008; Yang et al., 2008). They catalyze the phosphorylation of phosphoinositols to form signaling intermediates such as phosphatidylinositol-3,4,5-triphosphate (PIP3). The formation of these intermediates leads to activation of several serine-threonine kinases including Akt (also known as Protein Kinase B). The well-known role of Akt in cell survival has led several investigators to hypothesize and demonstrate the importance of this pathway in estradiol-mediated neuroprotection (Du et al., 2004; Suzuki et al., 2006; Morissette et al., 2007). More recently, the McEwen lab and others have begun to elucidate a specific role for Akt in estradiol regulation of spine synapse remodeling and synaptic protein expression. Estradiol activates Akt in the NG108-15 neuroblastoma cell line, and PI3K/Akt signaling in these cells is important for estradiol induction of PSD-95 expression (Akama and McEwen, 2003; Yuen, 2006). This induction occurs rapidly via an increase in protein translation. Estradiol also activates Akt in the CA1 stratum radiatum of the rat hippocampus. Immunoreactivity for phosphorylated Akt

(pAkt), the activated form, increases in the CA1 stratum radiatum of proestrus and estradiol-replaced rats relative to ovariectomized rats and rats in other estrous cycle stages (Znamensky et al., 2003).

BDNF/TrkB. BDNF, part of the neurotrophin family, is a peptide critical for neuronal differentiation, growth, and survival in the developing nervous system. In the adult hippocampus, BDNF supports protein-synthesis-dependent LTP, spine structure modification, and memory (Kang and Schuman, 1995; Messaoudi et al., 2002; Bekinschtein et al., 2008; Tanaka et al., 2008). The numerous similarities between the effects of estradiol and BDNF on hippocampal physiology and behavior have led several investigators to hypothesize a role for BDNF in estrogen effects on this brain region (Scharfman et al., 2003; Scharfman and Maclusky, 2005, 2006; Sohrabji and Lewis, 2006). Estradiol increases BDNF mRNA and protein expression in the hippocampus of ovariectomized rats (Gibbs, 1998; Jezierski and Sohrabji, 2001; Scharfman et al., 2003; Zhou et al., 2005; Scharfman et al., 2007). In addition, levels of BDNF mRNA and protein fluctuate across the estrous cycle in female rats (Gibbs, 1998), with the highest protein expression during proestrus (Scharfman et al., 2003; Scharfman et al., 2007).

There are several possible mechanisms by which estradiol may increase BDNF expression. A putative ERE has been identified in the rat BDNF gene (Sohrabji et al., 1995), whose sequence is preserved in mouse and human, that binds estrogen receptor-ligand complexes in vitro. Estradiol could in this way activate transcription of the BDNF gene in hippocampal interneurons containing nuclear ERs. In hippocampal pyramidal cells that do not contain nuclear ERs, estradiol activation of another transcription factor could increase BDNF expression. One possibility is the cyclic AMP response element binding protein (CREB), which is phosphorylated by estradiol in hippocampal neurons (Abraham et al., 2004; Lee et al., 2004a; Szego et al., 2006). CREB activates transcription at cyclic AMP response element (CRE) sites in DNA, one of which is contained in the BDNF promoter region (Tao et al., 1998). Estradiol activation of CREB via membane-associated ERs could therefore activate BDNF gene transcription via its CRE site.

BDNF signals through high-affinity interaction with its specific receptor, tropomyosin-related kinase B (TrkB), and low-affinity interaction with the P75 neurotrophin receptor (P75NTR). Activation of TrkB is involved in spatial memory formation in rats (Mizuno et al., 2003a), raising the plausible hypothesis that TrkB signaling may mediate the effects of E-

induced BDNF expression on spatial memory. In addition, signaling through TrkB leads to Akt activation (Chao, 2003; Schratt et al., 2004), and postsynaptic knockout of TrkB decreases a mature subset of spines in the mouse hippocampal CA1 region (Chakravarthy et al., 2006), suggesting that TrkB activation could amplify estrogen effects on Akt activation and facilitate spine maturation. Indeed, a few reports provide evidence for TrkB signaling as a mediator of estradiol effects in the brain. In rat hippocampal slices, TrkB inhibitors block the estradiol-mediated increase in hippocampal excitability, PSD-95 expression, and dendritic spine formation (Scharfman et al., 2003; Sato et al., 2007). Taken together these findings demonstrate that in the female rat, estradiol increases BDNF expression, and that BDNF signaling through TrkB may contribute to estradiol enhancement of hippocampal synaptic plasticity.

Rodent models

Most of the *in vivo* research on the mechanism of estradiol effects in the hippocampal formation has been conducted in rats. Despite the historical utility of the rat for these investigations, the mouse is an attractive model for *in vivo* work because of the ease of genetic manipulation, which

affords opportunities for direct mechanistic investigation. For example, one group investigated the importance of ERs in CREB activation using estrogen receptor knockout mice. They found that ER beta, but not ER alpha,was necessary for estradiol to activate CREB in the CA1 stratum radiatum (Abraham et al., 2003). Despite the clear utility of these knockout mice, and the easy availability of many other mouse models with useful genetic manipulations, few investigators currently study estradiol effects in the mouse hippocampus.

Previous work has uncovered a few differences in the effects of estradiol in rat and mouse that may inhibit investigators from crossing species. For example, while estradiol increases synaptic protein expression most potently in the CA1 region of the rat hippocampus, it induces a widespread increase in synaptic protein expression throughout the mouse hippocampal formation (Brake et al., 2001; Li et al., 2004). Additionally, although estradiol increases the total dendritic spine density in the rat (Gould et al., 1990; Gonzalez-Burgos et al., 2005), it increases only the density of "mushroom" shaped spines in the mouse CA1 (Li et al., 2004). Finally, in the dentate gyrus, neurogenesis increases after estradiol treatment in the rat, but not the mouse (Lagace et al., 2007; Galea, 2008).

Though these mouse/rat differences in estradiol sensitivity may be real and important, the many similarities in estradiol effects on spine shape, synaptic protein expression, spatial memory, and ERK/CREB activation suggest that the mechanisms of estradiol effects may be similar in mouse and rat. Akt and BDNF are therefore very promising candidate mediators of estradiol effects in mice based on their sensitivity to estradiol in both hippocampal culture and rat hippocampus *in vivo*. I set out to investigate whether these two molecules are affected by estradiol in the mouse hippocampal formation.

Study design

In rats, estradiol effects on hippocampal Akt and BDNF have been seen with natural increases in circulating estradiol during proestrus, and with estradiol injection after ovariectomy (Scharfman et al., 2003; Znamensky et al., 2003; Scharfman et al., 2007). The primary goal of the work in this thesis was to understand the effects of estradiol on Akt and BDNF in the mouse hippocampal formation. Both naturally cycling and ovariectomized mouse models were used to investigate the effects of natural and pharmacologic increases in circulating estradiol. Akt activation, BDNF

expression, and TrkB expression and activation were measured in the dorsal hippocampal formation using immunocytochemistry and in situ hybridization. The relationship of estradiol effects on Akt and BDNF signaling to downstream effects on expression of the synaptic protein, PSD-95, was determined using immunocytochemistry. The studies took advantage of pre-existing transgenic mouse lines to explore the mechanisms of estradiol effects on these endpoints. Mice with targeted deletions of each receptor were used to determine role of estrogen receptors alpha and beta in these effects using. Electron microscopy was used to study the ultrastructural details of estradiol effects on TrkB activation. In the final study, I considered how a common human polymorphism in the BDNF gene, Val66Met, affects cognitive behavior and the natural fluctuation of these molecular endpoints across the estrous cycle.

Chapter 2

A murine model to study estradiol effects in the hippocampus

C57Bl/6J mice were chosen for all the following studies based on their common use for the back crossing of transgenic lines. For the most complete study of estradiol effects in the hippocampal formation, studies were designed using both naturally cycling mice and ovariectomized mice replaced with estradiol. However, there were no papers in the literature with a detailed description of the characteristics of the estrous cycle in this mouse strain. This was therefore the necessary starting place to be able to meaningfully study estradiol effects in the mouse hippocampal formation. For a detailed characterization of the estrous cycle in these mice, cycles were monitored for several weeks using vaginal smear cytology. The mice were then sacrificed in difference estrous cycle stages for measurements of uterine weights and circulating levels of estradiol and progesterone.

In addition to the characteristics of the estrous cycle, the location of estrogen receptors in the hippocampal formation is essential information in order to draw conclusions about how estradiol affects this brain region. The

first localization of estrogen receptors in the mouse hippocampus, using light microscopy, suggested similarities with the rat (Mitra et al., 2003). Extensive extranuclear ER beta was found, most notably in fibers in the CA1 stratum radiatum and CA3 stratum lucidum. In contrast, extranuclear ER alpha was light and not easily discernable using light microscopy, but scattered nuclear ER alpha was found throughout the hippocampal formation that could indicated expression in interneurons, similar to the rat. In order to make more meaningful comparisons between the species, I collaborated with Dr. Teri Milner and Katherine Mitterling at Weill Cornell Medical College to perform an ultrastructural localization of ERs alpha and beta in the mouse hippocampal formation.

C57BL/6J mice have regular cycles with predictable hormone levels and uterine weights

The mice had uniform estrous cycles with an average length of 6.1 +/- 0.30 days (Figure 2.1). Mice spent an average of 1.1 days in proestrus, 2.5 days in estrus, and 2.5 days in diestrus. Uterine weights and plasma hormone measurements were conducted on mice sacrificed in proestrus, estrus, and early or late diestrus, termed diestrus 1 and diestrus 2.

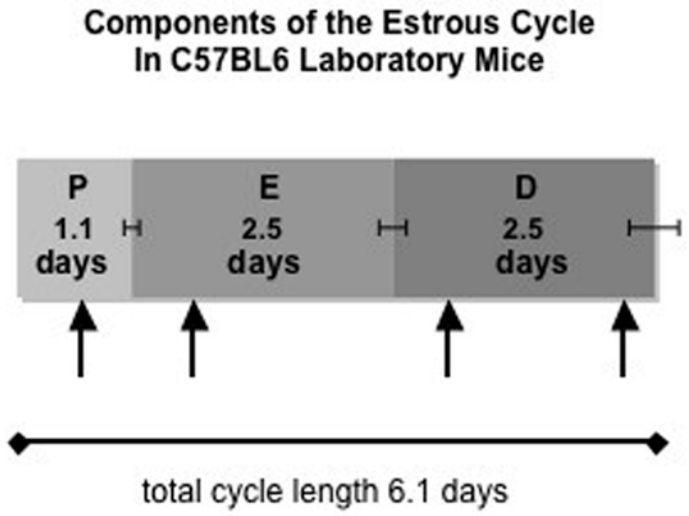


Figure 2.1 C57Bl/6J mice have regular estrous cycles. The C57BL6 female mice used in this study had regular, 6.1-day estrous cycles. As assessed by daily morning vaginal smears, 1.1 days (18%) of the cycle were spent in proestrus, 2.5 days (41%) in estrus, and 2.5 days (41%) in diestrus. Mice perfused as indicated with arrows in proestrus, early estrus, diestrus 1 and diestrus 2 for uterine weight and serum ovarian steroid measurements. Error bars show standard error of the mean.

Estradiol levels peaked during proestrus, and were low in all other stages (Figure 2.2). In contrast, progesterone levels rose during estrus and remained elevated until diestrus 2. Because of the large variation in circulating hormone levels across the cycle, circulating estradiol and progesterone averages are pictured as a schematic.

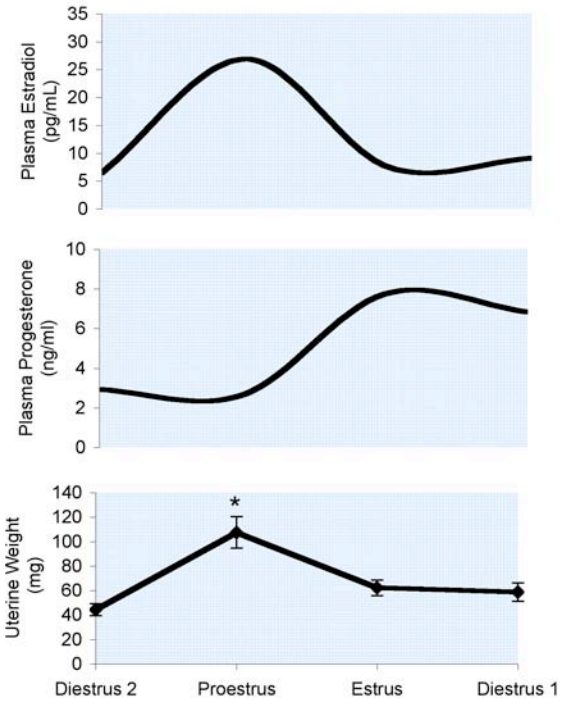


Figure 2.2 Hormone levels and uterine weights fluctuate across the mouse estrous cycle. Average plasma estradiol and progesterone levels are shown as a schematic, due to the large amount of variation among animals, while uterine weights are shown with error bars as standard error of the mean. Uterine weights were significantly increased in proestrus compared to all other cycle stages. N = 3 for diestrus 2, 6 for proestrus, 5 for estrus, and 5 for diestrus 1.

One-way ANOVA comparing uterine weights showed that uteri were significantly heavier in proestrus than in other cycle phases (Figure 2.2; $F(3,15) = 7.793$, $p = 0.0023$, post-hoc tests show proestrus greater than all other groups, $p<0.05$).

The mouse hippocampal formation contains extranuclear ERs

ERs alpha and beta were localized using isoform-specific antibodies with light and electron microscopy. The specificity of the ER antibodies has previously been confirmed using more than one method for each antibody (see Chapter 8, Materials and Methods). Specificity was not tested in ER knockout mice, as these mice were created using targeted disruptions of the ER genes and express some nonfunctional ER peptides that the antibody may recognize.

ER alpha and beta labeling was found almost exclusively outside the nucleus. Light microscopy revealed a few scattered pyramidal cell nuclei with ER alpha labeling, and electron microscopy revealed some ER beta labeling within principal cell perikarya. The electron microscopic localization revealed extensive extranuclear ER alpha and beta immunoreactivity (ir), with differences in the distribution of the two receptors (Tables 2.1 and 2.2).

TABLE 1. Distribution of ERα-Immunoreactive Profiles in the Hippocampal Formation

Hippocampal subregion	Dendritic shafts (n=12)	Dendritic spines (n=12)	Axons (n=12)	Terminals (n=12)	Glia (n=12)	Unknown (n=12)
CA1						
SO	1.00	1.50	2.17	1.67	2.25	1.50
SR (proximal)	1.00	1.92	2.67	1.58	2.08	1.50
SR (distal)	0.67	1.75	2.42	1.33	2.42	1.58
SLM	0.67	0.67	2.42	1.00	2.25	1.25
CA3						
SO	0.50	2.17	2.33	1.33	1.08	0.00
SR	0.25	1.33	1.83	1.08	0.75	0.00
Slu	0.25	0.75	3.58	0.50	1.00	0.08
DG						
OML	0.08	1.33	1.33	1.08	1.17	0.75
MML	0.67	0.92	0.75	1.00	2.08	0.33
IML	0.33	0.67	1.25	0.42	1.75	0.33
HIL	0.17	0.67	2.67	0.75	1.75	0.08
SGZ	0.33	0.50	1.83	0.83	1.83	0.17

Table 2.1 Distribution of ER alpha-immunoreactivity in the mouse hippocampal formation. ER alpha-labeled profiles were counted from a 2,646 μm^2 area in each hippocampal lamina of 12 male and female mice. Numbers represent the average number of each type of labeled profile in each lamina per mouse.

TABLE 2. Distribution of ERβ-Immunoreactive Profiles in the Hippocampal Formation

Hippocampal subregion	Dendritic shafts (n=12)	Dendritic spines (n=12)	Axons (n=12)	Terminals (n=12)	Glia (n=12)	Unknown (n=12)
CA1						
SO	3.33	5.00	4.17	2.33	5.25	0.75
SR (proximal)	3.92	2.92	3.33	1.42	4.42	0.92
SR (distal)	3.58	3.58	3.00	1.58	4.92	0.42
SLM	3.00	1.25	2.42	1.33	5.17	0.92
CA3						
SO	2.83	2.42	3.33	0.67	6.00	0.08
SR	2.08	2.33	3.25	0.83	4.17	0.00
Slu	2.17	1.25	4.08	0.67	2.25	0.00
DG						
OML	2.50	1.42	2.75	1.00	4.25	0.58
MML	2.58	1.00	2.33	1.25	5.00	0.58
IML	3.08	1.08	2.92	0.83	4.33	0.67
HIL	1.50	0.50	3.08	1.75	3.67	0.58
SGZ	1.83	0.83	2.42	1.17	3.75	1.08

Table 2.2 Distribution of ER beta-immunoreactivity in mouse hippocampal formation. ER beta-labeled profiles were counted from a 2,646 μm^2 area in each hippocampal lamina of 12 male and female mice. Numbers represent the average number of each type of labeled profile in each lamina per mouse.

Both ERs were found at synapses, including postsynaptic dendritic spines and presynaptic axon terminals (Figures 2.3 and 2.4). ER alpha was very prominent in axons and glia, and less prominent in dendrites. Dendritic ER beta-ir was much more common than dendritic ER alpha-ir. ER beta was also abundant in axons and glial profiles.

Figure 2.3 Extranuclear ER alpha-ir in mouse hippocampal formation. ER alpha immunoreactivity is found in select dendrites, axons, terminals, and glia in males and female mice at all stages of the estrus cycle. (A) ER alpha labeling is seen in a dendritic shaft at the base of a spine (ERa-S; arrowhead) in the middle molecular layer of the dentage gyrus. (B) An ER alpha-labeled dendritic spine is shown making a synapse (curved arrow) with an unlabeled terminal (uT) in CA1 stratum radiatum. An unlabeled spine is also shown (uS). (C) An ER alpha-labeled terminal (ERa-T; arrowhead) is shown making a synapse with an unlabeled dendritic spine (curved arrow) in CA3 stratum oriens. (D) ER alpha labeling is shown in a longitudinal view of an unmyelinated axon (ERa-A) in CA3 stratum radiatum. An unlabeled axon (uA) is also shown for comparison. (E) ER alpha labeling is found in an unmyelinated axon leading to an unlabeled terminal in CA3 stratum lucidum. (F) ER alpha labeling was also found in a myelinated axon (ERa-mA; arrowhead) in CA3 stratum oriens. G. An ER alpha-labeled glia (ERa-g) is found apposed to an unlabeled dendritic spine and an unlabeled terminal in CA1 stratum radiatum. Scale bar, 0.5 μm.

Figure 2.3

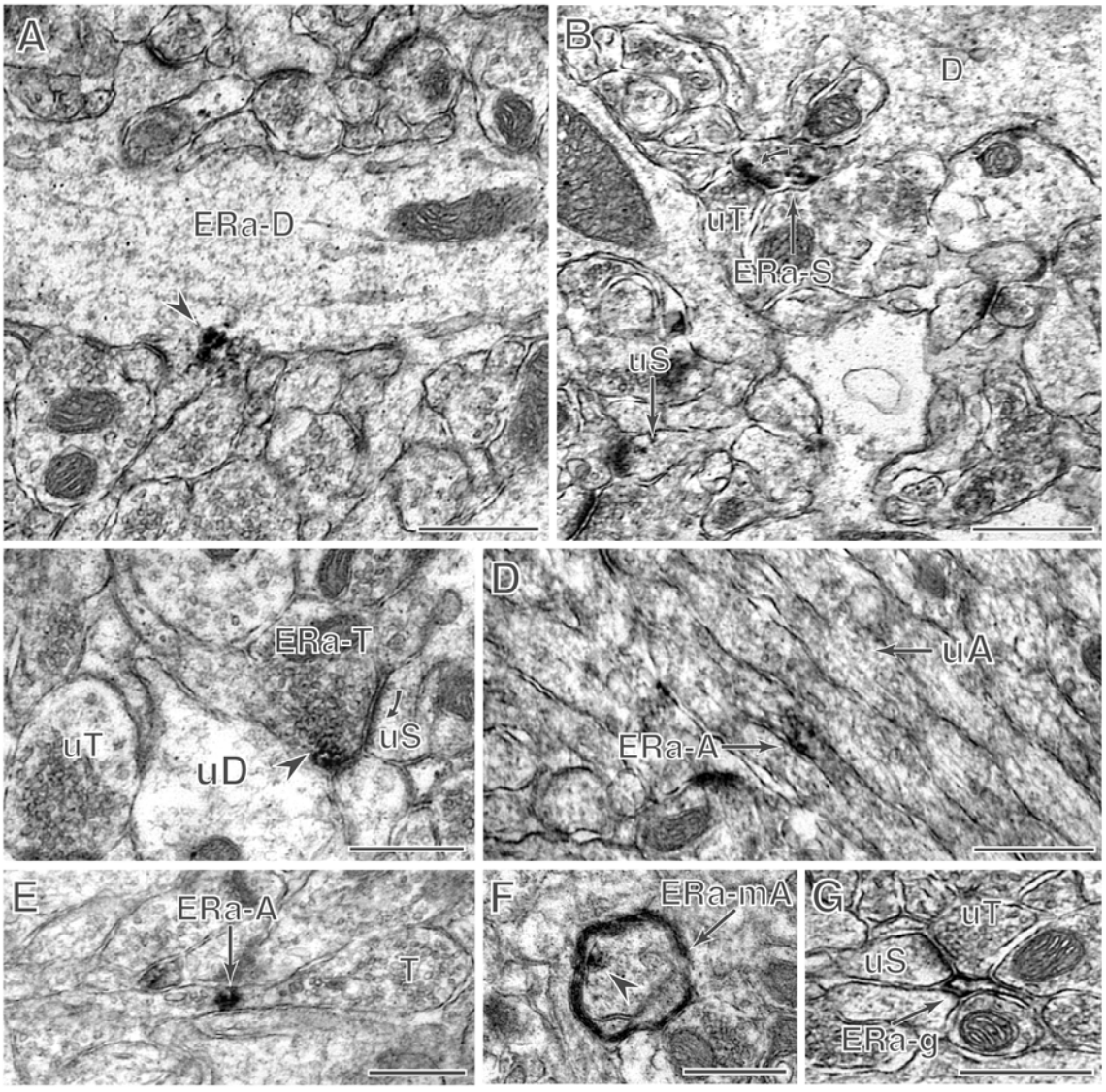
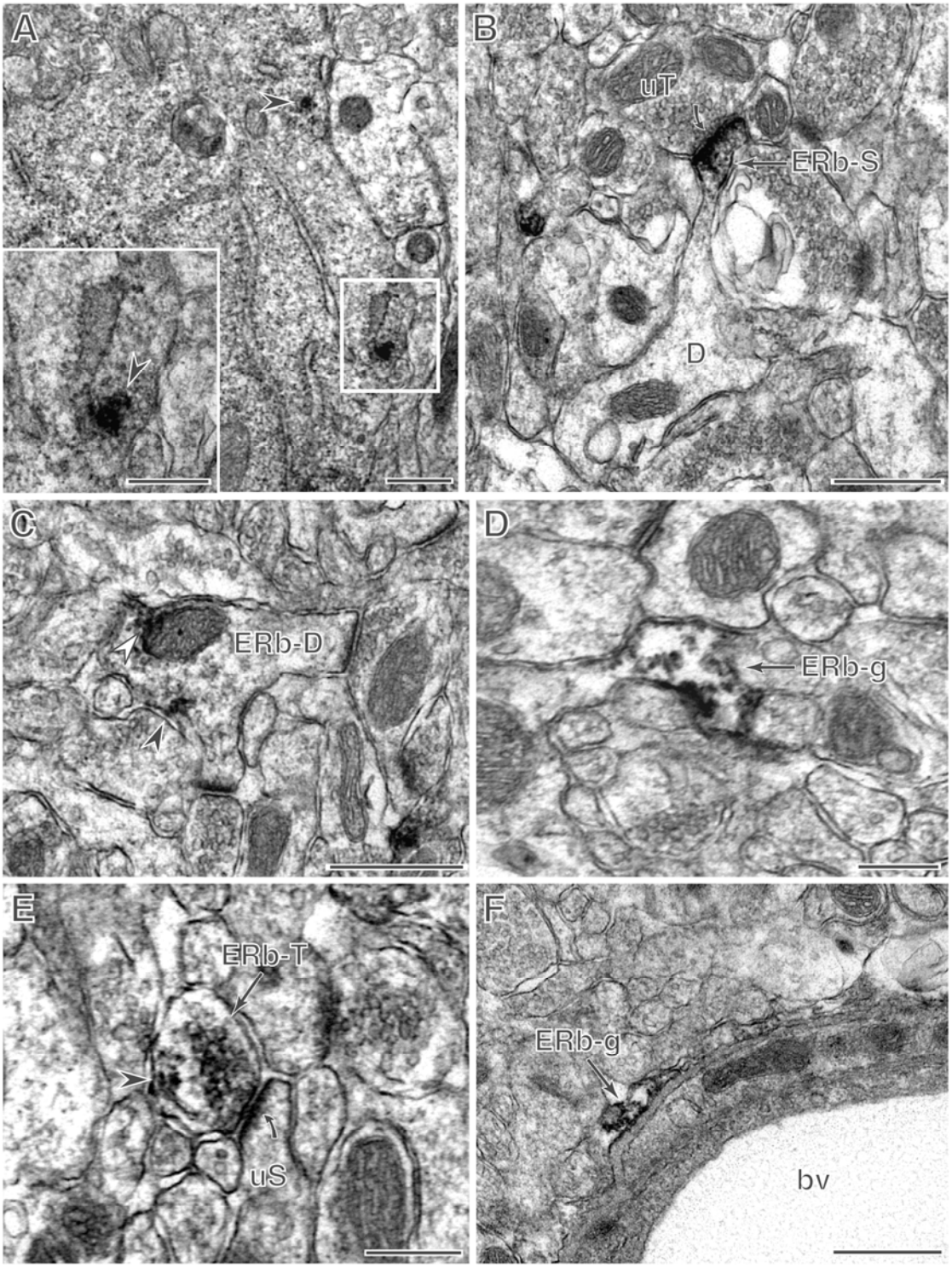

A
ERa-D
B
D
uT
ERa-S
uS
ERa-T
uT
uD
uS
D
uA
ERa-A
E
ERa-A
T
F
ERa-mA
G
uT
uS
ERa-g

Figure 2.4 Extranuclear ER beta-ir in mouse hippocampal formation. (A) ER beta labeling is seen throughout the perikarya (arrowhead) in CA1 stratum radiatum. Inset shows labeling at a higher magnification (Scale bar, 250 nm). (B) An ER beta-labeled spine (ERb-S) of an unlabeled dendrite (uD) makes a synapse with an unlabeled terminal (uT) in CA1 stratum oriens. (C) ER beta labeling is found in a dendrite (ERb-D), both in the cytoplasm (black arrowhead) and on a mitochondrion (white arrowhead), in CA1 stratum radiatum. (D) ER beta labeling is found in a glia (ERb-g) in CA1 stratum radiatum. Scale bar, 250 nm. (E) An ER beta-labeled terminal (ERb-T; arrowhead) is shown making a synapse with an unlabeled spine (uS; curved arrow) in CA1 stratum radiatum (CA1 sr). Bar 250nm F. An ER beta-labeled glia is shown near a blood vessel (bv). All images were taken from CA1 stratum radiatum except where noted. Scale bars, 0.5 μm except where noted. Scale bars, 0.5 μm (A-C) and 0.25 μm (D-F).

Figure 2.4



Discussion

These data suggest that the C57Bl/6J mouse is a suitable animal model for studies based on natural fluctuations in ovarian steroids. Cycle lengths are consistent between and within animals, facilitating the practical aspects of these studies. By definition, an estradiol surge occurs during proestrus that makes the mouse receptive for sexual activity (Turner and Bagnara, 1971a). On the late morning when vaginal smears showed exclusively the nucleated epithelia characteristic of proestrus, plasma estradiol levels in the mice were increased (but variable) and uterine weights were uniformly increased. When circulating estradiol increases, it acts directly on the uterus to increase water content and blood flow within six hours, followed by endometrial cell proliferation and hypertrophy within 12-24 hours (Couse et al., 1995). These two responses were not distinguished in this study, allowing the conclusion that in these mice, estradiol levels began to increase 6-24 hours prior to the late morning of proestrus, when uterine weights were clearly elevated. In rats and CD-1 mice, circulating estradiol begins to rise during late diestrus (Walmer et al., 1992; Staley and Scharfman, 2005). In the current study, late diestrus vaginal cytology was observed 24 hours before proestrus in C57Bl/6 mice. Thus circulating

estradiol likely increased gradually over the 24 hours prior to the late morning of proestrus. This makes the late morning an appropriate time point for the observation of both rapid estradiol effects on signaling pathways and more delayed actions requiring new gene transcription.

The ultrastructural localization of ERs alpha and beta in the mouse hippocampal formation showed that, as in the rat, these receptors are located almost exclusively outside the nucleus (Milner et al., 2001; Milner et al., 2005). The receptors had a distinct but overlapping distribution that could be important for the downstream effects of estradiol in this brain region. The synaptic localization of both receptors positions them for local modulation of synaptic transmission and spine morphology. The relatively greater amount of ER alpha in axons, found in both mouse and rat, suggests that estradiol may activate different pathways in axons and dendrites due to the ratio of ERs found in these processes. Finally, the localization of ERs to glial profiles suggests that distinctions between estradiol effects in neurons and glia would be useful to explore in future studies. Overall, the similarities in the distribution of ER alpha and beta in the mouse and rat hippocampal formation suggest that the effects of estradiol in this brain region will be similar. Any species differences in estradiol effects in the

hippocampal formation may not be attributable to different receptor localizations.

In sum, this chapter puts forward the C57Bl/6J mouse as a useful model for studies of estradiol effects in the hippocampal formation. These mice have regular estrous cycles with expected vaginal cytology, circulating hormone levels, and uterine weights, which will enable studies of natural fluctuations of estradiol. ERs alpha and beta are found primarily in extranuclear locations, well position for membrane-initiated steroid signaling in both neurons and glia.

Chapter 3

Rapid and physiological effects of estradiol on Akt

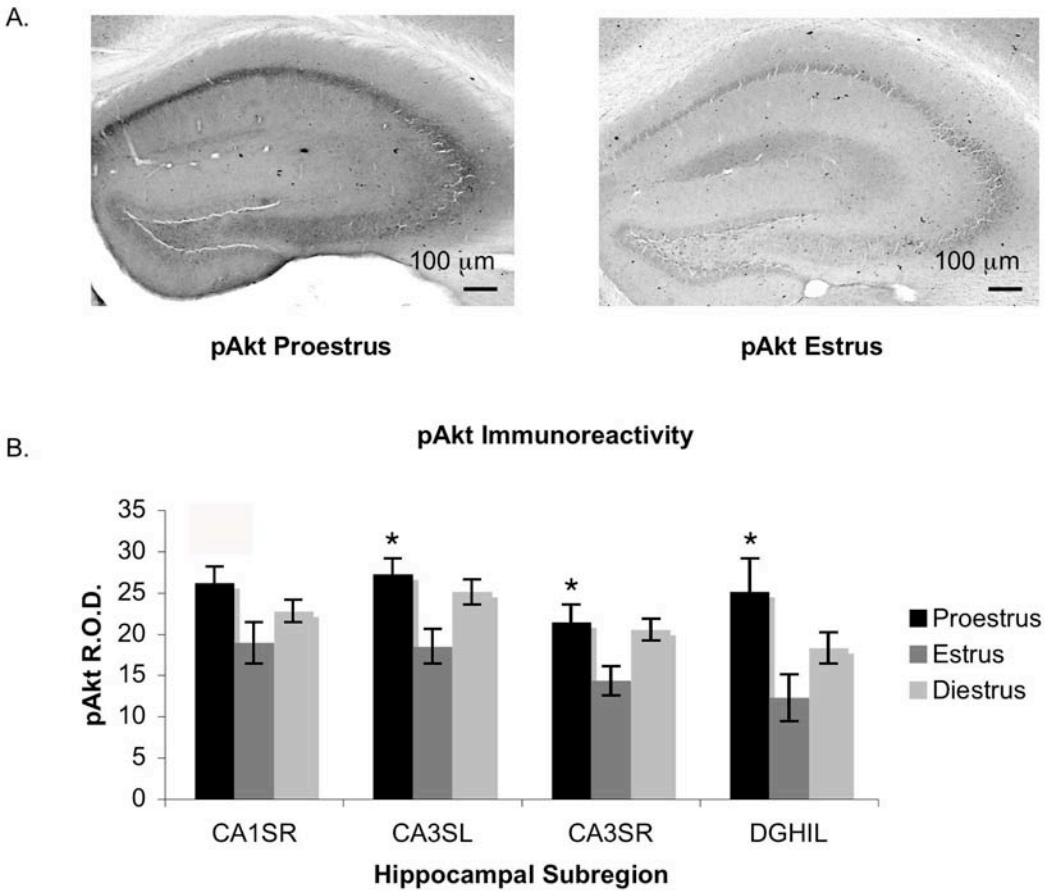
The central hypothesis addressed in this chapter is that estradiol, via membrane-initiated steroid signaling, rapidly activates the PI3K/Akt pathway in the mouse hippocampal formation. Furthermore, this estradiol activation of Akt is associated with a rapid increase in PSD-95 protein expression.

Three approaches were used to test this hypothesis. First, mice in the proestrus, estrus and diestrus stages of the estrous cycle were assayed to determine the effects of natural fluctuations of ovarian steroids on Akt activation. Second, ovariectomized mice, sacrificed 6 hours after injection with estradiol benzoate or vehicle, were assayed to determine the time course of estradiol affects on Akt, and the relationship of Akt activation to PSD-95 expression. Finally, the requirement of ERs alpha and beta in Akt activation was assessed using transgenic mice with targeted disruptions of each receptor. Akt activation was measured as phosphorylated Akt immunoreactivity (pAkt-ir) and PSD-95 expression as PSD-95-ir in sections through the dorsal hippocampal formation. This assay does not account for

any change in total Akt protein after estradiol treatment, which was considered unlikely given that estradiol does not affect Akt expression *in vitro* (Akama and McEwen, 2003). Because estradiol increases synaptic protein expression throughout the mouse hippocampal formation (Li et al., 2004), pAkt-ir and PSD-95-ir measurements were taken from several hippocampal subregions: CA1 stratum radiatum, CA3 strata radiatum and lucidum, and hilus of the dentate gyrus.

Akt activation fluctuates across the estrous cycle

To determine whether Akt activation fluctuates across the mouse estrous cycle, pAkt-ir was assayed in the hippocampal formation of mice in proestrus, estrus, and diestrus. The pattern of pAkt-ir in the mouse hippocampal formation by light microscopy (Figure 3.1A) was similar to that in rats (Znamensky et al., 2003). pAkt-ir localized to cell bodies and processes of principle cells. The densest labeling was seen in pyramidal cell bodies and strata lucidum and lacunosum-moleculare.



3.1 Hippocampal pAkt-ir fluctuates across the estrous cycle

(A) Images of peroxidase labeling of phosphorylated Akt in the dorsal hippocampal formation of representative sections from one proestrus and one estrus mouse. pAkt-ir is darker throughout the hippocampus in proestrus than in estrus. (B) quantification of pAkt-IR in four hippocampal subregions across the estrous cycle. pAkt-ir was significantly higher in proestrus compared to estrus, and in diestrus compared to estrus. *, $p<0.05$. R.O.D., relative optical density. SR, stratum radiatum; SL, stratum lucidum; DG HIL, hilus of the dentate gyrus. N = 4 for proestrus, 6 for estrus, and 11 for diestrus.

pAkt-ir fluctuated across the estrous cycle, with the highest levels during proestrus (Figure 3.1). Consistent with a previous finding in rats (Znamensky et al., 2003), pAkt-ir in the CA1 stratum radiatum was higher in proestrus than estrus or diestrus. However, one-way ANOVA showed only a trend for an effect of cycle phase in this subregion ($F(2, 17) = 2.702$, $p = 0.0957$). The effect of estrous cycle was much more pronounced in other hippocampal subregions, which were not examined in the rat study. There was a significant effect of cycle phase in the CA3 stratum radiatum ($F(2, 17) = 4.919$, $p = 0.0206$), CA3 stratum lucidum ($F(2, 17) = 5.231$, $p = 0.0170$), and dentate hilus ($F(2, 16) = 4.426$, $p = 0.0295$),. Post-hoc tests revealed that pAkt-ir was significantly higher in proestrus than in estrus in all three of these subregions ($p<0.05$ for all). Additionally, pAkt-ir was significantly higher in diestrus than in estrus in CA3 strata lucidum and radiatum ($p<0.05$ for both). Thus Akt activation in the mouse hippocampal formation is highest during proestrus, lowest during estrus, and these fluctuations are most pronounced in the CA3 and dentate subregions.

To determine whether the fluctuation of pAkt-ir was specific to the hippocampal formation rather than a global cerebral response to vascular or metabolic changes across the cycle, pAkt-ir was measured in the somatosensory cortex overlying the dorsal hippocampus in the same image

from which hippocampal measurements were taken. The relative optical density of pAkt-ir in the somatosensory cortex, normalized to background labeled in corpus callosum, was 24.5 +/- 2.2 in proestrus, 27.0 +/- 1.3 in estrus, and 24.0 +/- 1.1 in diestrus, with no significant differences between cycle phases.

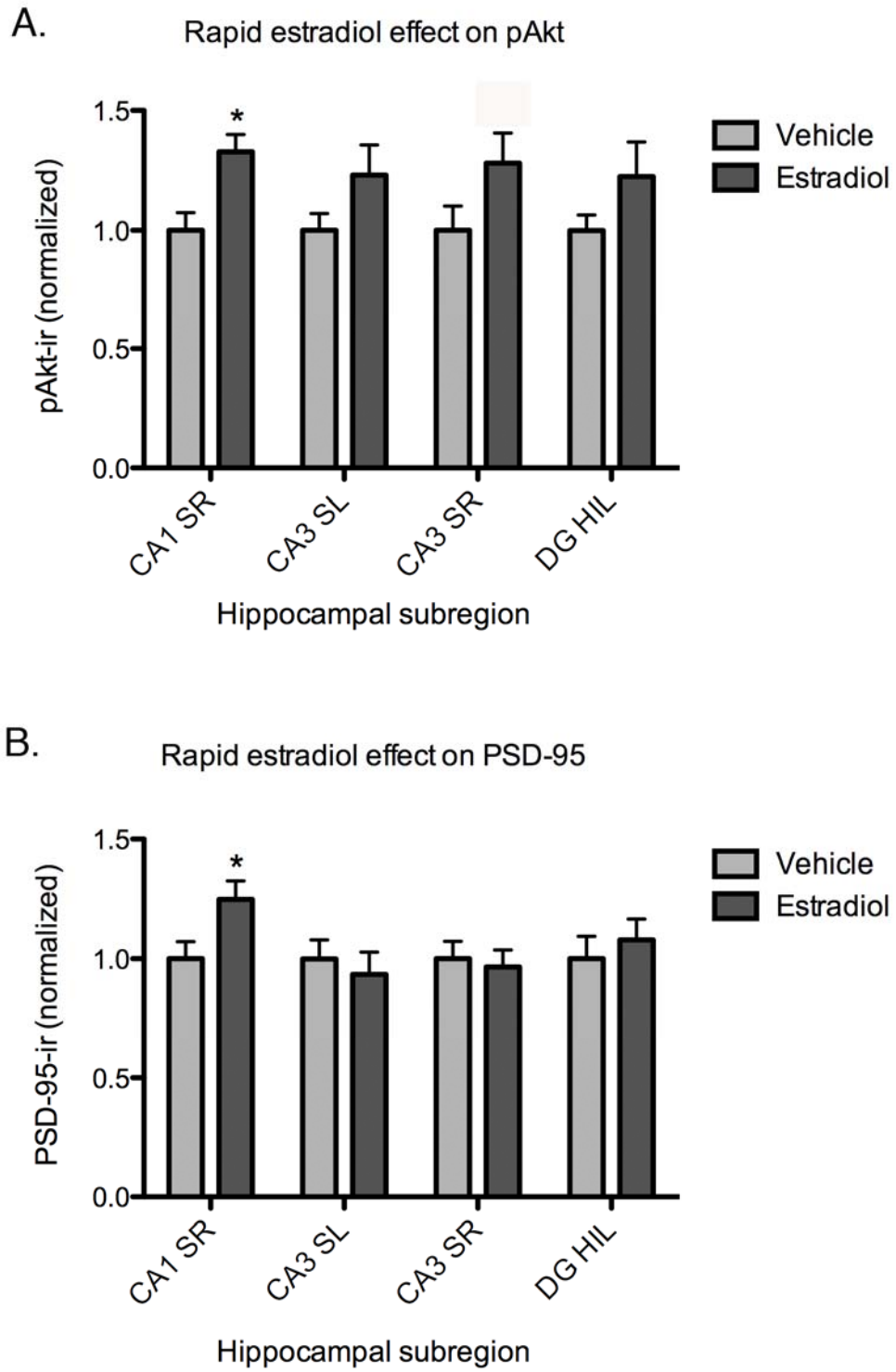
Estradiol rapidly activates Akt and increases PSD-95 expression

The increase in pAkt-ir during proestrus suggests that the endogenous increase in circulating estradiol during this cycle phase activates Akt. To assess directly whether estradiol rapidly activates Akt, ovariectomized mice sacrifice 6 hours after injection with oil vehicle or 5 μg estradiol benzoate were assayed for pAkt-ir in the dorsal hippocampal formation. The time point of 6 hours was chosen for its relevance to membrane-initiated signaling. 5 μg estradiol benzoate, a slowly metabolized form of estradiol, yields high physiologic levels of estradiol in mice sacrificed 24 hours after injection (Russell Romeo, personal communication).

Estradiol increased pAkt-ir in every hippocampal subregion, but most strongly in the CA1 stratum radiatum (Figure 3.2A).

Figure 3.2 Estradiol increases hippocampal pAkt-ir and PSD-95-ir. pAkt-ir and PSD-95-ir were measured from several different hippocampal subregions in wild-type mice sacrificed six hours after injection with vehicle or estradiol benzoate. Estradiol significantly increased pAkt-ir and PSD-95-ir in CA1 stratum radiatum. *, $p<0.05$. SR, stratum radiatum; SL, stratum lucidum; DG HIL, hilus of the dentate gyrus. N = 9.

Figure 3.2


A.
Rapid estradiol effect on pAkt
pAkt-ir (normalized)
1.5
1.0
0.5
0.0
*
CA1 SR
CA3 SL
CA3 SR
DG HIL
Hippocampal subregion
Vehicle
Estradiol
B.
Rapid estradiol effect on PSD-95
PSD-95-ir (normalized)
1.5
1.0
0.5
0.0
*
CA1 SR
CA3 SL
CA3 SR
DG HIL
Hippocampal subregion
Vehicle
Estradiol

T-tests between vehicle and estradiol-treated groups in each hippocampal subregion showed a significant effect of estradiol in CA1 stratum radiatum ($p = 0.0053$) and a trend in CA3 stratum radiatum ($p=0.0972$).

Previous work using a neuronal cell line suggested that estradiol also increases PSD-95 protein translation on a rapid time course that depends on Akt activation. If this is the case, 6 hours of estradiol treatment should also increase PSD-95 expression. Indeed, estradiol significantly increased PSD-95-ir in the CA1 stratum radiatum ($p = 0.0330$; Figure 3.2B).

Rapid estradiol effect on Akt requires estrogen receptors

The above study demonstrated that 6 hours of estradiol treatment increases pAkt-ir in the CA1 stratum radiatum. To determine whether estrogen receptor alpha or beta mediates these effects, the effect of estradiol was assessed in mice with targeted disruptions of each receptor. As in the above study, ovariectomized mice were sacrificed 6 hours after vehicle or estradiol injection. pAkt-ir was measured in the CA1 stratum radiatum.

Estradiol did not affect pAkt-ir in ERKO or BERKO mice (Figure 3.3A). Thus estradiol activation of Akt requires both ERs alpha and beta.

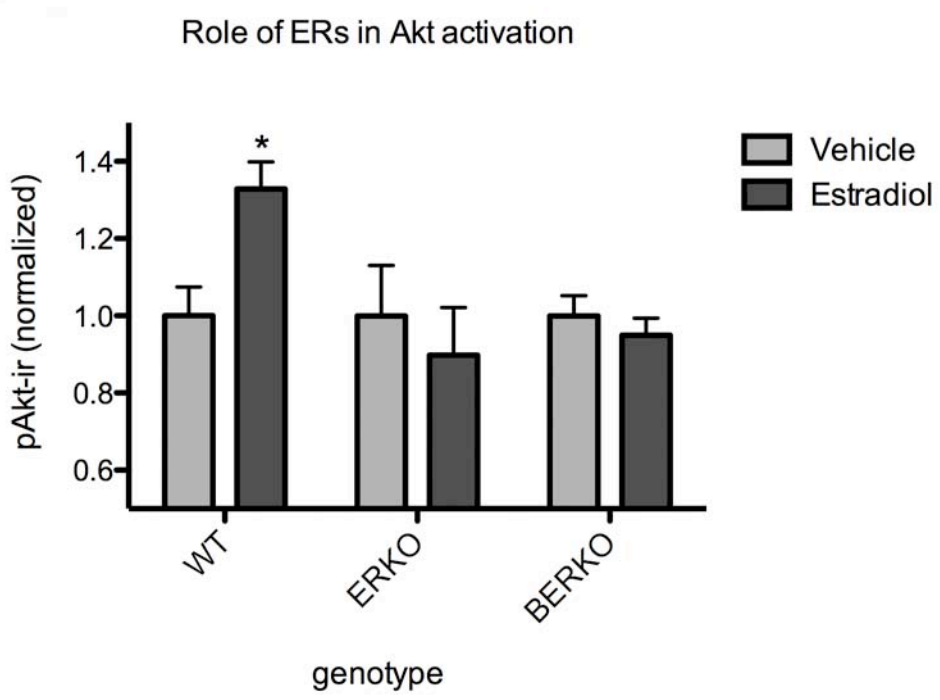


Figure 3.3 Estradiol effects on pAkt depend on ERs. pAkt-ir was measured from the CA1 stratum radiatum of wild-type, ERKO, and BERKO mice sacrificed 6 hours after injection with vehicle or estradiol benzoate. Estradiol had no effect on pAkt-ir in ERKO of BERKO mice. For comparison, results from wild-type littermates are shown, where estradiol significantly increased pAkt-ir. *, $p<0.05$. N = 9 for wild-type mice, 6 for ERKO vehicle, 5 for ERKO estradiol, and 7 for BERKO.

Discussion

The data presented in this chapter demonstrate that physiological and pharmacological increases in circulating estradiol activate Akt in the mouse hippocampal formation. Akt activation influences actin-remodeling and cell-

survival pathways that may be important for estradiol effects on spine synapse density and neuroprotection. The entire hippocampal formation exhibited this estradiol sensitivity, with the strongest effect of estrous cycle in the CA3 and dentate, and the strongest effect of estradiol treatment in the CA1. The involvement of the entire hippocampal formation is consistent with the finding that estradiol increases synaptic protein expression throughout the mouse hippocampus (Li et al., 2004), but whether it represents a species difference between mouse and rat in estradiol activation of Akt remains to be seen.

The finding that pAkt-ir increased slightly (but significantly) during diestrus relative to estrus was surprising and not previously reported in any species. This may be explained by considering the changing levels of circulating progesterone in addition to estradiol in different estrous cycle phases. Progestins can rapidly activate Akt in breast, endometrial, leiomyoma, and cerebral cortical cells (Ballare et al., 2006; Kaur et al., 2007; Hoekstra et al., 2009). The increase in circulating progesterone during diestrus (see Chapter 2) could therefore account for the increase in Akt activation during this cycle stage. Alternatively, increased pAkt-ir during diestrus might represent a recovery of baseline Akt signaling after the

precipitous drop in estradiol levels and concomitant decrease in Akt signaling during estrus.

The increase in pAkt-ir six hours after estradiol injection is consistent with membrane-initiated estradiol signaling. After a six-hour treatment in cultured neurons, estradiol activation of signaling pathways can still be observed (Akama and McEwen, 2003; Yuen et al., 2004), while effects on gene transcription are typically not yet seen (Gottfried-Blackmore et al., 2007). It is therefore reasonable to conclude that estradiol activates Akt by signaling through membrane-associated ERs, at least in the CA1 stratum radiatum. This is the first report of a rapid action of estradiol on Akt *in vivo*. The same estradiol treatment also increased PSD-95 expression in the CA1 stratum radiatum, consistent with the finding that estradiol rapidly enhances PSD-95 protein translation via Akt signaling *in vitro* (Akama and McEwen, 2003).

Extranuclear ERs alpha and beta can both mediate rapid membrane-initiated estradiol signaling (Razandi et al., 1999), but the specific pathways activated by each receptor have not been elucidated. In the current study, knockout of each estrogen receptor, alpha and beta, eliminated the increase in pAkt-ir after six hours of estradiol treatment in ovariectomized mice. This suggests that estradiol activates Akt by a mechanism involving both

ERs. It is consistent with previous studies in the NG108-15 cell line from the McEwen laboratory, which showed that both ERs alpha and beta can mediate estradiol activation of actin remodeling pathways downstream of Akt (Yuen, 2006). The importance of ER alpha for estradiol activation of Akt was not surprising. ER alpha binds *in vitro* to the p85 regulatory subunit of PI3K in rat primary cortical neurons (Mannella and Brinton, 2006), and could therefore participate in Akt activation through direct interaction with its upstream activator. ER beta does not interact with p85 in vitro (Simoncini et al., 2000), but aside from this its role in Akt activation has not been investigated. The finding that ER beta knockout blocks Akt activation suggests that ER beta has a hitherto unrecognized role in estradiol activation of Akt.

In sum, both physiologic and pharmacologic increases in estradiol activate Akt in the mouse hippocampal formation. Estradiol activation of Akt is rapid, correlates with an increase in PSD-95 expression, and requires ERs alpha and beta.

Chapter 4

Physiological effect of estradiol on TrkB activation

The TrkB ligand, BDNF, has attracted much attention for its role in mood and cognition. BDNF and estradiol target similar pathways and proteins in the hippocampal formation, including the PI3K/Akt pathway and PSD-95 protein (Mizuno et al., 2003b; Jia et al., 2008; Luikart et al., 2008). In addition, estradiol increases BDNF expression in the rat hippocampus (Gibbs, 1998, 1999; Scharfman et al., 2003; Scharfman et al., 2007). I therefore hypothesized that BDNF would be an important player in estradiol actions in the mouse hippocampal formation.

The central hypothesis addressed in this chapter is that increased circulating estradiol leads to increased BDNF expression and release, thereby enhancing activity of the BDNF receptor, TrkB. Two approaches were used to test this hypothesis. First, mice in the proestrus, estrus and diestrus stages of the estrous cycle were assayed to determine the effects of natural fluctuations of ovarian steroids on TrkB activation. Second, ovariectomized mice sacrificed 6 hours or 48 hours after vehicle or estradiol benzoate injection were assayed to determine the time course of estradiol

effects on BDNF expression and TrkB activation. TrkB activation was measured as pTrkB-ir. Because this assay does not account for changes in TrkB expression, TrkB mRNA was measured using in situ hybridization to assess any changes in TrkB expression across the estrous cycle.

TrkB activation fluctuates across the estrous cycle

By light microscopy, pTrkB-ir localized to cell bodies and neuropil throughout the hippocampal formation (Figure 4.1A). In addition, pTrkB strongly labeled cells in the subgranular zone (SGZ) of the dentate gyrus, an area that gives rise to newly born neurons that integrate into hippocampal circuits (Doetsch and Hen, 2005). Finally, pTrkB labeled glial cells, which could be identified by the distinctive morphology of their processes within the neuropil.

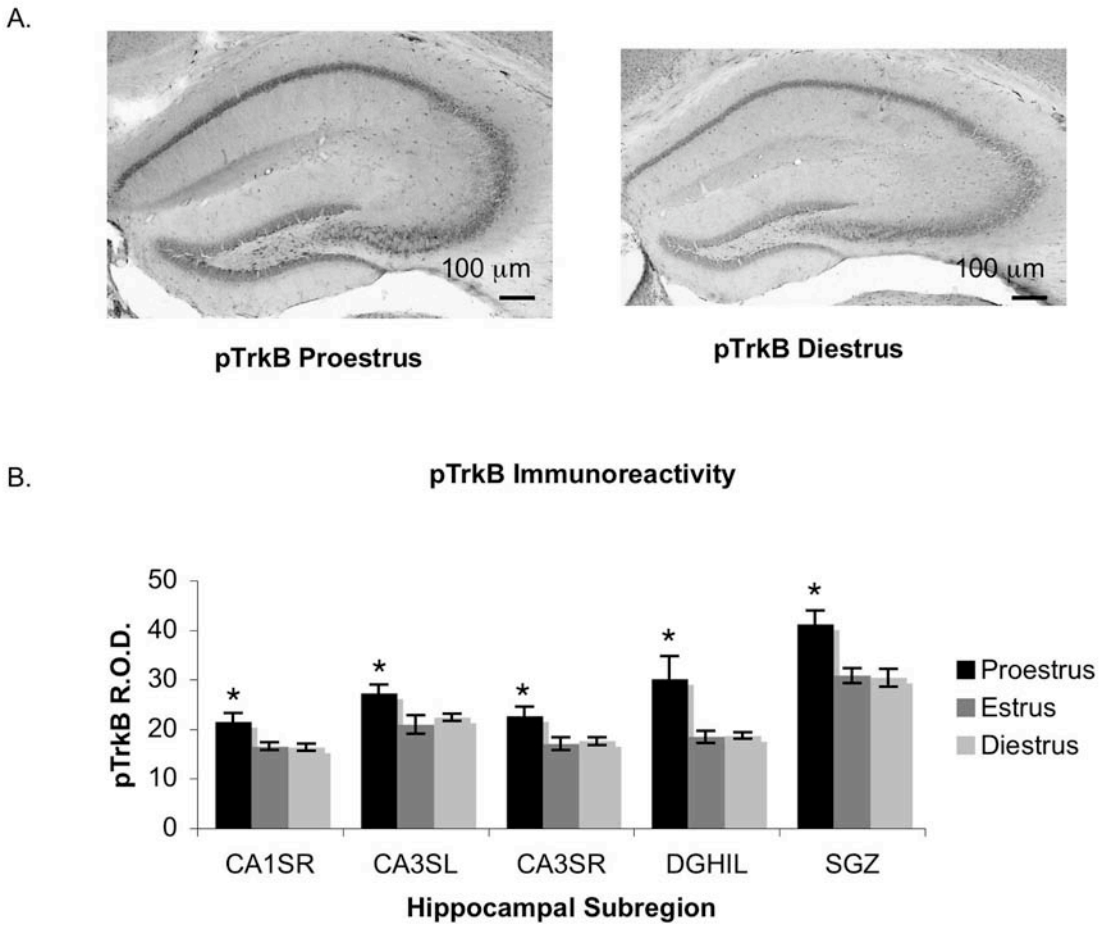


Figure 4.1 pTrkB-ir fluctuates across the estrous cycle (A) Images of peroxidase labeling of pTrkB in the dorsal hippocampal formation of representative sections from one proestrus and one diestrus mouse. pTrkB-ir is darker throughout the hippocampal formation in proestrus than in diestrus. (B) quantification of pTrkB-ir in five hippocampal subregions. pTrkB-ir was significantly higher in proestrus than estrus and diestrus. . SR, stratum radiatum; SL, stratum lucidum; DG HIL, hilus of the dentate gyrus; SGZ, subgranular zone. *, $p<0.05$. N = 4 for proestrus, 6 for estrus, and 11 for diestrus.

pTrkB-ir was highest during proestrus in all hippocampal subregions (Figure 4.1B). One-way ANOVA showed a significant effect of cycle phase in all hippocampal subregions: CA1 stratum radiatum ($F(2, 17) = 7.378$, $p = 0.0041$), CA3 stratum lucidum ($F(2, 17) = 5.494$, $p = 0.0144$), CA3 stratum radiatum ($F(2, 17) = 5.559$, $p = 0.139$), dentate hilus ($F(2, 16) = 10.21$, $p = 0.0014$), and subgranular zone ($F(2\ 16) = 6.377$, $p = 0.0092$). Post-hoc tests revealed that pTrkB-ir was significantly higher in proestrus than in estrus or diestrus in all subregions ($p < 0.05$ for all).

To determine whether the fluctuation of pTrkB-ir was specific to the hippocampal formation, pTrkB-ir was measured in the sensory cortex overlying the dorsal hippocampus in the same images from which hippocampal measurements were taken. The relative optical density of pTrkB-ir in the cortex was 22.6 +/- 3.4 in proestrus, 23.6 +/- 2.1 in estrus, and 20.0 +/- 1.9 in diestrus, with no significant differences between cycle phases.

Hippocampal TrkB mRNA does not fluctuate across the estrous cycle

The proestrus increase in TrkB activation is presumably caused by an increase in BDNF expression during proestrus, as has been demonstrated in

rats (for mouse, see Chapter 6). However, it is also possible that the expression of TrkB itself fluctuates across the estrous cycle. Although no studies have examined the effects of estradiol on TrkB expression *in vivo*, estradiol increases TrkB expression in hypothalamic neurons *in vitro* (Carrer et al., 2003). To test whether natural fluctuations in ovarian steroids affect TrkB expression in female mice, TrkB mRNA was measured using in situ hybridization on tissue sections from mice in proestrus or diestrus (Figure 4.2). Optical density measurements were taken from the CA1 pyramidal cell layer and the ventromedial hypothalamus (VMH). TrkB mRNA increased significantly during proestrus in the VMH ($p = 0.0182$), but not the CA1 ($p = 0.3503$). This suggests that circulating estradiol increases TrkB expression in the hypothalamus, but not in the hippocampus.

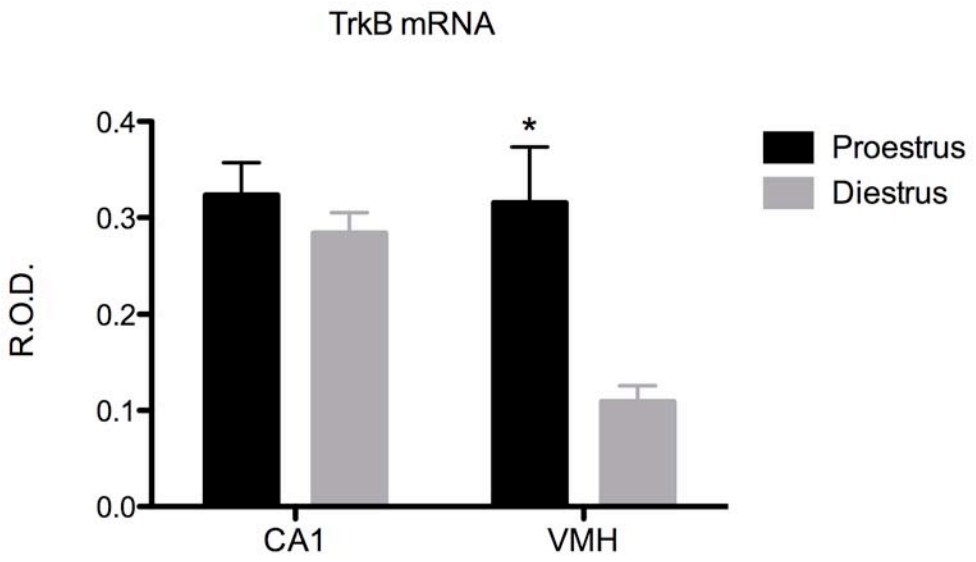


Figure 4.2 Hippocampal TrkB mRNA does not fluctuate across the cycle. Optical density of TrkB mRNA from in situ hybridization films was measured from the CA1 pyramidal cell layer of the hippocampus and the ventromedial hypothalamus and normalized to background density. TrkB mRNA was significantly higher in proestrus than diestrus in hypothalamus, but not hippocampus. . SR, stratum radiatum; SL, stratum lucidum; DG HIL, hilus of the dentate gyrus; VMH, ventromedial hypothalamus; R.O.D., relative optical density. * $p<0.05$. N = 5.

Estradiol increases BDNF expression and TrkB activation

Although estradiol most likely increases TrkB activation by increasing BDNF gene transcription, more rapid mechanisms could be involved. ERs are known to couple to several other growth factor receptors (such as the

IGF-1 receptor), increasing their activation (Mendez et al., 2006). In addition, the glucocorticoid family of steroid hormones has been shown to activate TrkB through a glucocorticoid receptor-dependent, BDNF-independent mechanism, suggesting that rapid ER-dependent TrkB activation could also occur (Jeanneteau et al., 2008). To determine whether estradiol rapidly activates TrkB independent of BDNF expression, BDNF mRNA and pTrkB-ir was measured in the hippocampal formation of ovariectomized mice sacrificed 6 hours after estradiol benzoate or vehicle injection (Figure 4.3). Estradiol had no effect on BDNF mRNA or pTrkB-ir at this time point, suggesting that the effect of estradiol on TrkB activation occurs over a longer treatment time and depends on an increase in BDNF expression. To test this hypothesis, BDNF mRNA and pTrkB-ir were measured in mice sacrificed after two daily injections of estradiol benzoate or vehicle, for a total treatment time of 48 hours (Figure 4.4). BDNF mRNA was assayed using in situ hybridization with an oligonucleotide probe specific for the coding region of the BDNF gene. In the hippocampus, BDNF expression is strongest in the dentate granule cell and CA3 pyramidal cell layers; for this study, optical density was measured from the CA3 pyramidal cell layer.

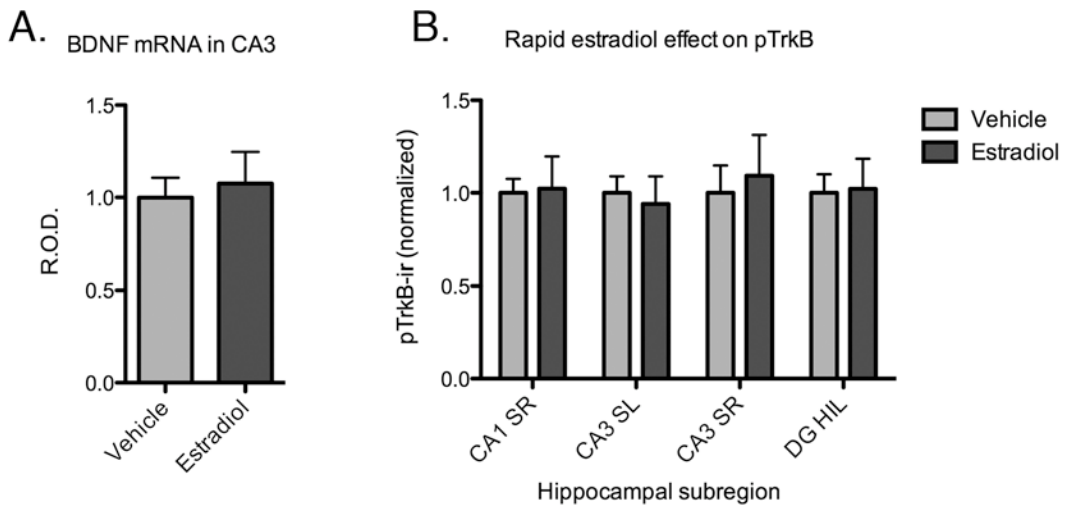


Figure 4.3 Six hours of estradiol treatment does not alter BDNF/TrkB signaling (A) BDNF mRNA was measured in the CA3 cell layer from mice sacrificed 6 hours after vehicle or estradiol benzoate injection. Estradiol had no effect on BDNF mRNA. (B) pTrkB-ir was measured from several different hippocampal subregions in the same mice. Estradiol had no effect on pTrkB-ir in any hippocampal subregion. SR, stratum radiatum; SL, stratum lucidum; DG HIL, hilus of the dentate gyrus. N = 9.

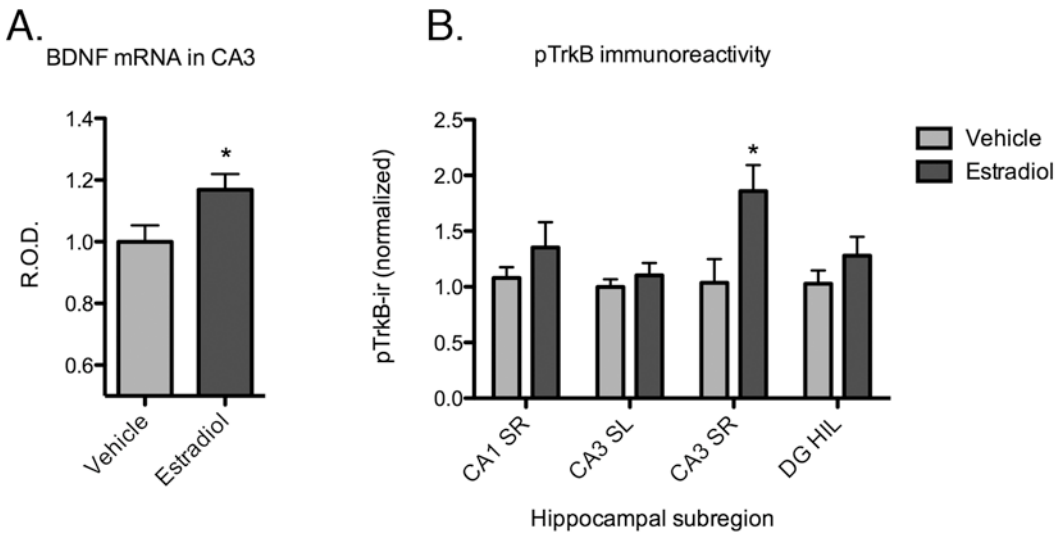
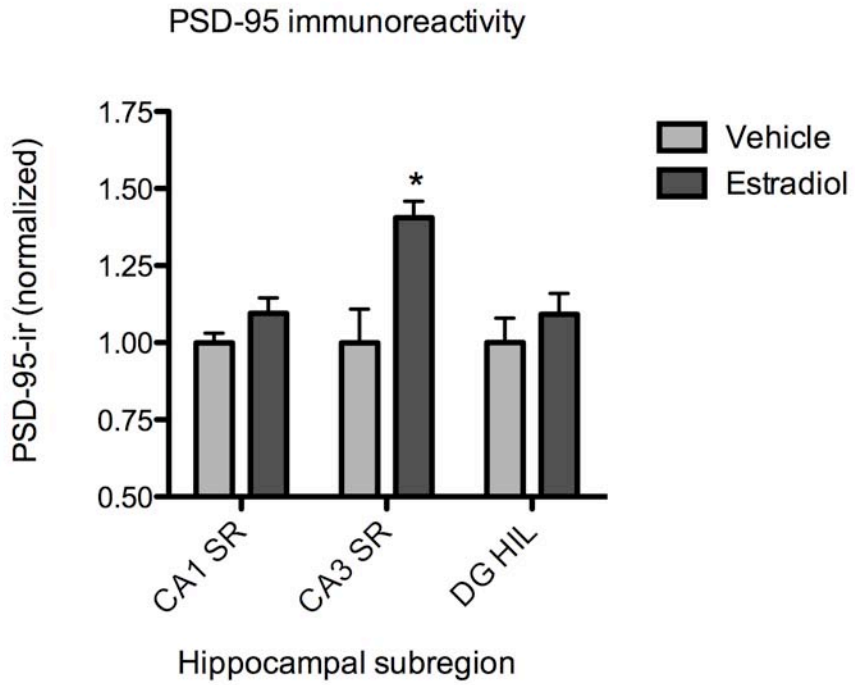


Figure 4.4 Forty-eight hours of estradiol treatment increases BDNF/TrkB signaling. (A) BDNF mRNA was measured in the CA3 pyramidal cell layer from wild-type mice sacrificed after two daily injections of estradiol benzoate, for a total treatment time of 48 hours. Estradiol significantly increased BDNF mRNA. (B) pTrkB-ir was measured from several different hippocampal subregions in the same mice. Estradiol significantly increased pTrkB-ir in the CA3 stratum radiatum. SR, stratum radiatum; SL, stratum lucidum; DG HIL, hilus of the dentate gyrus. R.O.D., relative optical density. *$p<0.05$. N = 8 (vehicle), 9 (estradiol benzoate).

Forty-eight hours of estradiol treatment significantly increased BDNF mRNA in the CA3 pyramidal cell layer ($p = 0.0375$) and pTrkB-ir in the CA3 stratum radiatum ($p = 0.02$).

Previous work in slices from rat hippocampus suggested that estradiol increases PSD-95 expression in the CA3 subregion, and that this effect depends on TrkB receptors (Sato et al., 2007). If this mechanism operates in the mouse hippocampus, then 48 hours of estradiol treatment should increase PSD-95 expression in concert with TrkB activation in the CA3 stratum radiatum. To test this hypothesis, PSD-95-ir was assayed in the dorsal hippocampal formation of mice treated for 48 hours with vehicle or estradiol benzoate (Figure 4.5). Estradiol significantly increased PSD-95-ir only in the CA3 stratum radiatum ($p = 0.0152$).

In sum, estradiol does not rapidly activate TrkB, but rather increases TrkB relatively slowly in concert with an increase in BDNF expression. The increase in TrkB activation in the CA3 stratum radiatum after estradiol treatment correlates with an increase in PSD-95 protein expression.



4.5 Forty-eight hours of estradiol treatment increases PSD-95 immunoreactivity. PSD-95-ir was measured in several different hippocampal subregions from wild-type mice sacrificed after two daily injections of estradiol benzoate, for a total treatment time of 48 hours. Estradiol significantly increased PSD-95-ir in the CA3 stratum radiatum. . SR, stratum radiatum; DG HIL, hilus of the dentate gyrus. *$p<0.05$. N = 8 (vehicle), 9 (estradiol benzoate).

Estradiol induction of BDNF requires ER beta

To determine whether estrogen receptor alpha or beta mediates estradiol induction of BDNF, the effect of estradiol on BDNF expression was assessed in mice with targeted disruptions of each receptor. As in the above study, ovariectomized mice were sacrificed after two daily injections of vehicle or estradiol benzoate, for a total treatment time of 48 hours. BDNF mRNA was assayed using in situ hybridization and measured in the CA3 pyramidal cell layer (Figure 4.6).

Estradiol significantly increased BDNF mRNA in ERKO mice ($p = 0.0352$), similar to its effect in wild-type mice. In contrast, estradiol had no effect on BDNF mRNA in BERKO mice. Thus estradiol induction of BDNF expression requires ER beta, but not ER alpha.

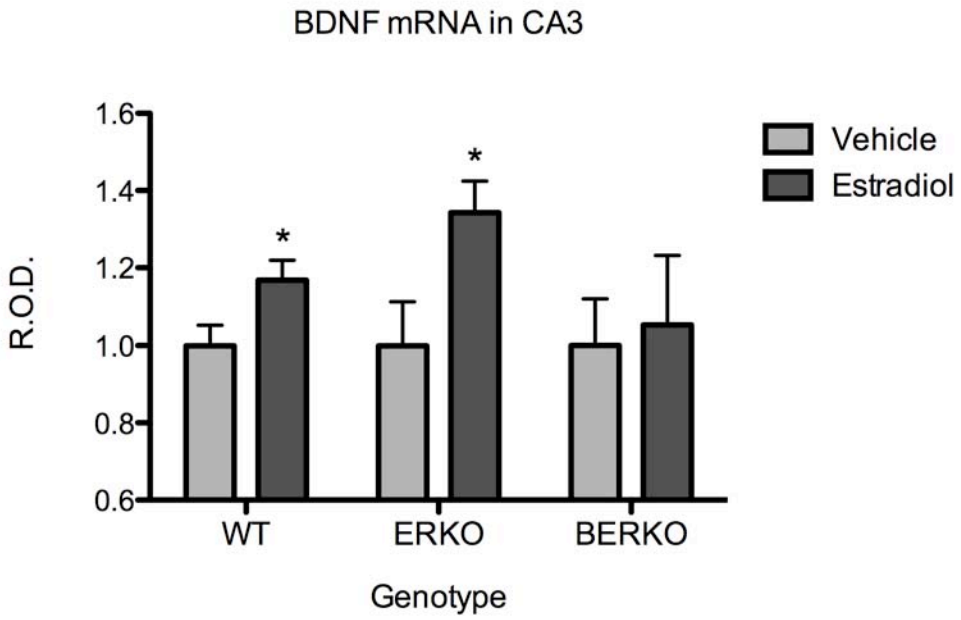


Figure 4.6 Estradiol induction of BDNF depends on estrogen receptor beta. BDNF mRNA was assayed using in situ hybridization and measured from the CA3 pyramidal cell layer of wild-type, ERKO, and BERKO mice sacrificed 48 hours after injection with vehicle or estradiol benzoate. Estradiol significantly increased BDNF mRNA in ERKO, but not BERKO, mice. For comparison, results from wild-type littermates are shown, where estradiol significantly increased BDNF mRNA. *, $p<0.05$. N = 8 for wild-type vehicle, 9 for wild-type estradiol benzoate, 7 for ERKO vehicle, and 6 for the remaining groups.

Discussion

The data presented in this chapter demonstrate that physiologic and pharmacologic increases in circulating estradiol increase TrkB activation. This effect of estradiol is most likely mediated by increased BDNF expression, as estradiol increased BDNF but not TrkB mRNA. The downstream effects of TrkB on local synaptic signaling and hippocampal gene expression likely contribute to estradiol enhancement of synaptic plasticity.

In contrast to the hippocampus, TrkB mRNA did fluctuate across the estrous cycle in the ventromedial hypothalamus, increasing dramatically during proestrus. Thus estradiol has distinct effects in different brain areas. There are many possible explanations for this difference, including the estrogen receptors and cofactors expressed by hypothalamic and hippocampal neurons, the location of the receptors (e.g. nuclear or extranuclear), and level of exposure to circulating factors including steroids. That a hormone such as estradiol may act so differently in two brain areas highlights the importance of directly investigating its specific effects in the hippocampal formation.

Although estradiol may affect BDNF expression via membrane-initiated signaling, this mechanism still requires new BDNF gene transcription, which likely requires at least 12 hours of estradiol exposure (see Chapter 1). Consistent with this idea, 6 hours of estradiol treatment did not significantly alter BDNF mRNA or pTrkB-ir in ovariectomized mice, but 48 hours of estradiol treatment increased both BDNF mRNA and TrkB activation in the CA3 stratum radiatum. The same estradiol treatment also increased PSD-95 expression in the CA3 stratum radiatum. This correlation suggests that BDNF signaling through TrkB may be involved in estradiol induction of PSD-95 in the CA3 of the mouse hippocampus, as was previously found in rat hippocampal slices in vitro (Sato et al., 2007). The limitation of this increase in pTrkB-ir and PSD-95-ir to the CA3 contrasts with the hippocampus-wide increase in pTrkB-ir during proestrus. As with Akt in Chapter 4, the involvement of the whole hippocampal formation during proestrus may be related to the timing of estradiol exposure in these different models.

This is the first study to investigate the contribution of estrogen receptors to estradiol effects on BDNF expression. The estradiol-mediated induction of BDNF expression was eliminated in BERKO mice missing the ER beta, suggesting that ER beta mediates this effect. In contrast, the ERKO

mice missing the ER alpha showed an exaggerated estradiol-mediated induction of BDNF. This further suggests that ERs alpha and beta have opposing effects on BDNF expression, with ER alpha mitigating the enhancing effect of ER beta on BDNF expression. Opposing transcriptional effects of ERs alpha and beta on specific genes have been described in several different cell types (Matthews and Gustafsson, 2003). This opposition could serve as a homeostatic mechanism to dampen or control hormone actions – in the hippocampus, too much BDNF could increase neuronal excitability to toxic levels.

In sum, both physiologic and pharmacologic increases in circulating estradiol increased BDNF expression and TrkB activation in the dorsal hippocampal formation of female mice. Increased BDNF expression and TrkB activation in the CA3 subregion correlated with an increase in PSD-95 expression. Estradiol induction of BDNF required estrogen receptor beta.

Chapter 5

Ultrastructural distribution of pTrkB depends on sex and cycle stage

Up to this point, this thesis has addressed widespread estradiol effects on signaling molecule activation and protein expression in the hippocampal formation that can be observed at the light microscopic level. The ultimate goal of identifying these estradiol effects is to understand the mechanism of estradiol enhancement of synaptic plasticity. Although widespread changes are likely important for this process, synaptic plasticity involves activity-dependent changes to specific hippocampal synapses. Therefore, if estradiol enhances synaptic plasticity in the hippocampus, then it must facilitate these types of synapse-specific changes, which are unobservable by conventional light microscopy.

The TrkB receptor could be important for communicating the widespread effects of estradiol in the hippocampus to specific synapses. Increased BDNF release at active synapses may serve to "tag" these individual synapses for modification via TrkB activation (Frey and Morris, 1998; Boulanger and Poo, 1999; Nagappan and Lu, 2005; Reymann and Frey, 2007). By increasing BDNF expression, estradiol could facilitate the

ability of an active synapse to release enough BDNF to tag its synapse. In this way, the hippocampus would experience a targeted increase in TrkB activation at locations of activity-dependent BDNF secretion during periods of high circulating estradiol.

By light microscopy, it is impossible to determine the precise location of the TrkB receptors that "see" increased BDNF when circulating estradiol increases. Investigation of this hypothesis relies on the precise localization of activated TrkB under conditions of higher and lower circulating estradiol. TrkB is expressed almost ubiquitously in the brain (Yan et al., 1997), yet the precise sites of TrkB activation in the intact organism are still unknown. The best way to identify these sites in the brain is with electron microscopy. In addition to its importance for investigating estradiol actions, identification of the cell types and subcellular locations of activated TrkB under different conditions will provide crucial information about experience-dependent plasticity in general.

For this experiment, I collaborated with Dr. Teresa Milner in her laboratory at Weill Cornell medical college. We conducted immunocytochemistry and performed a quantitative analysis of the sites of pTrkB-ir in the mouse hippocampal formation using electron microscopy. To assess changes in the distribution of pTrkB under conditions of higher

and lower estradiol, the distribution of pTrkB-ir was compared among different phases of the estrous cycle and also between sexes. By comparing proestrus females, where pTrkB-ir was enhanced overall by light microscopy, to lower-estradiol females and males, we uncovered shifts in the distribution of pTrkB-ir that will be important to the mechanism of estradiol and BDNF-dependent plasticity.

Light microscopic immunolocalization of pTrkB

In sections from mice perfused with acrolein in preparation for electron microscopy, the light microscopic localization of pTrkB-ir was consistent with the previously described pattern from mice perfused with paraformaldehyde (Figure 5.1A; see Chapter 4). Pre-adsorption of the primary antisera with pTrkB blocking peptide eliminated all labeling (Figure 5.1B).

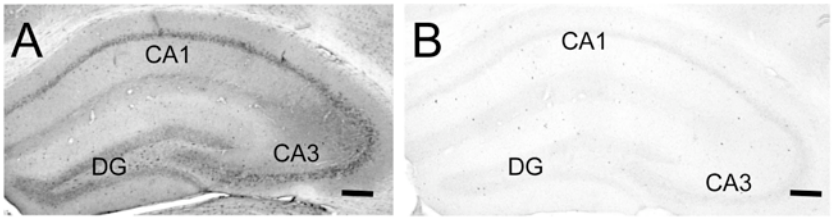


Figure 5.1 Light microscopic localization of pTrkB. (A) By light microscopy, the pTrkB antiserum labels neuronal cell bodies and neuropil in the CA1, CA3 and dentate gyrus in acrolein- and paraformaldehyde-fixed tissue. (B) Pre-adsorption with pTrkB blocking peptide completely eliminates all labeling.

Presynaptic profiles contain the most abundant pTrkB-ir

To determine the distribution of pTrkB in neurons and glia, pTrkB-ir was identified using electron microscopy in the dorsal hippocampal formation of 9 cycled female and 3 male mice. Profiles were counted from 12 different hippocampal laminae lacking principal cell perikarya in CA1, CA3, and dentate, for a total area of 2,646 μm^2 per mouse (Table 6.1). An average of 388.3 +/- 19.03 labeled profiles were counted for each animal, with no difference between estrous cycle stages or sexes. Labeled profiles were identified as dendrites, dendritic spines, axons, axon terminals, or glia. Less than 1% of labeled profiles were unidentifiable, and these unknowns were not included in the analyses.

The majority of pTrkB-ir was in presynaptic profiles regardless of sex or estrous cycle stage, with 36.0 +/- 1.3% in axons and 17.3 +/- 0.6% in axon terminals. Postsynaptic profiles contained one quarter of the pTrkB-ir, with 16.7 +/- 0.9% in dendrites and 10.7 +/- 0.7% in dendritic spines. Glial profiles accounted for one fifth of the total pTrkB-ir, 19.3 +/- 0.9%.

Table 1. Distribution of pTrkB-IR in the hippocampal formation of female cycle and male mice

Subregion	Cycle/ Sex	% Dendrites	Spines	Terminals	Axons	Glia	Total
CA1	Proestrus	6.1 +/- 1.0	3.6 +/- 0.8	9.2 +/- 0.5	16.1* +/- 0.7	9.9 +/- 0.3	44.9 +/- 2.1
	Estrus	8.0 +/- 1.2	5.2 +/- 0.8	7.9 +/- 2.4	11.8 +/- 1.9	5.7 +/- 0.3	38.7 +/- 2.4
	Diestrus	6.5 +/- 0.6	3.4 +/- 1.2	6.0 +/- 1.7	13.8 +/- 2.3	8.6 +/- 1.6	38.3 +/- 3.4
	Male	7.3 +/- 0.01	3.4 +/- 0.8	6.0 +/- 0.6	10.9 +/- 0.8	6.0 +/- 0.6	33.5 +/- 1.3
CA3	Proestrus	2.5 +/- 0.6	2.6 +/- 0.8	3.6 +/- 0.08	8.9 +/- 0.9	3.1 +/- 0.3	20.7 +/- 2.0
	Estrus	3.0 +/- 0.7	4.3 +/- 0.5	5.4 +/- 0.3	7.5 +/- 0.9	4.2 +/- 0.04	24.3 +/- 2.5
	Diestrus	2.6 +/- 0.1	3.7 +/- 1.5	5.0 +/- 1.0	9.4 +/- 1.1	3.3 +/- 0.9	24.1 +/- 1.7
	Male	4.3 +/- 0.3	3.1 +/- 0.1	6.4 +/- 1.0	6.7 +/- 0.7	3.3 +/- 0.4	23.8 +/- 0.4
DG	Proestrus	5.2 +/- 0.5	2.4 +/- 0.7	5.2 +/- 0.6	14.0 +/- 3.0	7.6 +/- 0.72	34.4 +/- 1.5
	Estrus	6.6 +/- 0.8	4.4 +/- 0.5	4.5 +/- 0.8	13.8 +/- 1.6	7.6 +/- 1.5	37.0 +/- 2.2
	Diestrus	6.8 +/- 0.4	3.1 +/- 0.5	5.0 +/- 1.0	14.1 +/- 2.2	8.6 +/- 1.8	37.6 +/- 3.7
	Male	7.9 +/- 0.6	3.6 +/- 0.7	5.0 +/- 1.0	16.8 +/- 0.7	9.2 +/- 1.7	42.6 +/- 1.0
total		16.7 +/- 0.9	10.7 +/- 0.7	17.3 +/- 0.6	36.0 +/- 1.3	19.3 +/- 0.9	

A total area of 2,646 μm^2 was analyzed per animal (N=3). Figures represent percent of all counted profiles in the hippocampal formation +/- standard error of the mean. *$p<0.05$ relative to male mice. DG, dentate gyrus.

Table 5.1 Distribution of pTrkB-ir in the hippocampal formation of cycling female and male mice

Axons and axon terminals, including mossy fibers, contain pTrkB-ir

Throughout the hippocampal formation, pTrkB-ir was affiliated with the plasma membrane of small unmyelinated axons (Figure 5.2A). Occasionally, larger myelinated axons contained pTrkB-ir, usually affiliated with endomembranes or mitochondria (Fig. 5.2B). Diffuse pTrkB-ir filled the cytoplasm of some small-caliber axons (Figure 5.2B). pTrkB-ir also was localized using silver-intensified gold particles (SIG), allowing more discrete localization of labeling. With this method, pTrkB SIG-ir was on or near the axon plasma membrane (Figure 5.2C).

In axon terminals, pTrkB-ir was in clusters most often affiliated with small synaptic vesicles opposite the synaptic contact (Figure 5.3A). Discrete patches of pTrkB-ir localized to small synaptic vesicles of large terminals with morphological characteristics of mossy fibers in the CA3 stratum lucidum (Figure 5.3B). Labeled mossy fiber terminals often contained several patches of immunolabeling. Occasionally, these labeled terminals contacted pTrkB-labeled spines (Figure 5.3B). SIG labeling revealed pTrkB-ir adjacent to the presynaptic contact, or on mitochondria inside the axon terminal (Figure 5.3C).

Figure 5.2 pTrkB-ir is most prominent in axons throughout the hippocampal formation. (A) In the CA1 stratum radiatum, pTrkB-ir (arrowheads) is found in clusters within axonal profiles (a). (B) In the stratum lucidum, pTrkB-ir is found in axon bundles most likely belonging to the mossy fiber pathway, and in myelinated axons (ma). (C) In CA1 stratum radiatum, SIG pTrkB-ir (arrowheads) is found in axons near the plasma membrane. Scale bars, 0.5 µm.

Figure 5.2

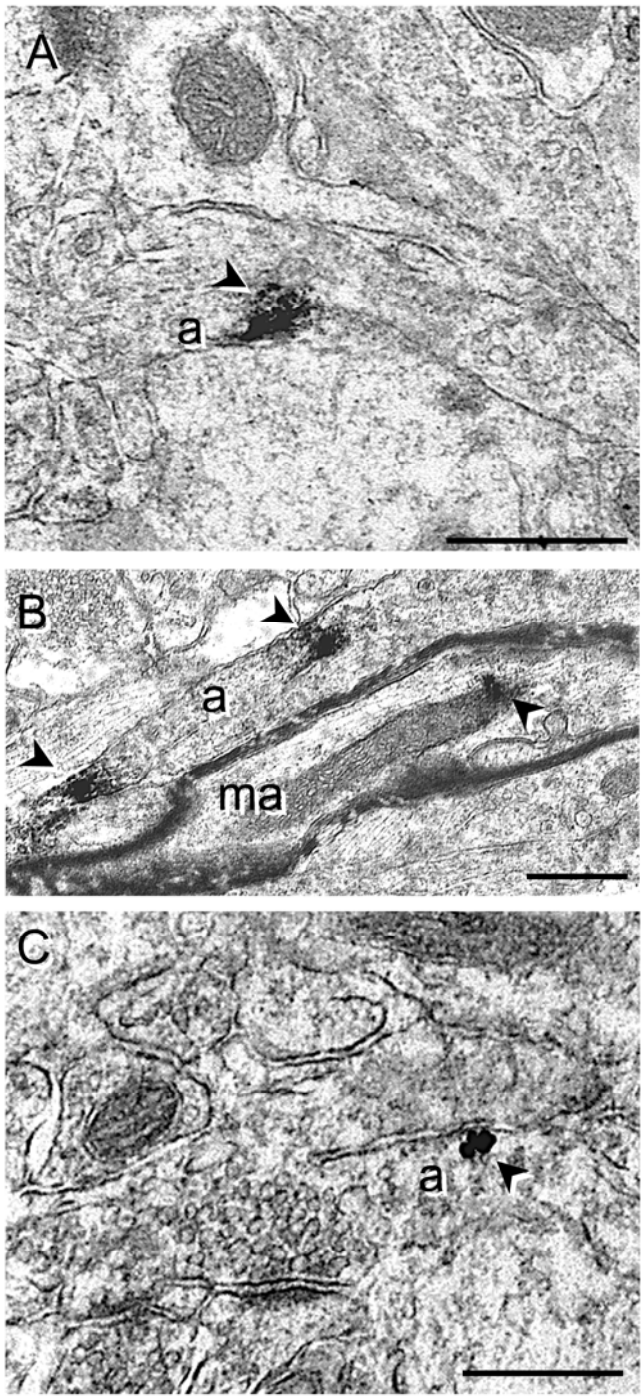
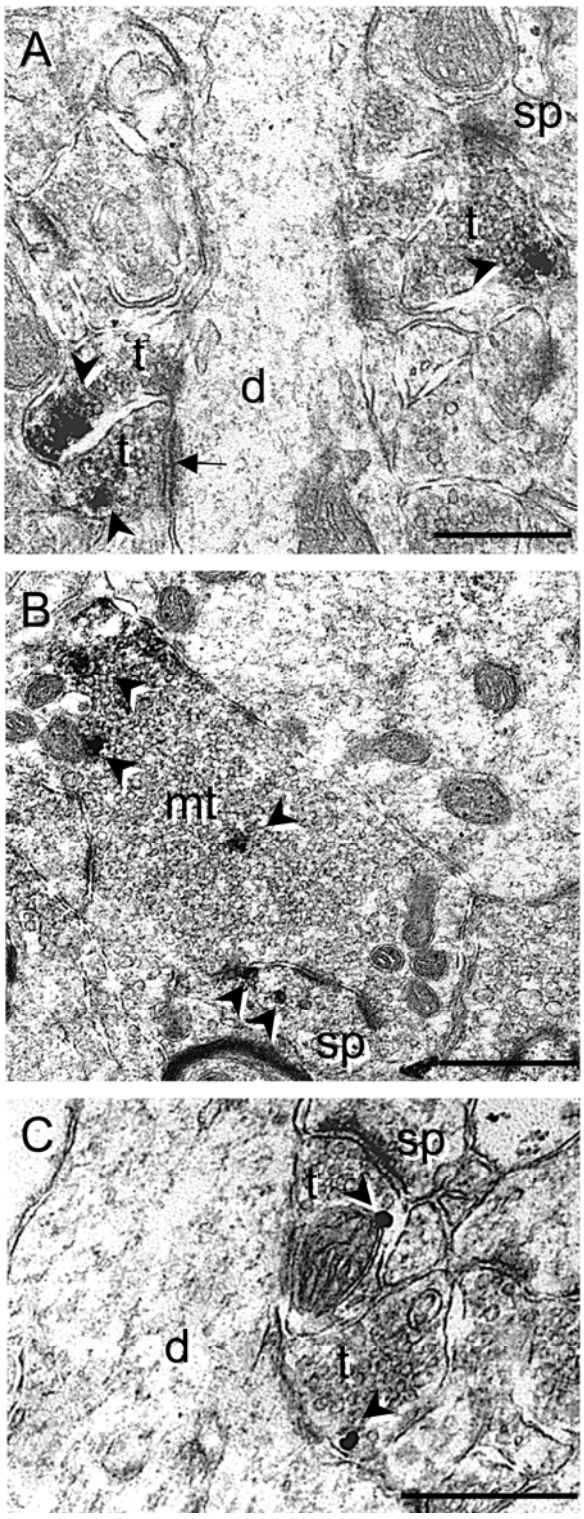


Figure 5.3 pTrkB-ir is found in axon terminals throughout the hippocampal formation. (A) In the CA1 stratum radiatum, pTrkB-ir (arrowheads) clusters among small synaptic vesicles in axon terminals (t). One of the pTrkB-labeled terminals (lower left) forms an asymmetric synapse (arrow) with the shaft of an unlabeled dendrite (d). (B) A mossy fiber terminal (mt) in the CA3 stratum lucidum contains pTrkB-ir associated with small synaptic vesicles and mitochondria. The pTrkB-labeled mossy fiber terminal contacts a dendritic spine (sp) containing pTrkB-ir. (C) In the CA1 stratum radiatum, SIG pTrkB-ir (arrowheads) is in axon terminals, on the membrane of a mitochondrion and affiliated with small synaptic vesicles adjacent to the synaptic contact. Scale bars, 0.5 μm.

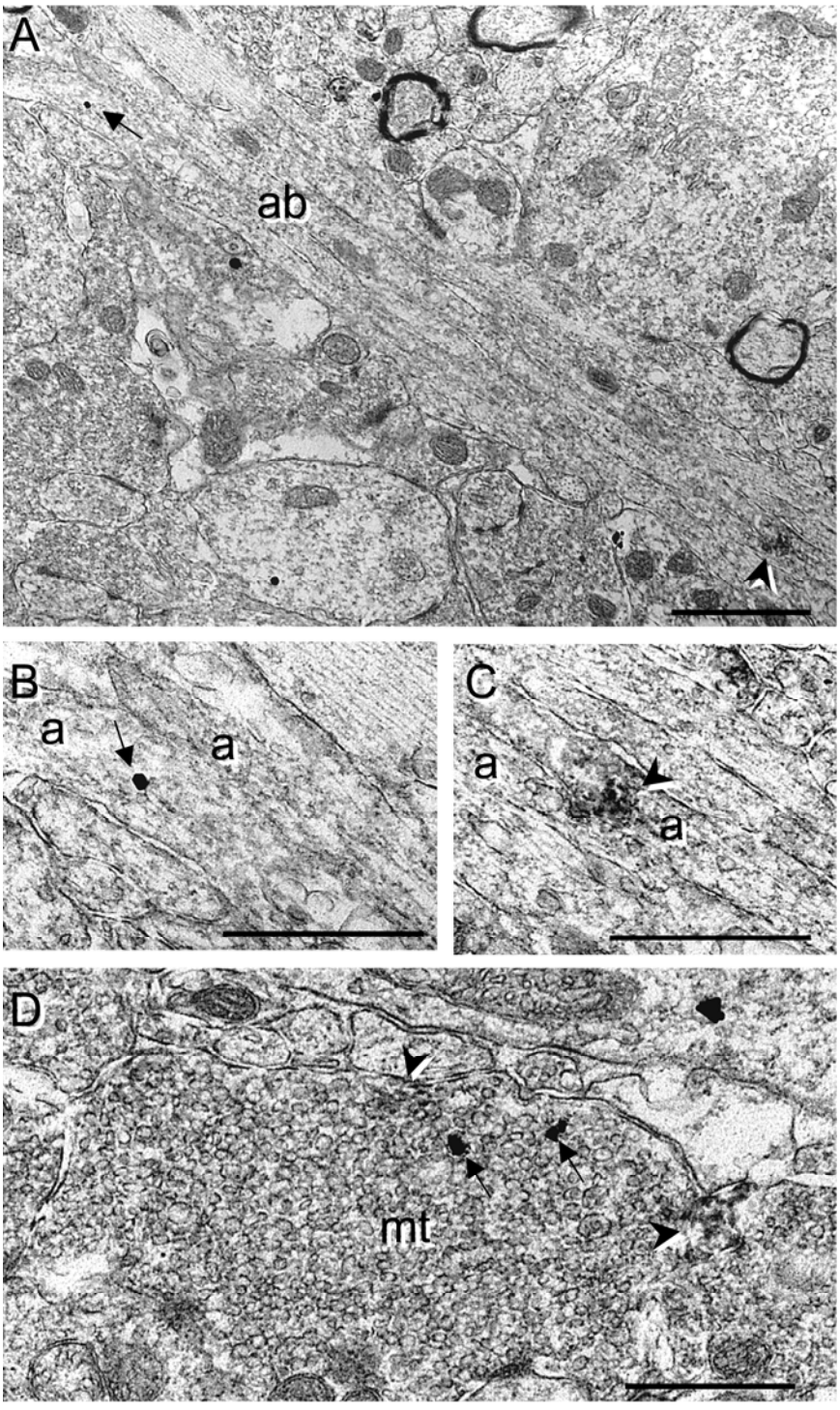
Figure 5.3



To confirm the localization of pTrkB-ir to mossy fibers and terminals, a few sections were dually labeled for pTrkB and the opioid peptide dynorphin, which is exclusively found in mossy fibers in the stratum lucidum (Drake et al., 2007). Indeed, dynorphin-immunoreactive axon bundles and mossy fiber terminals also contained pTrkB-ir (Figure 5.4), confirming that pTrkB localizes to the mossy fiber pathway in the mouse.

Figure 5.4 pTrkB-ir colocalizes with dynorphin in the mossy fiber pathway. (A) pTrkB-ir (peroxidase; arrowhead) and dynorphin-ir (SIG, black particles; arrow) localizes to the same axon bundle (ab) in the CA3 stratum lucidum. (B and C) Higher magnification shows dynorphin-ir and pTrkB-ir affiliated with vesicles in the axon (a). (D) A large mossy fiber terminal (mt) with dynorphin-ir (SIG, arrows) shows a cluster of pTrkB-ir (arrowhead) associated with vesicles near the plasma membrane. Scale bars, 0.5 μm.

Figure 5.4



Dendrites and dendritic spines contain pTrkB-ir

In dendrites, both labeling methods showed pTrkB-ir affiliated with the plasma membrane and endomembranes (Figure 5.5). Although more pTrkB-ir localized to axons than dendrites, a single dendrite often contained several patches of pTrkB-ir. In contrast to the pTrkB-ir contained in axon terminals, which was usually affiliated with vesicles opposite the synaptic contact, pTrkB-ir in dendritic spines was often affiliated with the spine apparatus adjacent to the synaptic contact (Figure 5.6). Occasionally, a labeled dendritic spine contacted a labeled axon terminal (Figure 5.6 A and B).

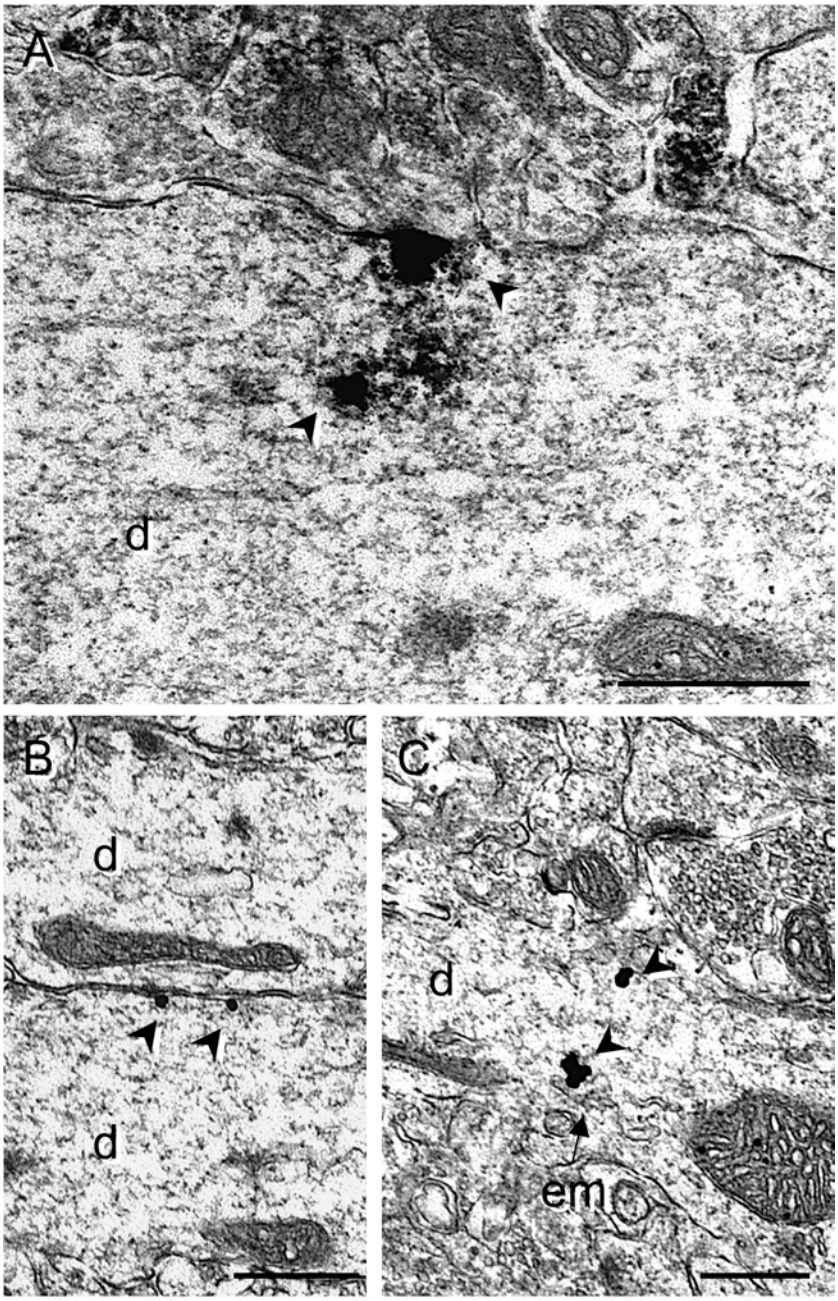
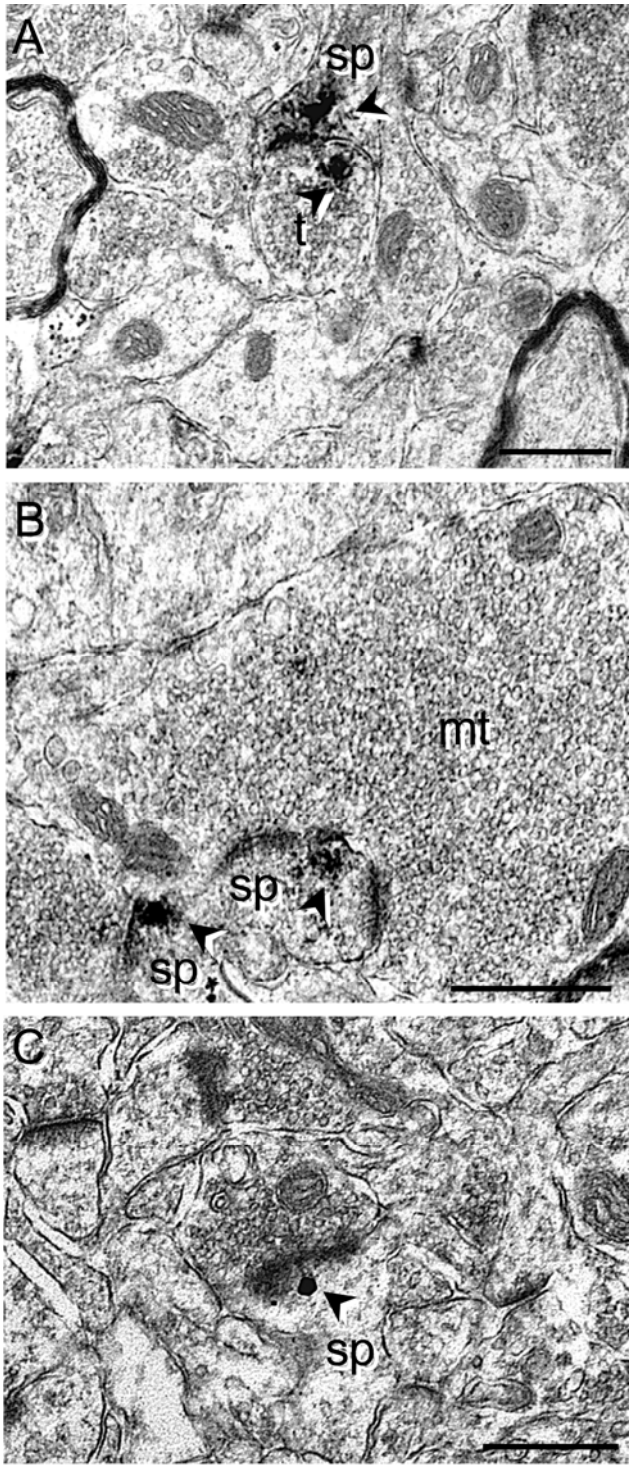


Figure 5.5 pTrkB-ir is found in dendritic shafts throughout the hippocampal formation. (A) In the CA1 stratum radiatum, clusters of pTrkB-ir (arrowheads) are found near the plasma membrane in dendritic shafts (d). (B and C) Immunogold labeling reveals pTrkB-ir (arrowheads) on the plasma membrane and affiliated with endomembranes (em, arrow) within dendrites. Scale bars, 0.5 μm.

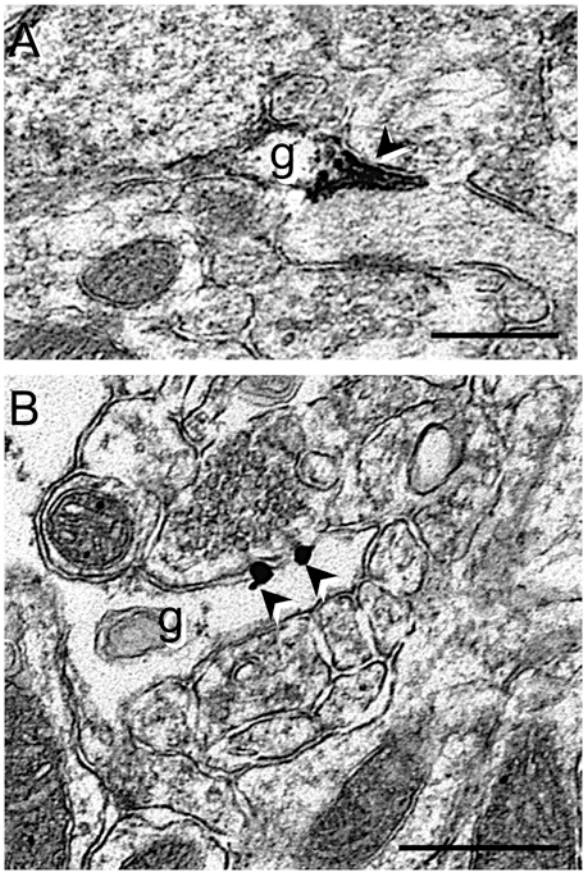
Figure 5.6 pTrkB-ir is found in dendritic spines throughout the hippocampal formation. (A) In the CA1 stratum radiatum, a dendritic spine (sp) containing pTrkB-ir (arrowheads) is contacted by a labeled axon terminal (t). (B) In the CA3 stratum lucidum, the same unlabeled mossy fiber terminal (mt) contacts two separate pTrkB-labeled spines. (C) SIG labeling shows pTrkB-ir (arrowheads) clustered next to the postsynaptic density. Scale bars, 0.5 μm.

Figure 5.6



Glial profiles contain pTrkB-ir

In addition to the neuronal pTrkB described above, many glial profiles contained pTrkB labeling. Diffuse pTrkB-ir was seen in glial profiles from sections labeled using the ABC-peroxidase method (Figure 5.7A). SIG pTrkB-ir was primarily on the plasma membrane of glia (Figure 5.7B). With these methods, it was not possible to distinguish among different types of glia containing pTrkB-ir.



5.7 pTrkB-ir is found in glial profiles throughout the hippocampal formation. (A) In the CA1 stratum radiatum, diffuse pTrkB-ir (arrowhead) is found in glial profiles (g). (B) SIG pTrkB-ir (arrowheads) is affiliated with the plasma membrane of a glial profile. Scale bars, 0.5 μm.

Few synaptic profiles contain pTrkB-ir

To determine the proportion of synaptic contacts containing pTrkB labeling, unlabeled axon terminals and dendritic spines in the distal CA1

stratum radiatum were counted. In this lamina, axon terminals were present at a density of 0.59 per μm^2, and dendritic spines at 0.47 per μm^2. The greater density of terminals reflects the presence of some axon terminals making asymmetric contacts with dendritic shafts, and the relative ease of identifying terminals outside the plane of the synaptic contact. pTrkB immunoreactivity was discerned in only 6.1 +/- 0.7% of axon terminals and 4.7 +/- 0.8% of dendritic spines. This suggests that at a given time, TrkB is activated in only a very small number of the total axon terminals and dendritic spines in the distal CA1 stratum radiatum of the mouse.

pTrkB-ir in axons increases in proestrus

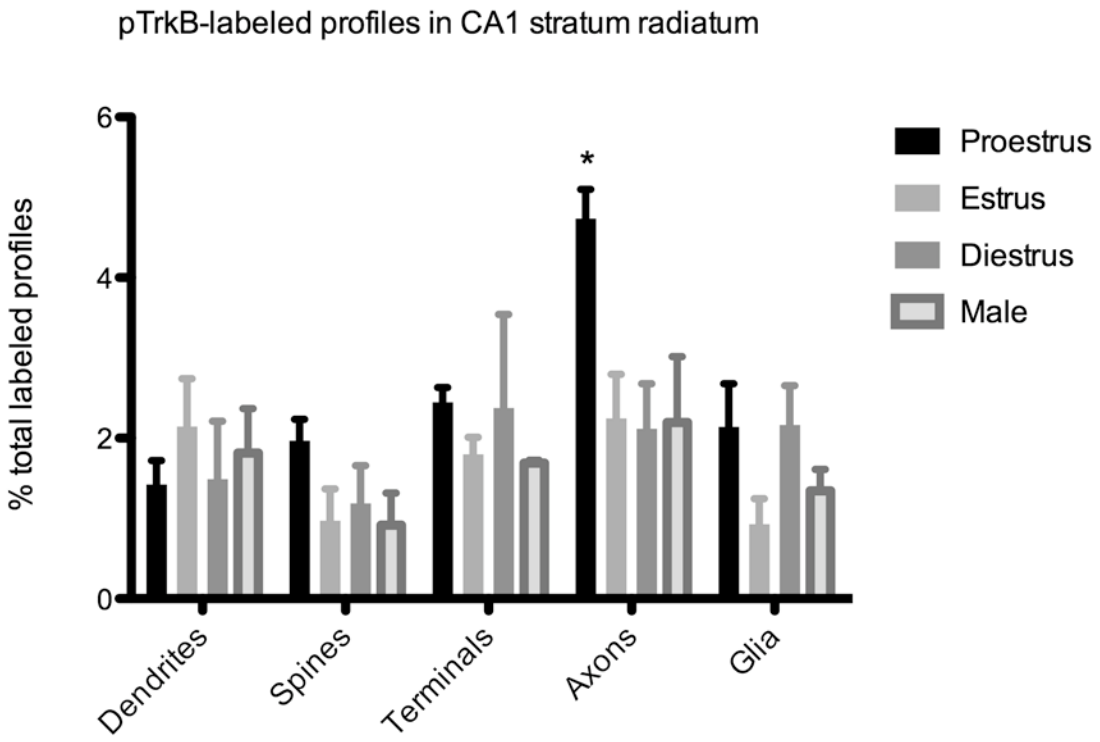
Chapter 5 of this thesis demonstrated that pTrkB-ir increases during proestrus relative to estrus and diestrus in the mouse hippocampal formation. To determine whether a shift in the distribution of pTrkB-ir accompanies this proestrus increase in total pTrkB-ir, the distribution of pTrkB-ir in proestrus females was compared with its distribution in estrus and diestrus females as well as males.

In proestrus animals, the relative number of pTrkB-labeled profiles increased in the CA1 subregion. Relative to the number of labeled profiles in

the entire hippocampal formation, there was more pTrkB-ir in the CA1 of proestrus females than in other groups of females or males (44.9 +/- 2.1%; Table 5.1). This was evident statistically as an overall effect of cycle phase on profile percent in the CA1 region ($F(3, 40) = 3.047$, $p = .0396$). The most striking difference was between sexes: proestrus females showed 34% increase in pTrkB-ir in the CA1 relative to males. In particular, post-hoc tests revealed that proestrus females had significantly more pTrkB-labeled axons in CA1 than males ($p<0.05$). Therefore, on top of the widespread increase in pTrkB-ir in the hippocampal formation during proestrus, axons in the CA1 may show particularly robust TrkB phosphorylation when circulating estradiol levels increase.

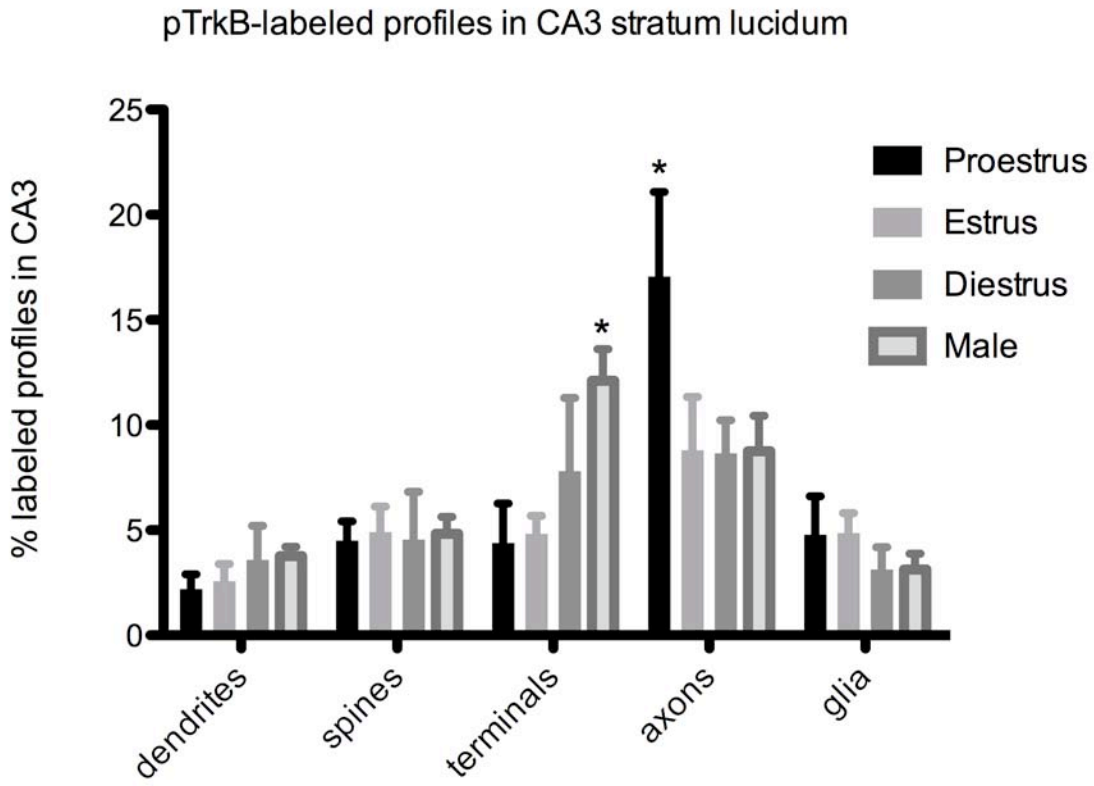
The increase in pTrkB-ir in the CA1 during proestrus may occur throughout this subregion, or in specific hippocampal laminae. After comparing the distribution of pTrkB-ir in each of the CA1 laminae, we found that the increase in labeled axons during proestrus occurred exclusively in the distal stratum radiatum of the CA1 (Figure 5.8). Two-way ANOVA revealed an overall effect of cycle/sex ($F(3, 40) = 3.606$, $p = 0.0214$). Post-hoc tests showed that pTrkB-labeled axons were significantly increased in proestrus females relative to estrus and diestrus females and males ($p<0.05$ for all). This indicates that the overall increase in pTrkB-ir in

the CA1 during proestrus resulted primarily from an increase in pTrkB-labeled axons in the distal CA1 stratum radiatum. Proestrus females also had more pTrkB-labeled dendritic spines and axon terminals in CA1 stratum radiatum than other females or males, but these differences were not statistically significant in post-hoc analyses.



5.8 Axonal pTrkB-ir in the distal CA1 stratum radiatum is affected by estrous cycle stage. The number of profiles in each cellular compartment was expressed as a percentage of the counted profiles counted in all sampled hippocampal areas. Females in the proestrus phase of the estrous cycle had significantly more pTrkB-labeled axons than estrus and diestrus females and males. *, $p<0.05$. N = 3.

Shifts in the distribution of pTrkB-ir also may occur within each hippocampal subregion, without any change in the overall percentage of pTrkB-labeled profiles in that region. Analysis of the distribution of pTrkB-labeled profiles within each hippocampal subregion revealed this type of redistribution in the CA3 stratum lucidum (Figure 5.9).



5.9 Axonal pTrkB-ir in the CA3 stratum lucidum is affected by estrous cycle stage and sex. The number of profiles in each cellular compartment was expressed as a percentage of the counted profiles in the CA3 region. Females in the proestrus phase of the estrous cycle had significantly more pTrkB-labeled axons than estrus and diestrus females and males. Male mice had significantly more pTrkB-labeled axon terminals than proestrus and estrus females. *, $p<0.05$. N = 3.

Two-way ANOVA revealed an interaction between cycle/sex and profile type ($F(12, 40) = 2.254$, $p = 0.0272$). Post-hoc tests showed that, similar to the findings in CA1 stratum radiatum, proestrus females had more pTrkB-labeled axons in stratum lucidum than estrus and diestrus females males ($p<0.05$ for all). In contrast, males had more pTrkB-labeled axon terminals than proestrus and estrus females ($p<0.05$), but not diestrus females. This indicates that when circulating estradiol increases during proestrus, the proportion of pTrkB-ir decreases in axons and increases in axon terminals.

Discussion

This is the first study to precisely localize activated TrkB in the mouse hippocampal formation. The extensive quantitative analysis is relevant not only to estradiol effects on TrkB, most pertinent to this document, but also for its general relevance to TrkB signaling and synaptic plasticity. This section will therefore discuss the findings with these specific and general implications in mind.

Previous immunocytochemical localization of TrkB in the rat hippocampal formation, performed by the Milner lab using electron

microscopy, showed extensive labeling of axon initial segments (Drake et al., 1999). In that study, the authors suggested that this labeling indicated extensive trafficking of TrkB to axons. The current study extends that finding, showing that the majority of activated TrkB, measured as pTrkB-ir, is in axons and axon terminals in the mouse hippocampal formation. Furthermore, this abundant presynaptic pTrkB was sensitive to natural fluctuations of ovarian steroids across the estrous cycle.

Methodological considerations

The pTrkB antibody used in these studies was tested for specificity using several methods (see Chapter 8), but future studies should confirm this in sections from a TrkB conditional knockout mouse. The antibody does not recognize the pTrkA neurotrophin receptor, but does recognize the pTrkC receptor in Western Blots from HEK293 cells (Moses Chao, personal communication). This cross-reactivity is unlikely to confound the present immunocytochemistry studies, as we saw no colocalization between pTrkB and TrkC antibodies in fluorescent immunocytochemistry on hippocampal sections (Joanna Spencer, unpublished observations).

pTrkB is positioned to locally regulate synaptic transmission and neurite growth

In vitro, TrkB modulates pre- and postsynaptic properties, including synaptic vesicle dynamics, neurotransmitter release, calcium influx, actin dynamics, and dendritic spine remodeling (Ji et al., 2005; Gomes et al., 2006; Magby et al., 2006; Rex et al., 2007). The localization of pTrkB-ir to both axon terminals and dendritic spines in the current study suggests that TrkB may indeed locally modulate synaptic function in the intact mouse hippocampus. In most cases, the pTrkB-ir in spines and terminals was not affiliated with the plasma membrane. This suggests that the rapid endocytosis of activated TrkB that has been described in cultured neurons may also occur *in vivo* (Du et al., 2003). In spines, pTrkB-ir was often adjacent to the synaptic density, positioning the receptor for local degradation or recycling back to the plasma membrane (Chen et al., 2005; Zheng et al., 2008). In contrast, in axon terminals, pTrkB-ir was often affiliated with small synaptic vesicles distal to the synaptic contact, suggesting that this internalized pTrkB may be in transit down the axon. Such retrograde transport of pTrkB has been previously described, and may

function to communicate TrkB signaling from the synapse to the cell body (Bhattacharyya et al., 1997; Watson et al., 1999).

Despite the important role ascribed to TrkB activation at the synapse, less than 10% of the terminals and spines in the CA1 stratum radiatum contained pTrkB-ir. The pre-embedding immunocytochemical method used to localize pTrkB in this study may slightly underestimate the total amount of pTrkB contained in the tissue (Milner et al., 2005). Still, this finding is striking, as TrkB itself is most likely present in every glutamatergic hippocampal neuron. The specific complement of synapses containing activated TrkB at any given time may be meaningful for the encoding or retrieval of information, perhaps participating in the "tagging" of individual synapses for sustained potentiation (Frey and Morris, 1998; Boulanger and Poo, 1999; Nagappan and Lu, 2005; Reymann and Frey, 2007).

In addition to its synaptic effects, TrkB may locally regulate axonal and dendritic arborization (Alsina et al., 2001; Horch and Katz, 2002; Horch, 2004). Indeed, pTrkB-ir was often affiliated with the plasma membrane in axons and dendrites, suggesting local activation of TrkB in these neuronal processes.

pTrkB is positioned to regulate glial cell function

The role of TrkB in glial cell function in the adult has received very little attention. It is widely believed that glia express only the truncated TrkB isoform lacking the intracellular catalytic domain (Alderson et al., 2000; Rose et al., 2003; Ohira et al., 2005; Ohira et al., 2007). However, one study found that cortical glia in culture express a small amount of functional full-length TrkB (Roback et al., 1995). The current study found that glial profiles contained one fifth of all pTrkB-ir, using an antibody that recognizes only the full-length TrkB. This suggests that not only do glial cells in the adult hippocampal formation express the full-length TrkB, but that this TrkB can be activated and may be an important aspect of neurotrophin functions in vivo.

Presynaptic pTrkB is implicated in estrous cycle fluctuation of BDNF signaling

In Chapter 4, I demonstrated that pTrkB-ir increases in the mouse hippocampal formation during proestrus. The current study revealed increased pTrkB-ir in axons likely contributed to these changes in two

specific subregions, the CA1 stratum radiatum and CA3 stratum lucidum. The anatomical specificity of this finding is notable, as these two hippocampal laminae contain crucial synaptic contacts for hippocampal function and synaptic plasticity. Mossy fiber synapses on CA3 pyramidal cell dendrites in stratum lucidum are critical for spatial learning in mice (Stupien et al., 2003; Florian and Roullet, 2004; Gartner et al., 2006; Kesner, 2007). Schaffer collateral synapses of CA3 axons on CA1 pyramidal cell dendrites, in CA1 stratum radiatum, are the most well characterized site of hippocampal LTP (Kerchner and Nicoll, 2008). TrkB activation at these synapses is crucial for LTP and hippocampal-dependent associative learning (Gruart et al., 2007). Given the importance of these two laminae for hippocampal function, increased TrkB activity in these areas likely contributes to the increased hippocampal excitability and plasticity during proestrus (Warren et al., 1995; Scharfman et al., 2003).

The proestrus increase in axonal pTrkB-ir, as opposed to pTrkB-ir in other cell compartments, has several possible implications. Increased axonal TrkB activation could increase arborization to affect synaptic plasticity indirectly through the availability of presynaptic contacts. Although an effect of estradiol on hippocampal axons has not been reported, estradiol increases axonal growth of cultured hypothalamic neurons through a TrkB-

dependent mechanism (Carrer et al., 2003; Brito et al., 2004). Furthermore, in rat hippocampal slices, estradiol does indeed increase the number of presynaptic sites (Sato et al., 2007).

Alternatively, increased axonal pTrkB-ir in proestrus could reflect increased retrograde transport of pTrkB, perhaps due to increased TrkB activation at axon terminals. Increased retrograde transport of pTrkB might decrease pTrkB-ir in axon terminals, as occurred in CA3 stratum lucidum during proestrus. A role for presynaptic TrkB in synaptic potentiation was first described in studies of the developing neuromuscular junction (Lohof et al., 1993; Stoop and Poo, 1996). These findings have since been extended to hippocampal neurons (Kang and Schuman, 1995; Pozzo-Miller et al., 1999; Xu et al., 2000; Tyler and Pozzo-Miller, 2001; Bamji et al., 2006; Madara and Levine, 2008). The current finding that presynaptic pTrkB-ir increased during proestrus is consistent with these studies that point to the importance of presynaptic TrkB in BDNF-mediated synaptic plasticity.

Estradiol may cause the increase in axonal pTrkB by several different mechanisms. As described in Chapter 4, estradiol increases BDNF expression, which could increase TrkB activation in proportion to BDNF availability. Estradiol also increases BDNF release in the CA3 of the rat hippocampus, which could increase presynaptic TrkB activation (Sato et al.,

2007). In addition, estradiol enhances retrograde transport of BDNF in the rat basal forebrain (Jezierski and Sohrabji, 2003). If this occurs in the mouse hippocampus, increased pTrkB transport from axon terminals could directly increase pTrkB-ir in axons. Finally, estradiol may affect TrkB signaling indirectly by augmenting calcium influx (Wu et al., 2005; Zhao et al., 2005). Such increases in neuronal activity rapidly enhance TrkB insertion into the membrane, kinase activity, and pTrkB endocytosis (Du et al., 2000; Du et al., 2003), events which may be involved in the "tagging" of active synapses (Nagappan and Lu, 2005). Without knowledge of the relative role of each of these mechanisms, the results of the current study suggest that the coordinated increase in neuronal activity and BDNF availability during proestrus specifically increase presynaptic TrkB activation in the CA1 stratum radiatum and CA3 stratum lucidum. The downstream effects of this increased presynaptic pTrkB could include enhanced synaptic plasticity through more efficient synaptic "tagging," and the creation of new presynaptic sites.

Sex differences in the distribution of pTrkB may reflect the influence of estradiol

The distribution of pTrkB-ir in male mice resembled that of low-estradiol (estrus or diestrus) females. Specifically, males had less pTrkB-ir in axons in the CA1 stratum radiatum and CA3 stratum lucidum than proestrus females, and more pTrkB-ir in axon terminals in the stratum lucidum than proestrus or estrus females. This similarity between males and low-estradiol females means that estradiol likely mediates any sex differences in BDNF/TrkB signaling. Indeed, the behavioral effects of BDNF reduction in mice differ significantly in males and females, and some of these differences may be explained by the presence or absence of estradiol (Monteggia et al., 2006; Ren-Patterson et al., 2006). In females, the ability of estradiol to increase presynaptic TrkB activation may protect females against some of the effects of BDNF reduction.

Summary

This ultrastructural localization of pTrkB in the mouse hippocampal formation shows pTrkB well positioned to carry out local effects of BDNF

in synapses and neurites, and more sustained effects via retrograde transport and activation of gene transcription. It provides evidence that presynaptic TrkB is important for the maintenance of hippocampal function, as the majority of pTrkB-ir was found in axons and terminals. Furthermore, this axonal pTrkB-ir increased during the high-estradiol stage of the estrous cycle, proestrus. This is strong evidence for the importance of presynaptic TrkB activation in estradiol effects on hippocampal activity and synaptic plasticity.

Chapter 6

BDNF variant Val66Met modifies the estrous cycling of hippocampal function

The molecular endpoints studied in this thesis were chosen because of their importance to hippocampal synaptic function, and therefore their possible relevance to estradiol effects on hippocampal-dependent learning and memory. For this reason, it is important to relate these molecular effects of estradiol to behavior. To do this, I studied estrous cycle fluctuation of behavior in wild-type mice, and mice with a specific disruption in BDNF signaling.

The BDNF variant Val66Met is a single nucleotide polymorphism in the pro region of the BDNF gene, found commonly in certain human ethnic groups; it is carried by 20-30% of Caucasians (Shimizu et al., 2004). Neurons expressing the Met variant show impaired trafficking of pro-BDNF and approximately 30% less activity-dependent BDNF secretion (Egan et al., 2003; Chen et al., 2004; Chen et al., 2006). The study of estradiol effects in these mice will therefore provide information about the importance of

activity-dependent BDNF secretion in these effects. Furthermore, because of the high prevalence of this BDNF variant in certain human populations, the findings are potentially clinically relevant.

For these studies, I collaborated with Dr. Francis Lee in his laboratory at Weill Cornell Medical College to conduct experiments on transgenic mice expressing the BDNF Val66Met variant. Behavioral experiments were conducted using wild type (Val/Val) and homozygous variant (Met/Met) female mice, which were then sacrificed in proestrus and diestrus.

This was the first thorough investigation of behavior in female Val66Met mice. Because of this, the first set of studies sought to identify overall genotype differences in non-mnemonic and mnemonic behaviors. To study fluctuation of hippocampal function across the estrous cycle, spatial memory was tested in several difference phases of the estrous cycle. Estrous cycles were carefully monitored and followed up with molecular studies to discover any major genotype effects on reproductive function that could confound study results. To relate the behavioral and molecular findings, immunocytochemistry and in situ hybridization assays were conducted for the following endpoints in sections through the dorsal hippocampal formation: BDNF mRNA, TrkB mRNA, pTrkB-ir, pAkt-ir, and PSD-95-ir.

Met/Met mice are reproductively normal

The hormonal control of reproduction is centered on the hypothalamic-pituitary-gonadal (HPG) axis, which regulates circulating hormone levels through complex positive and negative feedback loops. The resulting fluctuations in ovarian steroids across the estrous cycle induce measurable changes in reproductive behavior, vaginal cytology, and hypothalamic gene expression.

Met/Met females had regular estrous cycles, with characteristic length and vaginal cytology, which were indistinguishable from Val/Val cycles. These mice reproduce normally, with no obvious differences in reproductive or maternal behavior. This suggests no significant changes in HPG axis function in Val/Val and Met/Met mice. To substantiate these qualitative assessments with a quantitative measure, progesterone receptor (PR) expression was examined in the medial preoptic nucleus (MPO) of the hypothalamus, where neurons release gonadotropin-releasing hormone in response to hormonal stimuli. Estradiol induces PR expression in this region, most likely via nuclear ERs (Kudwa et al., 2004). Proestrus mice

should therefore show increased PR expression in the MPO relative to diestrus mice.

In sections through the MPO labeled with anti-PR antibody, cells expressing PR were counted in a fixed polygon of area 6556.91 μm^2 in the MPO from each hemisphere (Figure 61).

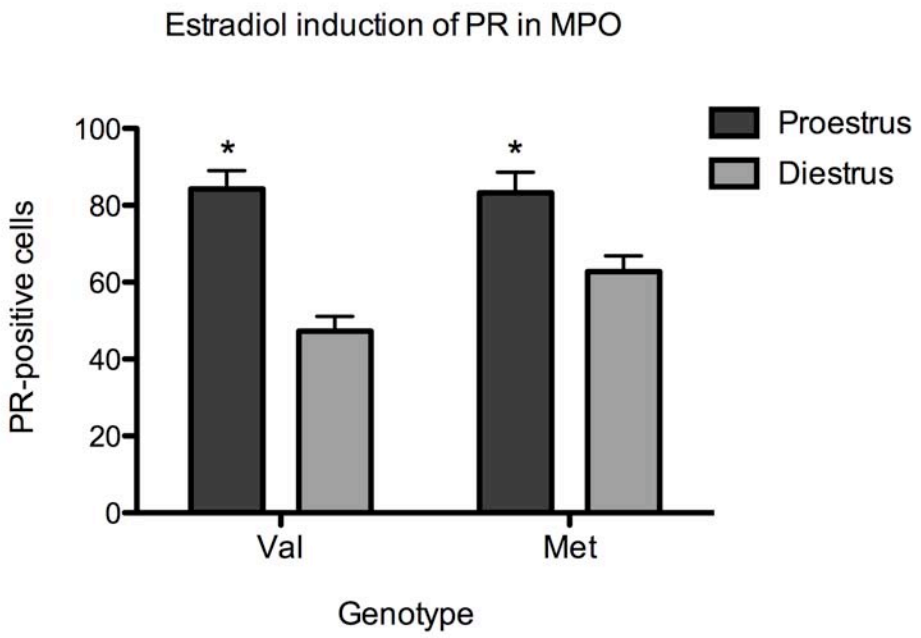


Figure 6.1 PR expression increases during proestrus in BDNF Val66Met mice. PR-positive cells were counted from a fixed area in the medial preoptic area of the hypothalamus. PR mRNA was significantly higher in proestrus than diestrus in both Val/Val and Met/Met mice. R.O.D., relative optical density. *, $p<0.05$. PR, progesterone receptor; MPO, medial preoptic area. N = 6 for Val/Val proestrus, 4 for Val/Val diestrus, 5 for Met/Met proestrus, and 4 for Met/Met diestrus.

The number of PR-labeled cells in this area was higher in proestrus than diestrus in both Val/Val and Met/Met genotypes (Figure 6.1). Two-way ANOVA with genotype and cycle phase as the independent factors showed a significant overall effect of genotype ($F(1, 16) = 32.42$, $p < 0.0001$). Post-hoc tests revealed that for both genotypes, the density of PR-labeled cells increased significantly during proestrus ($p<0.05$ for both).

In sum, BDNF Met/Met mice are reproductively normal, with no differences in vaginal cytology, reproductive behavior, or cyclic PR expression in the MPO. These findings suggest no differences in circulating ovarian steroid levels or nuclear ER activity between Val/Val and Met/Met mice.

Met/Met mice show differences in non-mnemonic behaviors

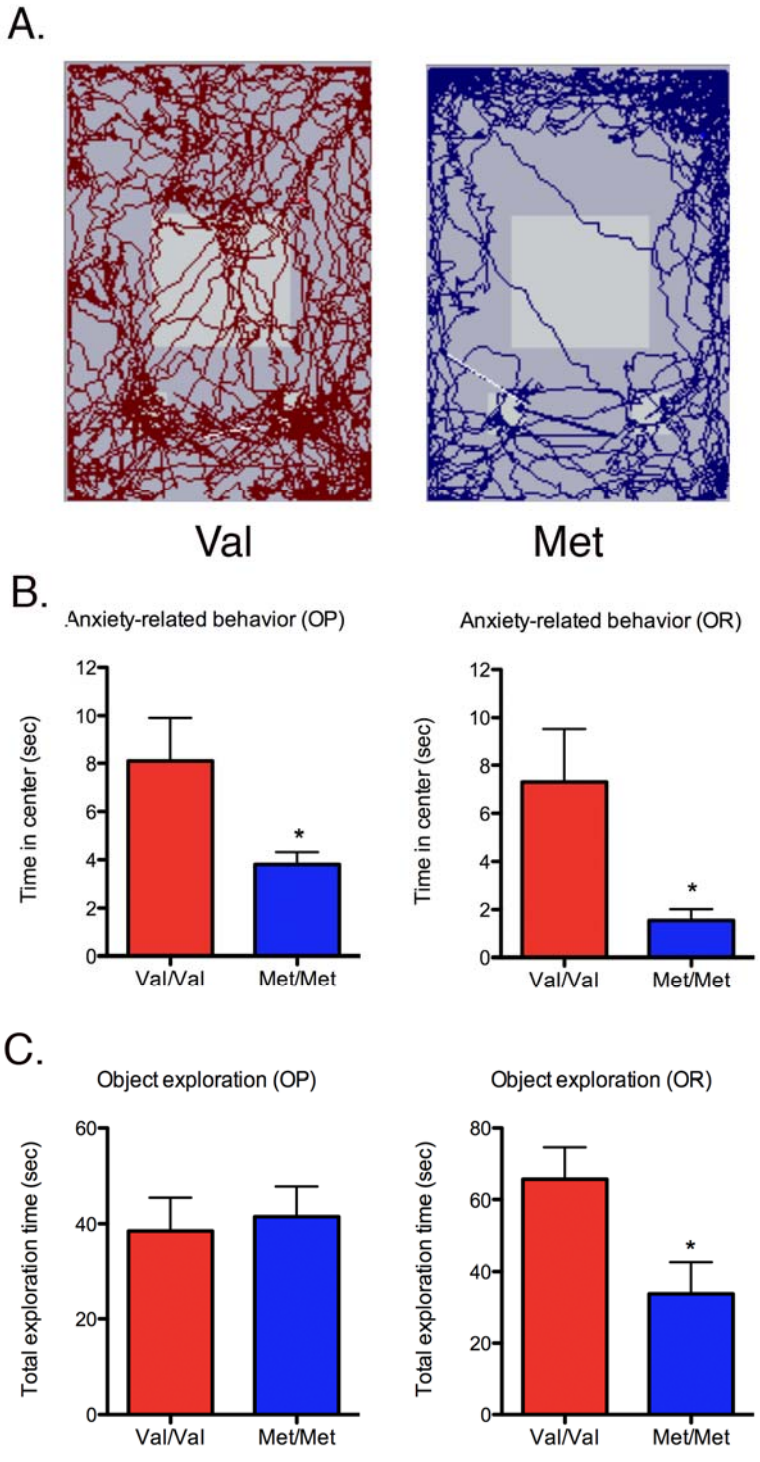
To test the effects of the BDNF variant Val66Met on behavior in females, female Met/Met and wild-type Val/Val littermates in mixed estrous cycle phases were tested on two tests of short-term memory, novel object placement recognition (OP) and novel object recognition (OR). Both tests consisted of a sample trial and a recognition trial separated by an intertrial delay. The OP test uses two familiar, identical objects to assess recognition

of a new object location relative to external cues. The OR test uses novel objects to assess recognition of a new object identity. Both tests are often used to assess hippocampal function in rodents, though other brain regions play a major role in OR performance (Luine, 2006; Good et al., 2007). In addition to object and place recognition, two non-mnemonic behaviors were recorded during the sample trials: the amount of time spent in the center of the open field, and total object exploration. The center time is a commonly used measure of anxiety behavior in rodents, where less time signifies increased anxiety-type behavior.

Met/Met females spent significantly less time in the center of the open field during the sample trial of both OP ($p = 0.0437$) and OR ($p = 0.0240$) tests (Figure 6.2A and B). There was no difference between genotypes in time spent exploring the two identical, familiar objects in the OP sample trial (Figure 6.2C). In contrast, Met/Met females spent significantly less time exploring the two identical, novel objects in the OR sample trial (6.2A and D; $p = 0.0222$). In sum, Met females exhibit increased anxiety behavior and decreased overall exploration of novel, but not familiar, objects.

Figure 6.2 BDNF Val66Met affects non-mnemonic behaviors in female mice. Anxiety and exploratory behavior were examined from the sample trials of the OP and OR test in BDNF Val66Met mice. (A) Diagram of the exact path of one representative mouse of each genotype during a sample trial of the object recognition test as recorded by the Noldus software. White circles represent objects, and white squares represent the center of the open field used for quantification. (B) Anxiety-related behavior was measured as the amount of time spent in the center of the open field, where less time in the center is interpreted as higher anxiety-related behavior. Met/Met females spent significantly less time in the center than Val/Val females during the sample trials of both OP and OR tasks. (C) Exploratory behavior was measured as the amount of time spent exploring both objects. Met/Met females spent significantly less time exploring the objects during the sample trial of the OR task, but not the OP task. *, $p<0.05$. N = 8 for OP, 5 for OR.

Figure 6.2


A.
Val
Met
B.
Anxiety-related behavior (OP)
Time in center (sec)
12
10
8
6
4
2
0
*
Val/Val
Met/Met
Anxiety-related behavior (OR)
Time in center (sec)
12
10
8
6
4
2
0
*
Val/Val
Met/Met
C.
Object exploration (OP)
Total exploration time (sec)
60
40
20
0
Val/Val
Met/Met
Object exploration (OR)
Total exploration time (sec)
80
60
40
20
0
*
Val/Val
Met/Met

Met/Met mice exhibit memory impairment

The OR and OP tests rely on a mouse's tendency to explore a novel object (or object location) more than a familiar one, thereby testing recognition of the familiar object or object placement. Object recognition relies on the hippocampal formation, parahippocampal cortices, and other brain regions in rodents, while object placement recognition is particularly dependent on hippocampal function (Dere et al., 2007; Squire et al., 2007). In order to successfully utilize these tasks to compare memory and hippocampal function in BDNF Val66Met mice, it was necessary to confirm that Met/Met females retain their preference for novelty when presented with one familiar and one novel object. This is especially important considering that Met/Met mice explored novel objects less than Val/Val females in the OR sample trial (Figure 6.2).

To determine novelty preference in both genotypes, mice were tested on the OR task using an easily differentiated pair of objects (chosen based on experience). The amount of time spent exploring the novel object during the recognition trial was expressed as a fraction of the time spent with both objects, termed "novel object fraction." If the mouse spent the same amount of time with both objects, a result consistent with no object preference, the

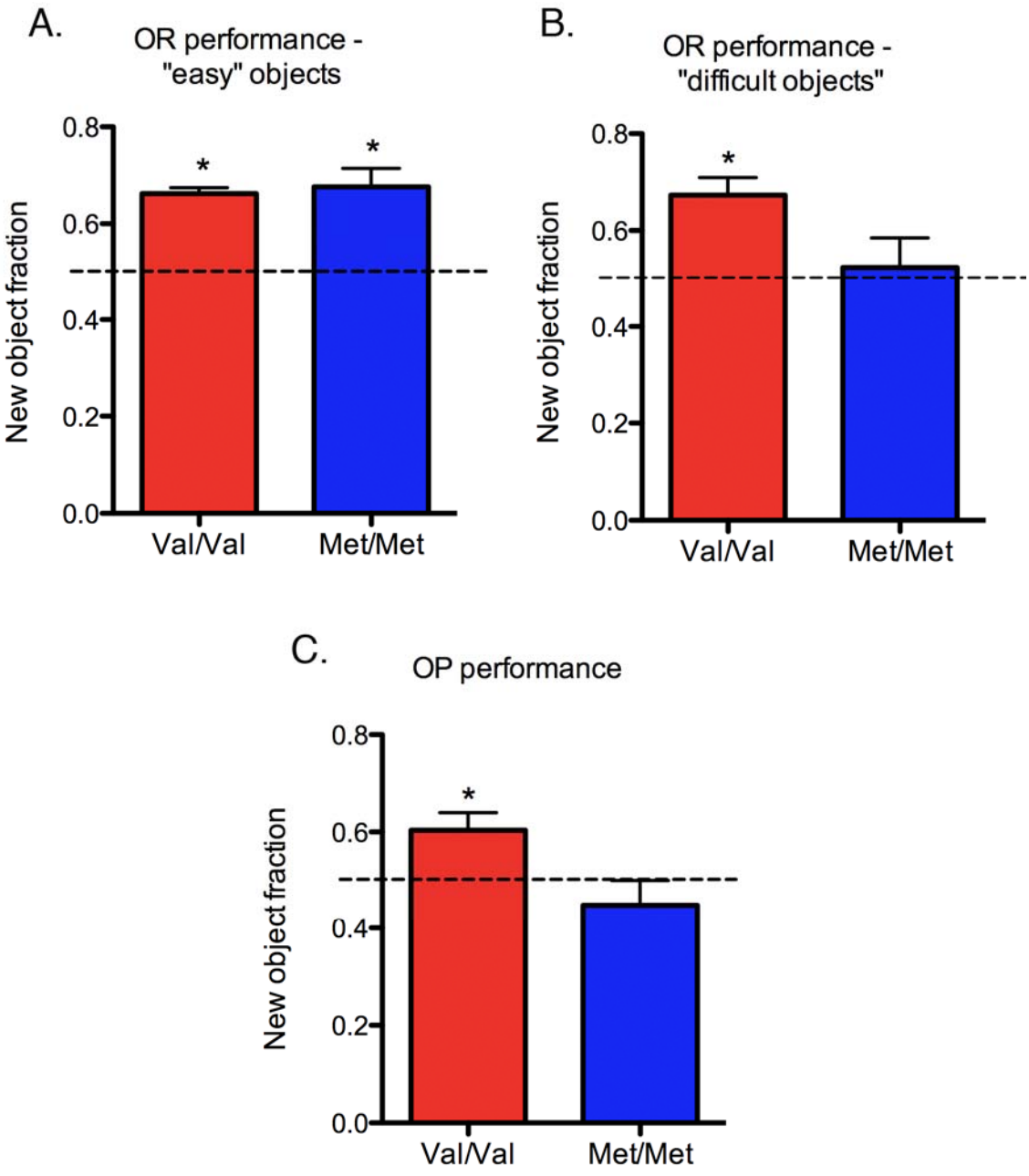
novel object fraction would be 0.5. Therefore, "chance" performance was defined as a fraction of 0.5, and "recognition" was defined as a fraction significantly greater than 0.5 (using a one-sample t-test).

Val/Val and Met/Met females both recognized the novel object (Figure 6.3A; $p < 0.0001$ for Val/Val and $p = 0.0103$ for Met/Met). This suggests that the Met/Met mice do indeed prefer novel objects, and therefore the OP and OR tasks are appropriate tests of memory.

To explore possible genotype differences in object recognition, mice were tested again on the OR task using a new, less easily differentiated, object pair (Figure 6.3B). This time, Val/Val mice recognized the novel object ($p = 0.0090$), while Met/Met mice did not. Mice were also tested on the OP task (Figure 6.3C). Again, Val/Vals recognized the novel object placement ($p = 0.0181$), while Met/Mets did not. Thus compared to wild type Val/Val females, Met/Met females exhibit impaired object and place recognition.

Figure 6.3 BDNF Val66Met impairs memory OP and OR performance was measured using the novel object fraction, where 0.5 (dotted line) represents chance performance. (A) Val/Val and Met/Met females recognized the novel object in an OR test using easily distinguishable objects, indicating that both genotypes show preference for novel objects. (B) Val/Val but not Met/Met females recognized the novel object in an OR test using less easily distinguishable objects. (C) Val/Val but not Met/Met females recognized the novel object placement. *, $p<0.05$ relative to chance. N = 8 for OP, 5 for OR.

Figure 6.3


A.
OR performance -
"easy" objects
New object fraction
0.8
0.6
0.4
0.2
0.0
*
*
Val/Val
Met/Met
B.
OR performance -
"difficult objects"
New object fraction
0.8
0.6
0.4
0.2
0.0
*
Val/Val
Met/Met
C.
OP performance
New object fraction
0.8
0.6
0.4
0.2
0.0
*
Val/Val
Met/Met

Estrous cycle affects the Val66Met cognitive phenotype

When mice are tested on the OP task repeatedly on different days, no overall change in performance is seen, suggesting no effect of practice on OP performance (personal experience and (Luine et al., 2003)). Val/Val and Met/Met mice were therefore tested repeatedly in each of four estrous cycle phases: proestrus, estrus, diestrus 1, and diestrus 2, to assess the effect of estrous cycle phase on spatial memory. Since all Val/Val females recognized the new object placement on their first OP test (Figure 6.3C), with a 5 minute intertrial delay, the task was made more difficult by increasing the intertrial delay to 30 minutes, to increase the likelihood of detecting differences in performance across the cycle.

OP performance fluctuated across the estrous cycle for both Val/Val and Met/Met females (Figure 6.4). The new object fraction was significantly greater than chance in diestrus for Val/Vals ($p = 0.0038$), and proestrus for Met/Mets ($p = 0.0318$). Spatial memory was therefore best during diestrus in Val/Val mice, and proestrus in Met/Met mice. Performance was also analyzed using two-way repeated measures ANOVA, with cycle phase and genotype as the independent variables. There was a significant interaction between genotype and cycle phase on object

placement test performance ($F(3, 36) = 2.962$, $p = 0.0450$), indicating that the effect of the Met variant on OP performance depended on estrous cycle phase. There were no differences in non-mnemonic behaviors between genotypes or across the cycle, including mobility and exploratory behavior. Thus, independent of non-mnemonic factors that could enhance performance, spatial memory peaks during different estrous cycle stages in wild type Val/Val and BDNF variant Met/met mice.

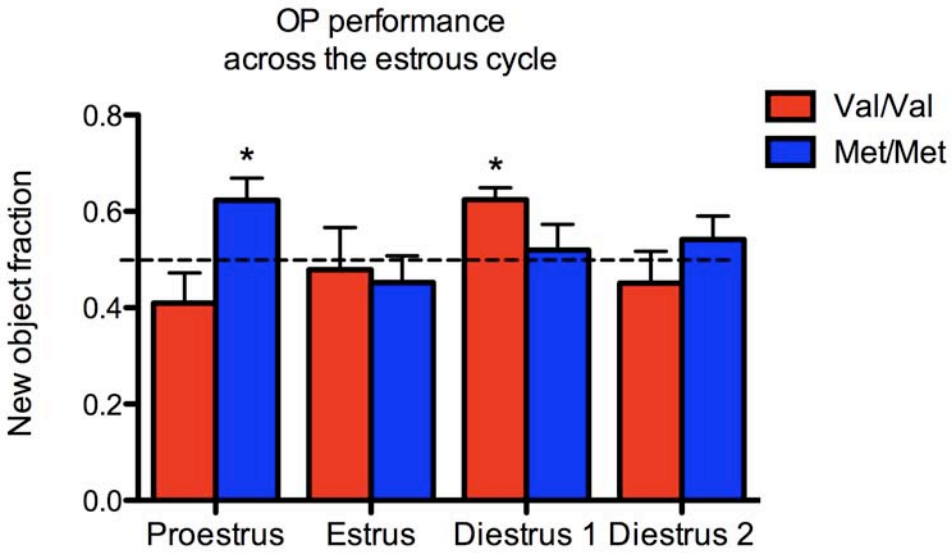


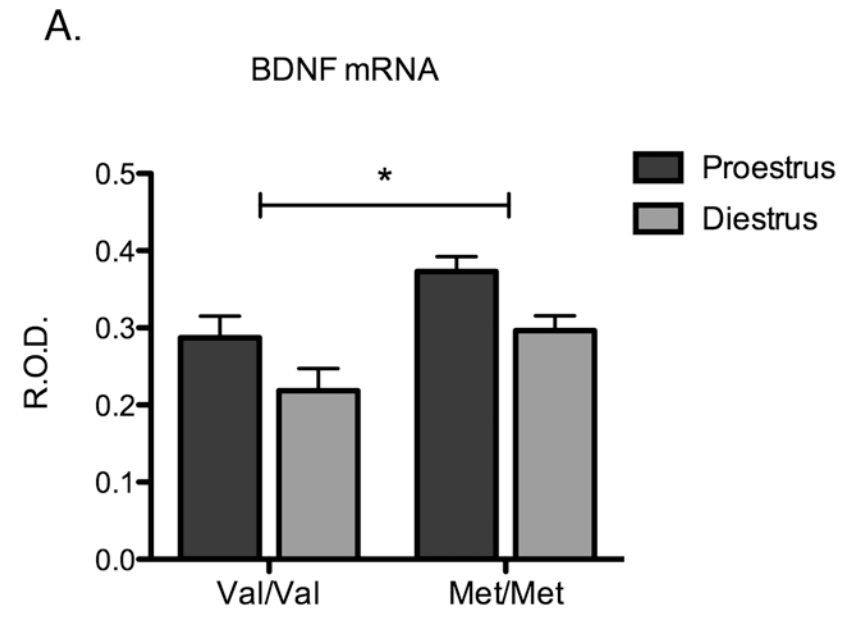
Figure 6.4 Estrous cycle and BDNF genotype interact to control OP performance. Val/Val and Met/Met mice were tested on the OP task in late morning of four different estrous cycle stages. Only Val/Val females in diestrus and Met/Met females in proestrus recognized the novel object placement, and two-way ANOVA showed a significant interaction between genotype and estrous cycle stage ($p<0.05$). *, $p<0.05$ relative to chance. N = 8.

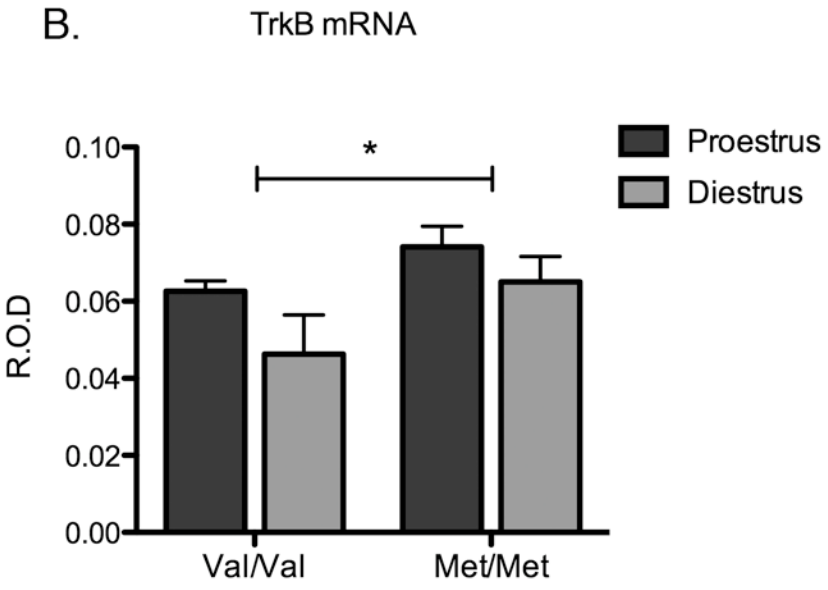
Met/Met hippocampi show increased BDNF and TrkB expression

Expression of BDNF and TrkB in the Val/Val and Met/Met mice was assayed using in situ hybridization in sections through the dorsal hippocampal formation (Figure 6.5). BDNF mRNA was measured in the CA3 pyramidal cell layer, where expression fluctuates across the estrous cycle in female rats (Scharfman et al., 2003). Similar to rats, BDNF mRNA was increased in proestrus compared to diestrus (Figure 6.5A). This was true for both Val/Val and Met/Met mice; one-way ANOVA showed a significant overall effect of cycle phase ($F(1, 15) = 7.994$, $p = 0.0127$). Furthermore, compared to the wild type Val/Vals, Met/Met mice had increased BDNF mRNA; one-way ANOVA showed a significant overall effect of genotype ($F(1, 15) = 10.17$, $p = 0.0061$).

TrkB mRNA was measured in the CA1 pyramidal cell layer, as in Chapter 4 (Figure 6.5B). Consistent with the results in Chapter 4, there was no significant fluctuation of TrkB mRNA across the estrous cycle. As with BDNF, TrkB mRNA was increased in Met/Mets compared to Val/Vals; one-way ANOVA showed a significant overall effect of genotype ($F(1, 13) = 6.576$, $p = 0.0235$).

Figure 6.5 BDNF Val66Met mice have increased hippocampal BDNF and TrkB expression (A) Optical density of BDNF mRNA from in situ hybridization films was measured in the CA3 pyramidal cell layer of the hippocampus and normalized to background density. BDNF mRNA was significantly increased during proestrus relative to diestrus in the combined sample. In addition, Met/Met females had significantly more BDNF than Val/Val females. (B) Optical density of TrkB mRNA from in situ hybridization films was measured in the CA1 pyramidal cell and normalized to background density. Met/Met females had significantly more TrkB mRNA than Val/Val females. R.O.D., relative optical density. * $p<0.05$. N = 6 for Val/Val proestrus, 4 for Val/Val diestrus, 5 for Met/Met proestrus, and 4 for Met/Met diestrus.





In sum, BDNF mRNA but not TrkB mRNA increased significantly during proestrus compared to diestrus. BDNF and TrkB mRNA were significantly increased in BDNF variant Met/Met compared to wild type Val/Val females.

Val66Met alters estrous cycle fluctuation of pAkt and PSD-95

The proestrus increase in BDNF mRNA in both Val/Val and Met/Met females suggests that estradiol increases hippocampal BDNF expression in both genotypes. However, since spatial memory peaks during different estrous cycle phases in Val/Val and Met/Met mice, there must be some difference in how estradiol affects hippocampal function in these genotypes. To address this, immunocytochemistry for pTrkB, PSD95, and pAkt was performed in tissue sections through the dorsal hippocampal formation. Based on previous chapters in this thesis, I expected that all of these endpoints would increase during proestrus in the wild type (Val/Val) females. To compare proestrus changes between genotypes, proestrus values for each endpoint were expressed as % change from diestrus, and these changes were directly compared between genotypes. Raw data (optical density normalized to background for each endpoint) was also compared between genotypes and cycle phases using two-way ANOVA.

Based on the proestrus increase in BDNF, it was not surprising that pTrkB-ir increased during proestrus in both Val/Val and Met/Met mice, with no difference between genotypes (Figure 6.6). Two-way ANOVA confirmed an overall effect of estrous cycle phase on pTrkB-ir in CA1

stratum radiatum (F (1,15) = 5.467, p = 0.0336) and dentate hilus (F (1, 15) = 8.502, p = 0.012).

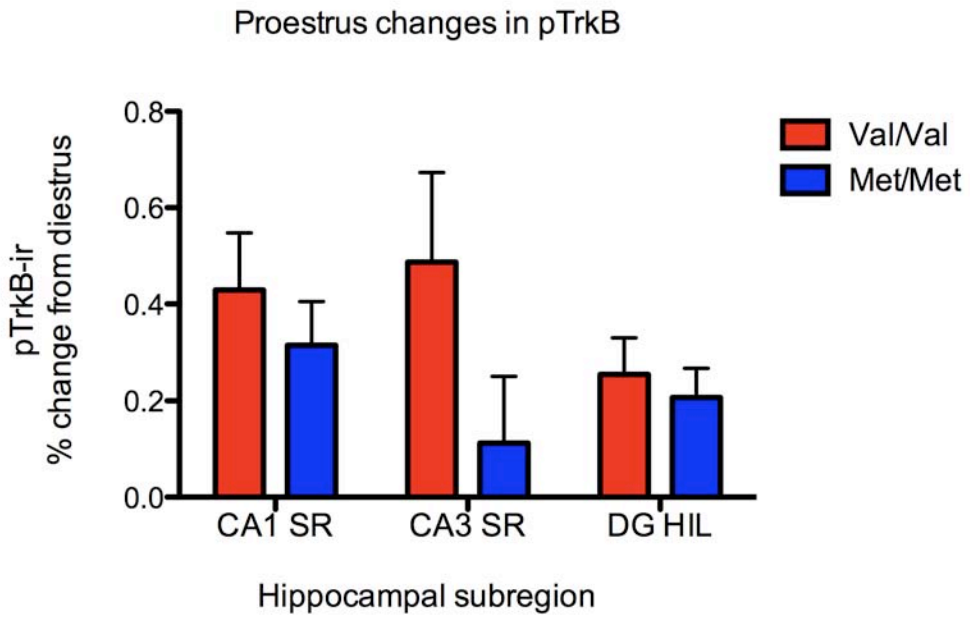
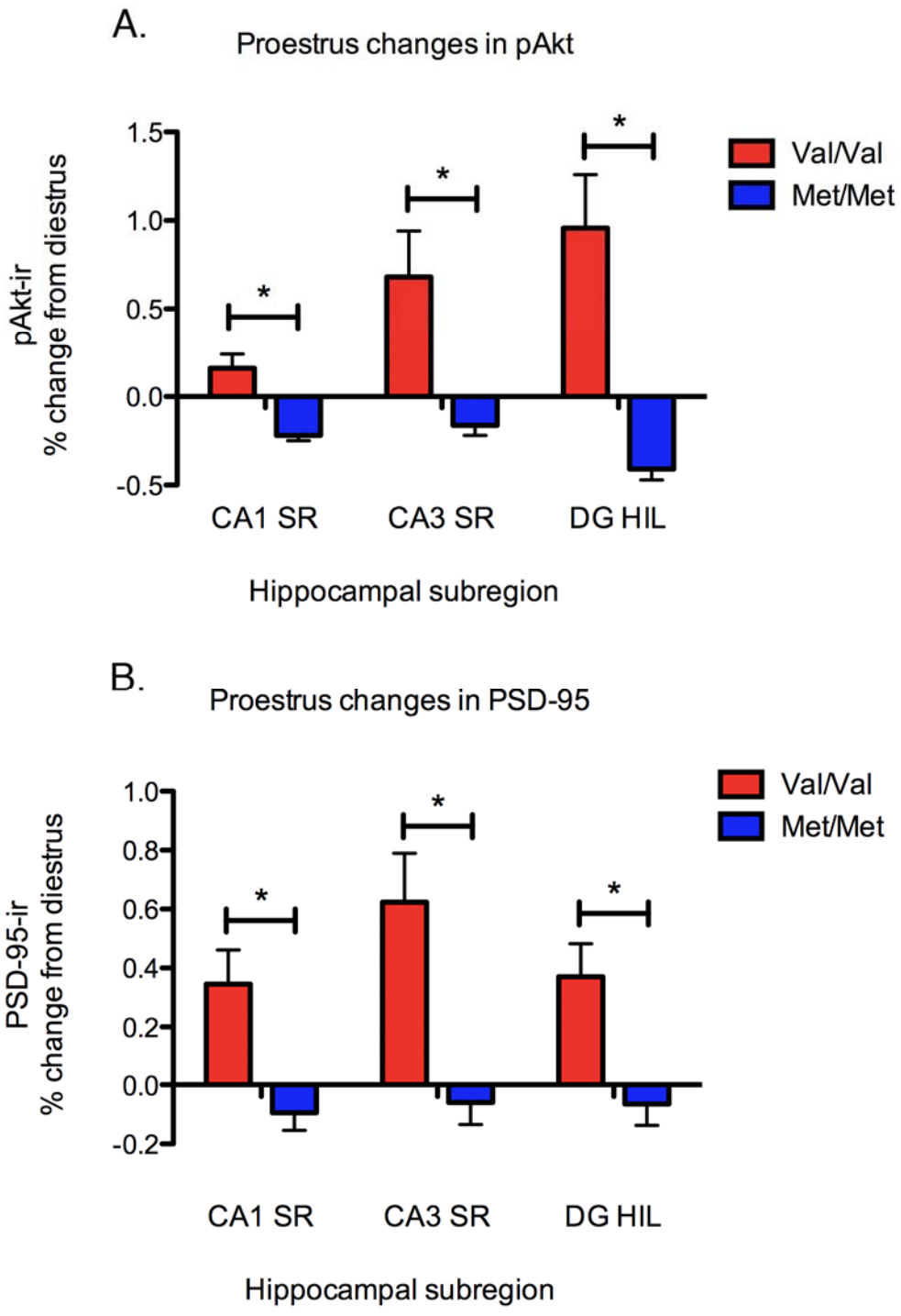


Figure 6.6 Normal estrous cycle fluctuation of pTrkB-ir in BDNF Val66Met mice. Proestrus changes in pTrkB-ir were expressed as a percent increase over diestrus pTrkB-ir in several hippocampal subregions. pTrkB-ir increased during proestrus in both Val/Val and Met/Met females. There was no significant difference in proestrus changes between the two genotypes. SR, stratum radiatum; SL, stratum lucidum; DG HIL, hilus of the dentate gyrus. N = 6 for Val/Val proestrus, 4 for Val/Val diestrus, 5 for Met/Met proestrus, and 4 for Met/Met diestrus.

As expected based on the estrous cycle fluctuation of, pAkt-ir in wild-type mice (Chapter 3), pAkt-ir increased during proestrus in Val/Val females (Figure 6.7A). However, pAkt-ir actually decreased during proestrus in Met/Met females. The proestrus changes in pAkt-ir differed significantly between Val/Val and Met/Met genotypes in all hippocampal subregions (CA1 stratum radiatum, $p = 0.0026$; CA3 stratum radiatum, $p = 0.0176$; dentate hilus, $p = 0.0029$). Two-way ANOVA confirmed that estradiol affected pAkt differently in Val/Val and Met/Met females, showing a significant interaction between cycle phase and genotype in CA1 stratum radiatum and dentate hilus, and a strong trend in the CA3 stratum radiatum (CA1 stratum radiatum, $F(1, 15) = 8.615$, $p = 0.0102$); CA3 stratum radiatum, $F(1, 15) = 4.372$, $p = 0.054$); dentate hilus, $F(1, 15) = 7.493$, $p = 0.0153$).

Figure 6.7 Abnormal estrous cycle fluctuation of pAkt-ir and PSD-95-ir in BDNF Val66Met mice. (A and B) Proestrus changes in pAkt-ir and PSD-95-ir were expressed as a percent increase over diestrus levels in several hippocampal subregions. pAkt-ir and PSD-95-ir increased markedly during proestrus in Val/Val mice, and decreased slightly during proestrus in Met/Met mice. Proestrus changes differed significantly between genotypes for both pAkt-ir and PSD-95-ir, in all hippocampal subregions. SR, stratum radiatum; SL, stratum lucidum; DG HIL, hilus of the dentate gyrus. *, $p<0.05$. N = 6 for Val/Val proestrus, 4 for Val/Val diestrus, 5 for Met/Met proestrus, and 4 for Met/Met diestrus.

Figure 6.7


A.
Proestrus changes in pAkt
pAkt-ir
% change from diestrus
1.5
1.0
0.5
0.0
-0.5
*
*
*
CA1 SR
CA3 SR
DG HIL
Hippocampal subregion
Val/Val
Met/Met
B.
Proestrus changes in PSD-95
PSD-95-ir
% change from diestrus
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
*
*
*
CA1 SR
CA3 SR
DG HIL
Hippocampal subregion
Val/Val
Met/Met

PSD-95-ir also increased during proestrus in Val/Val females (Figure 6.7B). Similar to pAkt-ir, PSD-95-ir did not increase during proestrus in Met/Met females, and even decreased slightly. The proestrus changes in PSD-95-ir differed significantly between Val/Val and Met/Met genotypes in all hippocampal subregions measured (CA1 stratum radiatum, $p = 0.0117$; CA3 stratum radiatum, $p = 0.007$; dentate hilus, $p = 0.0127$). Two-way ANOVA confirmed that estradiol affected PSD-95 differently in Val/Val and Met/Met females, with a significant interaction between cycle phase and genotype in all hippocampal subregions (CA1 stratum radiatum, $F(1, 15 = 9.088, p = 0.0087)$; CA3 stratum radiatum, $F(1, 15) = 6.457, p = 0.0226$; dentate hilus, $F(1, 15) = 7.161, p = 0.0173$).

In sum, compared to wild type Val/Val females, Met/Met females show similar estrous cycle fluctuation of TrkB activation, but opposite fluctuation of Akt activation and PSD-95 expression. These opposite effects on Akt and PSD-95 correlate with the opposite estrous cycle fluctuation of spatial memory in Val/Val and Met/Met females.

Discussion

This study of hippocampal function across the estrous cycle in BDNF variant Val66Met mice provides a wealth of new information about estradiol effects in the hippocampus. The findings pertain to ovarian steroid effects in wild-type mice, the role of activity-dependent BDNF secretion in these effects, and possible interactions between ovarian steroids and human gene variants in the control of behavior.

Behavioral and molecular effects of BDNF variant Val66met

Many studies have investigated the effects of BDNF Val66Met on behavior and neuropsychiatric disorders in humans. The behavioral characterization of the BDNF Val66Met mouse is just beginning, with the hope that it will be a useful model for the study of how this gene variant affects behavior. The current study revealed important behavioral phenotypes in anxiety, exploratory, and memory behaviors in female Val66Met mice.

Met/Met females had increased anxiety-type behavior compared to Val/Vals. A similar effect of the Met allele was found in male mice (Chen

et al., 2006). Human studies have shown variable effects of the Val66Met variant on anxiety, but several reports suggest that the Met allele predisposes to anxiety traits and disorders (Jiang et al., 2005; Gatt et al., 2009).

Met/Met females explored novel objects less than Val/Val mice, though they retained their preference for a novel object when that object was paired with a familiar one. This finding may reflect complex effects of the Met allele on motivational or novelty-seeking behavior that deserve further scrutiny. Human Met carriers have decreased susceptibility to substance abuse that could, with further study, be related to reduced novelty-seeking behavior in Met/Met mice (Gratacos et al., 2007).

Finally, Met/Met mice had impaired memory, measured as OP and OR performance, compared to Val/Vals. Consistent with these findings, human Met carriers have slightly lower IQ and poorer episodic memory (Egan et al., 2003; Hariri et al., 2003; Tsai et al., 2004). Overall, the behavioral phenotype of the female BDNF Met/Met mice in this study was generally consistent with the effects of the Met allele on human behavior, validating the utility of this mouse model for studying the behavioral effects of this polymorphism.

In situ hybridization studies showed increased BDNF and TrkB expression in the Met/Met female hippocampus. This suggests that in

Met/Met mice, hippocampal cells may attempt to compensate for the deficiency in activity-dependent BDNF release by an upregulation of BDNF and its receptor, thereby increasing the BDNF release pool and the sensitivity to secreted neurotrophin. This idea remains to be tested, but it is consistent with the finding of elevated BDNF serum concentrations in human Met carriers (Lang et al., 2009). A similar hypothesis was proposed to explain the upregulation of TrkB expression after stress, when BDNF levels are reduced (Nibuya et al., 1999). In the current study, there was no overall difference in hippocampal TrkB activation, measured as pTrkB-ir, between genotypes. This suggests that the increased BDNF and TrkB expression in Met/Met females at least partially compensate for the BDNF deficiency. But this apparent compensation did not prevent the Met behavioral phenotype.

Estradiol effects in wild type (Val/Val) mice

The current study confirmed the findings from Chapters 3 and 4 that TrkB and Akt activation increase during proestrus throughout the hippocampal formation, while TrkB expression remains unchanged. BDNF mRNA also increased during proestrus, consistent with a previous report in

rats (Scharfman et al., 2003). Combined with the estradiol induction of BDNF described in Chapter 4, this indicates that both endogenous and exogenous increases in circulating estradiol increase BDNF expression in the mouse hippocampus. This increased BDNF expression likely mediates the associated increase in TrkB activation. Finally, PSD-95 expression also increased during proestrus throughout the hippocampal formation. Although this finding is consistent with the effects of estradiol treatment in mice (Chapter 3 and (Li et al., 2004)) this is the first demonstration that natural fluctuations in ovarian steroids affect synaptic protein expression.

Hippocampal function across the estrous cycle was assessed in the mice using the object placement test of spatial memory. The wild type mice performed best during diestrus, when estradiol levels are low and progesterone levels high. This was surprising given that in female rats, several studies have shown an enhancement of spatial memory, including OP performance, during proestrus (Frye et al., 2007). In addition, the parameters that increased during proestrus in the mouse hippocampus, including BDNF activity, Akt activation, and PSD-95 expression, all function in excitatory synapse development, synaptic plasticity, and/or spatial memory (see Chapter 1). It was therefore expected that these

parameters would correlate positively with spatial memory across the estrous cycle.

There are several possible explanations for this finding. First, the OP task may depend on other brain regions besides the hippocampal formation. The hippocampus is crucial for spatial recognition memory in rats (Good et al., 2007), but its role in this behavior, and the relative role of the parahippocampal cortex and other brain regions, has not been investigated in mice. The balanced contribution of different brain regions to a behavior is important for the potential effects of estradiol on that behavior (Korol, 2004). Previous work has indeed demonstrated that mice and rats use different strategies to solve similar tasks of spatial navigation (Frick et al., 2000). Because of this, direct comparison of behavioral studies between mice and rats is not always appropriate.

A second possibility is that spatial memory is not enhanced during proestrus in the mouse. The OP testing took place in the late morning of proestrus, at the same time that the effects of circulating estradiol on Akt, PSD-95, and BDNF in the hippocampus were observed. These widespread molecular events could obscure the specific synaptic events required to form a short-term memory during the sample trial. For example, in rat hippocampal neurons, overexpression of PSD-95 dramatically increases

excitatory postsynaptic potentials, occluding the synaptic potentiation seen in control neurons (Stein et al., 2003). If this phenomenon is applicable to the proestrus mouse hippocampus, more long-term estradiol treatment or estradiol administration after the sample trial might still be expected to enhance memory formation or consolidation. Indeed, mice show enhanced spatial memory after six days of estradiol treatment, or post-training estradiol injections (Li et al., 2004; Gresack and Frick, 2006).

As indicated in the Introduction (Chapter 1), this is not the first study to find improved hippocampal-dependent memory when circulating estradiol is low. Previous studies showed varied estrous cycle fluctuation of different memory tasks in female rats, including water maze navigation and novel object recognition (Frick, 2009). The best conclusion based on all available data is that memory and hippocampal function do fluctuate across the estrous cycle, but the consequences of this fluctuation for behavior are complex and particularly task-dependent. With all this in mind, the current study demonstrates that natural fluctuations in ovarian steroids affect spatial memory in female mice. Object placement recognition increases during diestrus, and correlates inversely with hippocampal BDNF expression, TrkB activation, Akt activation, and PSD-95 expression.

Estradiol effects in BDNF variant Val66Met mice

The BDNF Val66Met mouse is an ideal model to assess the role of BDNF in estradiol effects because of the specific deficiency in activity-dependent BDNF secretion. Activity-dependent, or regulated, BDNF secretion occurs in response to neuronal activity through a specific neuronal secretory pathway (Lessmann et al., 2003). This regulation of secretion is at the heart of BDNF's role in activity-dependent synaptic modifications (Boulanger and Poo, 1999; Lim et al., 2003), and therefore may be pertinent to estradiol effects on synaptic plasticity. Because the Val66Met variant leaves the constitutive pathway of neurotrophin secretion intact, it is in some ways less dramatic than a BDNF knockout. At the same time, it allows for the specific assessment of the role of activity-dependent BDNF secretion in estradiol effects.

Using female mice homozygous for wild type BDNF or the Val66Met variant, the current study demonstrated that this variant does not eliminate the fluctuation of spatial memory across the estrous cycle, but alters it dramatically. OP performance peaked during the low-estradiol stage of diestrus in Val/Val mice, and during the high-estradiol stage of proestrus in Met/Met mice. In concert with their different estrous cycling of OP

performance, Val/Val and Met/Met females also showed a different fluctuation of Akt activation and PSD-95 expression across the cycle. Akt activation and PSD-95 expression in the hippocampal formation increased during proestrus as expected in Val/Val females, but decreased during proestrus in Met/Met females. Thus compared to Val/Vals, female Met/Mets exhibited a coordinated change in the estrous cycle fluctuation of spatial memory, Akt activation, and PSD-95 expression.

The effect of the BDNF variant Val66Met on estrous cycling of Akt and PSD-95 suggests that activity-dependent BDNF secretion plays a critical role in the widespread increase in hippocampal Akt activation and PSD-95 expression during proestrus. Since estradiol acts rapidly on Akt and PSD-95, (Chapter 3), this implicates activity-dependent BDNF secretion in rapid, membrane-initiated estradiol signaling. Furthermore, the correlation among spatial memory, Akt activation and PSD-95 expression in this study suggests that these specific rapid signaling events may be especially important for estradiol effects on hippocampal-dependent behavior. The BDNF variant Val66Met did not alter the more delayed effects of estradiol on PR and BDNF expression.

Surprisingly, Met/Met mice actually performed better than Val/Vals on the 30-minute OP test during proestrus. This was unexpected in light of

their overall impairment on the 5-minute OP task when tested in mixed estrous cycle stages. This suggests that in some cases, the effects of circulating ovarian steroids are robust enough to overcome the global behavioral phenotype of this BDNF variant. Alternatively, the 5- and 30-minute OP tasks may test different aspects of brain function. In rats, the role of the hippocampal formation in recognition memory depends on the intertrial delay (Hammond et al., 2004). If this holds true in mice, it would suggest that the memory impairment in Met/Met females depends on the specific memory task and its dependent brain regions.

In sum, the BDNF variant Val66Met leads to opposite effects of increased circulating estradiol on hippocampal function measured by Akt activation, PSD-95 expression, and OP performance in female mice. These different effects of circulating ovarian steroids in Val and Met genotypes may stem from developmental or adult differences in BDNF biology. Though the actual mechanism is unclear, activity-dependent BDNF secretion appears to play a critical role in how estradiol effects on hippocampal function.

Implications for human behavior

The BDNF variant Val66Met fascinates researchers because of its association with variations in human behavior, including susceptibility to several neuropsychiatric disorders and the course of these diseases. These effects clearly underscore the importance of BDNF in nervous system development and adult brain function. Studying this polymorphism will provide more information not only about basic BDNF biology, but also about genetic variation in human behavior.

Most women report mood and cognitive changes associated with the menstrual cycle, and 5-10% meet strict diagnostic criteria for premenstrual dysphoric disorder (PMDD) (Yonkers et al., 2008). Affective behavior, including mood, in humans depends on the cooperation of multiple brain areas including the hippocampal formation. Common genetic variation in some of the molecular mediators of estradiol effects on these brain regions could explain why some women develop severe behavioral symptoms associated with the menstrual cycle, while others experience little change in mood or cognitive function. Based on the current findings, the BDNF variant Val66Met is a strong candidate for one such genetic factor. Val/Val and Met/Met female mice showed different estrous cycle fluctuation of

behavior and several molecular endpoints in the hippocampal formation. This BDNF variant may therefore play a role in determining how the female brain responds to ovarian steroids, and may influence susceptibility to PMDD and other disorders associated with changes in circulating ovarian steroids.

The striking interaction between BDNF genotype and circulating ovarian steroids suggests that an interaction between the Met allele and estradiol may also contribute to gender differences in the effects of the Met allele on human behavior. A recent meta-analysis showed that female sex may protect Met carriers against an increased risk for major depressive disorder (Verhagen et al., 2008). In one study, the Met allele had opposing effects on the course of obsessive-compulsive disorder: it was associated with earlier onset in males but decreased severity in females (Hemmings et al., 2008). Future lab and clinical studies should be designed to specifically assess gender differences in the effects of the Met allele, and the contribution of sex hormones to these differences. This information will be critical to the successful application of clinical data to genetic risk assessment or treatment choices.

Chapter 7

Discussion

The goal of this thesis was to make progress toward understanding how estradiol affects hippocampal function in female mice. Two candidate molecular mediators of estradiol effects were investigated based on their relevance to synaptic plasticity and hippocampal function: the serine/threonine kinase Akt, and the neurotrophin BDNF. The effect of estradiol on these molecules was determined using intact cycling mice and estradiol replacement in ovariectomized mice. The studies also took advantage of several existing lines of transgenic mice to determine the role of estrogen receptors and BDNF in estradiol actions.

The results of these studies shed light on several key aspects of how estradiol affects hippocampal function: the upstream signaling pathways and target genes on which estradiol acts, which estrogen receptors mediate these effects, and the importance of these pathways in the downstream increase in synaptic protein expression. In addition to these important mechanistic points, these studies also demonstrate potentially important differences between the effects of physiologic and pharmacologic increases in circulating estradiol.

Mechanism of estradiol effects on hippocampal function

These studies clearly demonstrate that estradiol increases Akt and BDNF signaling in the mouse hippocampal formation. Estradiol activates Akt on a fairly rapid time course consistent with a membrane-initiated event. In contrast, estradiol increases BDNF expression on a more delayed time course consistent with a requirement for new gene transcription. Estradiol also increases TrkB activation, which occurs without any change in TrkB expression and is therefore likely mediated by estradiol effects on BDNF expression.

In these studies, estrogen receptors alpha and beta were both essential for estradiol effects in the mouse hippocampal formation. ER alpha was required for estradiol activation of Akt, while ER beta was required for both estradiol activation of Akt and induction of BDNF. The unique roles of these receptors will be important in the consideration of the effects of different estrogenic compounds, with distinct affinities for the two ERs, on brain function. Additionally, once the role of each receptor is clear, we may more clearly understand how changes in estrogen receptor expression across the lifespan influence estradiol sensitivity. For example, aging is associated

with decreased synaptic localization of ER alpha in the CA1 stratum radiatum of the hippocampus of female rats (Adams et al., 2002). Decreased synaptic ER alpha would impair the ability of estradiol to activate Akt in synapses, where Akt is linked to actin-remodeling pathways required for dendritic spine remodeling. This could explain the impaired estradiol regulation of spine morphology and density in the aging brain (Adams et al., 2001; Yildirim et al., 2008).

The choice to examine PSD-95 expression as a marker of downstream modulation of synaptic plasticity in this study was based primarily on two in vitro studies suggesting that Akt and BDNF are important for estradiol to increase PSD-95 expression. In the study supporting a role for BDNF, estradiol increased PSD-95 expression in rat hippocampal slices through a Trk receptor-dependent mechanism (Sato et al., 2007). In the study supporting a role for Akt, estradiol rapidly increased PSD-95 protein translation in the NG108-15 neuroblastoma cell line through a PI3K-dependent mechanism (Akama and McEwen, 2003). In the current study, there was a close relationship between the rapid effect of estradiol on Akt and PSD-95, in the CA1 stratum radiatum, and the more prolonged effect of estradiol on BDNF and PSD-95, in the CA3 stratum radiatum. Although these data are purely correlative, they support the findings of both in vitro

studies, suggesting that when circulating estradiol increases, both Akt and BDNF are involved in increasing PSD-95 expression in the mouse hippocampus.

Physiology vs. Pharmacology

In this thesis, consistent use of the same molecular assays allowed side-by-side comparison of the effects of physiologic increases in circulating estradiol during proestrus, and pharmacologic increases following estradiol injection. In general, these studies led to similar conclusions: estradiol increases Akt and BDNF signaling, and PSD-95 expression, in the mouse hippocampal formation. But there were some important differences, as physiologic and pharmacologic increases in circulating estradiol had anatomically and temporally distinct effects on Akt and BDNF signaling.

While the proestrus increase in Akt activation occurred throughout the dorsal hippocampal formation, it occurred only in the CA1 6 hours after a single estradiol injection. This suggests that rapid estradiol signaling is most robust in the CA1. The original description of estradiol effects on dendritic spine density in the rat hippocampus focused on this subregion, where the most robust changes occured (Woolley et al., 1990). Since then

investigators have acknowledged that estradiol does affect other hippocampal subregions (Rudick and Woolley, 2000; Scharfman et al., 2007). In particular, estradiol effects in the CA3 and dentate gyrus can be seen after a more prolonged estradiol exposure, as chronic estradiol increases PSD-95 expression throughout the hippocampal formation (Li et al., 2004). Indeed, in the current study a more prolonged 48 hour estradiol treatment increased BDNF expression, TrkB activation, and PSD-95 expression in the CA3 region. As with Akt, these effects occurred throughout the hippocampal formation during proestrus.

What all this suggests an anatomical and temporal separation of Akt and BDNF activity after estradiol treatment, but a convergence of these effects during proestrus. The rise and fall of circulating estradiol across the estrous cycle, is therefore uniquely timed for a convergence of rapid and delayed estradiol effects on the late morning of proestrus. This elegant orchestration may optimize cooperation between these two types of estradiol signaling (Hammes and Levin, 2007). For example, in numerous cell types, the rapid effects of estradiol lead to the phosphorylation of transcription factors (including ERs themselves) and coactivators important for the delayed effects on gene expression (Vasudevan et al., 2001; Levin, 2005). Estradiol activation of Akt could in this way support increase TrkB

activation in the CA1 during proestrus. Conversely, the gradual increase in circulating estradiol beginning in diestrus 2 may effect changes in gene transcription that support the rapid effects of peak circulating estradiol on proestrus morning (Scharfman et al., 2007). Estradiol induction of BDNF and the resulting increase in TrkB activation could in this way support Akt activation in the CA3 during proestrus.

In sum, the current study suggests that in mice, the precise timing of increased circulating estradiol ensures activation of rapid and delayed targets at the same time, in all hippocampal subregions. The convergence of these signals during proestrus may have important consequences for hippocampal function. One recent paper provided evidence for this possibility in female rats. Using a sequence of estradiol injections timed to mimic physiologic fluctuations of estradiol, the authors showed that the precise timing of increased circulating estradiol is important for the specific increase in hippocampal cell excitability seen during proestrus (Scharfman et al., 2007). In mice, such exaggerated changes during proestrus may not uniformly enhance hippocampal-dependent memory. In a test of object placement recognition across the estrous cycle, mice performed best during diestrus, indicating that the proestrus changes may actually impair spatial memory. Based on the current studies it is impossible to know how 6 or 48 hours of

estradiol treatment might differently impact performance on this test, although in a previous study, six days of estradiol treatment enhanced performance on the same test (Li et al., 2004).

Taking all this into account, it is clear that physiologic and pharmacologic models are complementary approaches to the study of estradiol effects in the brain, each reinforcing and informing the other. They should be considered equally important, but not equivalent, models for the study of basic ovarian steroid biology.

Working model

The new information acquired from this extensive body of work permits me to propose a new working model of how estradiol affects hippocampal function.

When circulating estradiol increases, the hormone acts rapidly on ERs alpha and beta to activate Akt in the CA1 stratum radiatum. This pAkt activates actin-remodeling pathways that allow synaptic activity to be translated into modifications of spine structure (Jaworski et al., 2005; Yuen, 2006), and increases PSD-95 protein translation. The increased PSD-95 protein is targeted to dendritic spines, where its recruitment of AMPA

receptors increases excitatory synaptic potentials, synergizing with the effects of Akt to enable dendritic spine maturation (El-Husseini et al., 2000; Beique et al., 2006). Meanwhile, the increase in estradiol triggers a more delayed increase in BDNF mRNA in CA3 pyramidal cells, via estrogen receptor beta activation of CREB or direct nucleus-initiated signaling. This BDNF is soon secreted at active hippocampal synapses throughout the hippocampal formation, where it activates TrkB, particularly in axon terminals in CA3 stratum lucidum and CA1 stratum radiatum. Some of this pTrkB at axon terminals is transported down the axon to activate signaling pathways in the cell body, leading to both local modifications at active synapses and also widespread changes in gene expression, including an increase in PSD-95. The increase in TrkB activation serves to sustain and amplify the rapid effects of estradiol on Akt, focusing them at active synapses to better serve hippocampal synaptic plasticity.

The effects of estradiol on hippocampal-dependent behaviors depend on the type of behavioral task, and the relationship of testing to circulating ovarian steroids. For example, mice show enhanced performance on an object placement test of spatial memory during diestrus, when estradiol levels are low. The acute increase in Akt activation and PSD-95 expression may be involved in this memory impairment during proestrus.

In transgenic female mice, the BDNF Val66Met polymorphism reverses the fluctuation of Akt activation, PSD-95 expression, and spatial memory across the estrous cycle. The mechanism for this reversal is unclear, but it suggests that activity-dependent BDNF secretion is important for estradiol activation of Akt. In addition, it points to a possible interaction between BDNF genotype and ovarian steroids in the control of human behavior.

Relevance to human health

In women, shifts in circulating estradiol during puberty, across the menstrual cycle, in pregnancy, and at the menopausal transition are all associated with changes in mood and cognition (Aloysi et al., 2006). The role of estradiol in these changes has been difficult to determine, but several human studies suggest a role for ovarian steroids in the cognitive impairment following gonadal suppression, and the mood disturbances in premenstrual dysphoric disorder (PMDD) (Sherwin and Tulandi, 1996; Schmidt et al., 1998). By exploring how estradiol regulates hippocampal function in rodents, researchers hope to gain insights that will be applicable to ovarian steroid effects on human behavior. Some evidence suggests that translation

of this work will be possible. Several of the major effects of estradiol – on spine density, synaptic protein expression, and cognition – are conserved among mice, rats, and nonhuman primates (see Chapter 1). The conservation of these effects in several mammalian species suggests that they serve an evolutionarily important role, and will probably be conserved in humans. One recent imaging study showed that gray matter volume in the right anterior hippocampal formation fluctuates across the menstrual cycle in women (Protopopescu et al., 2008). A previous study showed that postmenopausal women taking estrogen replacement therapy had increased hippocampal volumes compared to women taking the ER partial agonist, tamoxifen (Eberling et al., 2004). This is direct evidence that estradiol affects the hippocampus in women, fueling the hope that estradiol effects on the mouse hippocampus may be directly applicable to women.

Contributions from animal studies

Despite the many animal studies that have begun to shed light on estradiol effects in the mammalian brain, the translation of laboratory knowledge into bedside practice has so far been inefficient. This lack of discussion between bench and clinical scientists is especially damaging in

the realm of postmenopausal hormone replacement therapy. In 2003, the results from the large Women's Health Initiative (WHI) study showed that conjugated equine estrogens (CEE) with or without medroxyprogesterone acetate (MPA) actually *increased* the risk of dementia and cognitive decline in postmenopausal women (Rapp et al., 2003b; Shumaker et al., 2003; Craig et al., 2005). These findings were unexpected in light of the positive effects of estrogen on many cognitive tests in animal studies and smaller observational studies in humans. Guided by comparisons between human and animal studies, the research community has since come to a general agreement over two major pitfalls of the study: first, the timing of hormone replacement; and second, the type of hormone delivered (Sherwin, 2006; Brann et al., 2007).

First, most of the women in the WHI study began hormone replacement therapy more than ten years after the menopausal transition. Investigators hypothesized that the brain loses its responsivity to estrogen under a period of prolonged hypoestrogenicity, and that estrogen sensitivity can be maintained through timely hormone replacement after the onset of reproductive senescence. This idea, called the "healthy cell bias" or 'critical period" hypothesis, maintains that estrogen has positive effects on healthy brain structures, but that once the structures are already damaged, estrogen's

effects are detrimental (Brinton, 2005; Suzuki et al., 2007). This implies that there is a critical window of estrogen sensitivity after ovariectomy or menopause before the effects of long-term hypoestrogenicity become irreversible(Maki, 2006). Evidence for this critical period has been shown in rodents, nonhuman primates, and women (Gibbs, 2000; Daniel et al., 2006; Sherwin, 2006; Suzuki et al., 2007). Most of the women in the WHI study began taking hormones 10 or more years after menopause, long after the end of the hypothesized critical period (Maki, 2006; Sherwin, 2006). The effects of estradiol on Akt and BDNF signaling in the young, healthy brain explored in this thesis may not apply to the older, less healthy brain.

Second, different forms of estrogen and progesterone have been explored for hormone replacement therapy in women, and they should not be treated equally (Prestwood et al., 2004). The form chosen for the WHI study, CEE with or without MPA, is rarely used in laboratory studies. Scientists have begun to consider the different biological actions of these and other clinically relevant estrogenic compounds (Zhou et al., 2002; Bernardi et al., 2003; Ciriza et al., 2004; O'Neill et al., 2004; Rhodes and Frye, 2006; Zhao and Brinton, 2006). In particular, the important role of ER beta in estradiol effects in the hippocampus (Chapters 3 and 4 and (Liu et al., 2008)) supports the design of human trials of selective estrogen receptor

modulators with a higher affinity for ER beta than ER alpha. These drugs could maximize the cognitive benefits while minimizing unwanted side effects of estrogen replacement therapy.

In addition to the issues of timing and hormone preparation, the results in this thesis suggest that human studies may benefit from considering genetic variation in the effects of hormone replacement. If common gene variants such as the BDNF Val66Met polymorphism can so profoundly influence how estradiol acts in the brain, as suggested in Chapter 6, this knowledge could greatly increase the power of clinical trials. Only one such variant has so far been identified in humans, an ER alpha polymorphism associated with the risk of cognitive decline (Yaffe et al., 2002). Animal studies may prove to be valuable models for elucidating the specific roles of common human gene variants against an otherwise uniform genetic background.

In sum, increased communication between basic and clinical scientists in the wake of the WHI study has led to the development of several principles that will be valuable in the design of future large-scale trials. Hopefully, this experience will encourage continued conversation and more efficient translation of laboratory findings.

Concluding thoughts

With the burgeoning interest in translational medicine, laboratory scientists must choose animal models carefully, with a mind toward the clinical relevance of their experiments. Questions pertaining to cognitive decline or mood disorders following menopause or hysterectomy are best explored using models of reproductive senescence such as ovariectomy. Investigations of neurological or psychiatric disorders related to the menstrual cycle, such as catamenial epilepsy or premenstrual dysphoric disorder, benefit from the incorporation of naturally cycling animals. The most complete and informative studies will incorporate and compare several different animal models and/or *in vitro* systems.

In addition to the endpoints explored in this thesis, other signaling pathways and proteins surely play important roles in estradiol effects in the hippocampal formation. To study any of these players, reliable assays such as the in situ hybridization and immunocytochemistry used in this study are invaluable for the comparison of different hormone treatment paradigms and genotypes. In the future, more high-throughput methods would be welcome. To be successful tools, these must consistently detect the small changes in

protein expression or spine density, sometimes only 30% or 40%, which can translate into huge differences in synapse number, plasticity, and behavior.

The neuroendocrinology of sex hormones is mystifyingly complex. The effects of estradiol (or any hormone) in the brain are the result of multiple actions, through multiple receptors, in different cell types and brain regions. The most easily measured effect may not be the most important one, and no composite of molecular endpoints can substitute for a single behavioral test. But if we embrace the nuances of this system and seek to understand them, we will be equipped for the most effective translation of findings from the laboratory to that most intricate system, the human brain.

Chapter 8

Materials and Methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of The Rockefeller University, Weill Cornell Medical College, and/of the University of Tsukuba and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Animals were housed on a 12-hour light/dark cycle with food and water available *ad libitum* for the duration of the studies. All mice used for these studies were between the ages of 8 and 13 weeks.

Wild-type C57Bl6 mice were obtained from Taconic Farms, Inc. (Germantown, NY) for estrous cycle characterization (Chapter 2); Charles River Laboratories, Inc. (Wilmington, MA) for biochemical studies on cycling mice (Chapters 3 and 4); and Jackson Laboratory (Bar Harbor, ME) for electron microscopy studies (Chapter 5).

ER alpha knockout mice (ERKO) and ER beta knockout mice (BERKO) were previously generated by targeted disruption of the ER alpha

and beta genes. For ER alpha, a NEO gene was inserted into exon 2 to disrupt the reading frame (Lubahn et al., 1993). For ER beta, the NEO gene was inserted in reverse order into exon 3, introducing several stop codons to yield premature translation of the ER beta peptide (Krege et al., 1998). These mice were mated onto a C57Bl/6J background. The animals used for these studies were bred and genotyped in collaboration with Sonoko Ogawa at the University of Tsukuba, Japan. In every experiment, homozygous knockout mice were compared to wild-type littermates.

BDNF Val66Met mice were generated using a knock-in allele with the point mutation G196A in the BDNF coding region as previously described (Chen et al., 2006). This mutation changes the Valine in position 66 to a Methionine. The mice were crossed onto a C57Bl/6J background for at least 11 generations before these experiments, and were bred and genotyped in collaboration with Francis Lee at Weill Cornell Medical College. Homozygous Met/Met females were compared with Val/Val siblings.

Estrous cycling

Vaginal swabs were taken daily between 9:30 and 10:30 AM and vaginal cytology was observed under a microscope after staining with the Hema 3 Stain Set from Fisher Scientific Company L.L.C. (Kalamazoo, MI). Cycle phase was determined as proestrus, estrus, diestrus 1 or diestrus 2 according to published criteria (Turner and Bagnara, 1971b). Mice without progression through the distinct cytologic stages of proestrus, estrus and diestrus were excluded from the studies. Vaginal cytology was monitored for at least one complete cycle before sacrifice or behavioral testing. All cycling mice were sacrificed in late morning, between the hours of 10:30 AM – 12:00 PM. Uteri were dissected out and weighed. Trunk blood was collected into heparinized tubes, and plasma estradiol and progesterone were measured using enzyme immunoassays from Endocrine Technologies, Inc. (Newark, CA).

Ovariectomy and estrogen replacement

Mice were ovariectomized under isoflurane anesthesia and recovered for one week from the surgery. For the 6-hour treatment, subcutaneous

injections of 100 μl vehicle (sesame oil) or 5 μg estradiol benzoate (EB) in vehicle were then delivered six hours before sacrifice. For 48-hour treatment, two daily injections were given, and the mice sacrificed 24 hours after the second injection.

Behavioral testing

Recognition tests. BDNF Val66Met mice underwent behavioral testing on the novel object recognition (OR) and novel object placement (OP) tasks as described previously ((Li et al., 2004), adapted from (Luine et al., 2003)). Testing consisted of two five-minute trials, the sample and recognition trials, separated by a delay. During the sample trial of the OR test, a mouse explored two novel, identical objects at one end of an open field measuring 38.1 x 53.3. The mouse was then returned to her home cage for the delay period. During the recognition trial, the mouse explored two objects in the same locations. One of the objects was identical to the sample trial objects, and one was novel. For the OP test, the two identical objects used for the sample trial were familiar. In the recognition trial, the objects stayed the same, but one object was moved to a new location in the open field.

Mice were acclimated to the apparatus and OP objects before testing. Once testing began, the mice were tested no more than once each day and at least once every five days. Each mouse was tested on the object placement task once with a five-minute intertrial delay, and once with a thirty-minute intertrial delay in each of the following estrous cycle stages: proestrus, estrous, diestrus 1, and diestrus 2. The same two objects were used throughout OP testing. Each mouse was testing on the OR task with a 30-minute intertrial delay twice, with different object pairs. Starting objects (OR) or object start locations (OP) and new object locations (both OP and OR) were counterbalanced across mice and trials. In the initial OP test and the two OR tests, mice were in mixed estrous cycle stages. Trials were recorded and analyzed using the Noldus Ethovision XT software (Noldus Information Technology, Leesburg, VA). An area of 1 cm surrounding the objects was delineated using the software, and object exploration was defined as when the nose of the mouse was within this object surround area.

Quantitative and statistical analysis. To compare non-mnemonic behaviors that may influence task performance, the following parameters were measured from the sample trial: time spent in the center of the open field, and time spent exploring objects. Genotype differences on these parameters were analyzed using a two-tailed t-test. The effects of genotype

and estrous cycle on these parameters were examined using two-way ANOVA with genotype and cycle stage as the independent measures. Statistical tests for these and all other data sets in this document were run Prism GraphPad (version 5.0a for Macintosh). For the recognition trial, the time spent exploring each object was measured. The amount of time spent exploring the novel object (or object placement) during the recognition trial was expressed as a fraction of the time spent with both objects, termed "novel object fraction." A fraction of 0.5 is consistent with chance performance; therefore, "recognition" was defined as a fraction significantly greater than 0.5 (using a one-sample t-test). Novel object fractions across the estrous cycle were also analyzed using two-way ANOVA with genotype and cycle stage as the independent measures.

Sacrifice

Mice were anesthetized with an overdose of sodium pentobarbital (150 mg/kg intraperitoneal) and perfused through the aorta with saline/heparin followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4; PB). Brains were removed from the skull and postfixed in 4% paraformaldehyde for 1 hour. They were then sunk in 30% sucrose in PB

for 48 hours at 4°C, and frozen at -80°C until sectioning. Sections (40 μm thick) through the hippocampal formation were cut on a freezing microtome (Microm HM440E). Sections were stored in a cryoprotectant solution of 50% ethylene glycol, 15% sucrose in phosphate-buffered saline (PBS) until use in immunohistochemical procedures.

For electron microscopy studies (Chapter 5), mice were perfused with saline/heparin followed by 40 ml 3.75% acrolein and 2% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were removed from the skull and post-fixed for 30 minutes in 2% acrolein and 2% paraformaldehyde solution in PB. Coronal sections (40 μm thick) through the dorsal hippocampal formation were cut on a vibrating microtome (Leica Microsystems, Bannockburn, IL) and stored in 30% ethylene glycol and 30% sucrose in PB at -20°C.

For in situ hybridization studies on wild-type cycling mice (Chapter 4), mice were sacrificed via rapid decapitation and brains immediately removed and frozen on dry ice. Coronal sections (20 μm thick) were cut on a cryostat and stored on Superfrost Plus slides (Thermo Fisher Scientific, Inc., Waltham, MA) at -80°C.

In situ hybridization

Antisense oligonucleotides matching the TrkB (5' TGC GAC TGC GTC AGC TCG GTG GGC GGG TTC CCT CTG CCA TCA GCA CTG C 3') and BDNF exon IX (5'GGG TTA CAC GAA GGA AGG CTG CAG GGG CAT AGA CAA AAG GCA CTG GAA CT3') sequences were obtained from Integrated DNA Technologies (Coralville, IA). Control sense sequences were also generated and used as a control to confirm a low level of nonspecific interactions. For studies on fresh frozen brains (Chapter 4), the TrkB oligo was labeled with ^{35}S and in situ hybridization was performed on thawed slides containing sections through dorsal hippocampus and ventromedial hypothalamus as described previously (Hunter et al., 2005). Briefly, sections underwent the following washes: 4% paraformaldehyde, phosphate-buffered saline (PBS), deionized water (dipped), 0.1 M triethanolamine with 5ml/l acetic anhydride, salt–sodium citrate buffer (SSC), in ascending concentrations of ethanol, chloroform, and 95% ethanol. Sections were then air dried and incubated for 2 h with hybridization buffer without probe, followed by one wash in 2X SSC, and one in 95% ethanol. Sections were air dried and incubated overnight at 42°C with labeled probe (0.5×10^6 counts per slide) diluted in 50% deionized formamide in

hybridization buffer with dextran sulfate, denatured salmon testis DNA (Sigma), and 200 mM dithiothreital. After hybridization, sections were washed 4 times in SSC at 55 °C and left in SSC to cool to room temperature. Final washes were as follows: 50% ethanol, 0.3 M ammonium acetate, 85% ethanol, 0.3 M ammonium acetate, and 100% ethanol. Slides were then air dried and exposed to Kodak MR autoradiography films.

For studies using sections from paraformaldehyde-perfused mice (Chapter 6), floating sections containing dorsal hippocampus were mounted on Fisher Superfrost Plus slides, washed in 0.05 M PB, and dried in a desicator. The oligos were labeled with ^{33}P an in situ hybridization conducted as above, starting after the formaldehyde fix step.

After exposure to film for one week (TrkB) or two weeks (BDNF), films were developed and images taken on a light box using a CoolSnap camera. In the hippocampus, optical density was measured from the CA1 (TrkB) and CA3 (BDNF) pyramidal cell layers and normalized to background density (stratum radiatum). In the hypothalamus, optical density was measured from the ventromedial nucleus and normalized to background density (white matter). Values from proestrus and diestrus mice were compared using a two-tailed t-test; values from Val66met mice were compared using two-way ANOVA with genotype and cycle phase as the independent variables.

Immunocytochemistry and densitometry

Immunocytochemistry. Immunocytochemistry was performed on sections through the dorsal hippocampal formation level 29 of Paxinos and Watson (Paxinos and Watson, 1998) as previously described (Znamensky et al., 2003). For BDNF Val66Met mice, immunocytochemistry for PR was also performed on sections through the medial preoptic area of the hypothalamus. To ensure identical labeling conditions, tissue from each experimental conditions was marked with identifying punches and all conditions were pooled during labeling procedures. Moreover, tissue from all mice was processed together for each study (Pierce et al., 1999). Briefly, sections were washed in 0.1 M Tris buffer, blocked in 0.5% bovine serum albumin (BSA) and incubated in primary antiserum for 24 hours at room temperature followed by 48 hours at 4°C. Sections were then incubated in: (1) biotinylated goat anti-rabbit or anti-mouse secondary antibody in 0.1% BSA (1:400; Vector Labs, Burlingame, CA) for 30 minutes, avidin biotin complex (ABC) for 30 minutes (Vector Labs, Burlingame, CA), and diaminobenzidine with H_2O_2 (Aldrich, Milwaukee, WI) for 6 minutes, with Tris washes in between each incubation. Sections then were mounted on

Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA), air-dried, and coverslipped with DPX mounting medium (Aldrich, Milwaukee, WI). Images for densitometric analysis were captured on a Nikon E800 microscope using a Dage CCD C72 camera and control unit using preset gain and black levels, a Data Translation Quick capture card, and NIH Image 1.50 software. To compensate for uneven illumination, blank fields from a slide without tissue were subtracted. To prepare figures, the levels, brightness and contrast were adjusted in Adobe Photoshop 7.0 on a Macintosh computer. Final figures were assembled in Microsoft Powerpoint.

For tissue from mice perfused with acrolein (Chapter 5), sections were first washed in PB followed by 1% sodium borohydride in PB (30 minutes) before proceeding to the first set of Tris washes and blocking step. For pTrkB peptide blocking control, antiserum was pre-adsorbed with excess pTrkB blocking peptide.

Densitometry. Quantitative Analysis was conducted using NIH Image. The average pixel density (of 256 gray levels) was determined for each selected region. To compensate for background staining and control of variations in illumination level between images, the average pixel density for

3 small regions that lack labeling was determined within each captured image, and subtracted from all density measurements made on that image. The resulting relative optical densities (ROD) from were compared between cycle phases using Analysis of Variance or two-way Analysis of Variance. Post-hoc comparisons were made using Bonferroni tests.

Cell counting. To count PR-positive cells in the MPO of BDNF Val66Met mice, the MPO was identified on a light microscope. PR-positive cells were counted in a fixed-area polygon using Neurolucida software from both hemispheres.

Antibodies. The following antibodies were used for immunocytochemistry. Serial dilution tests of each antibody established that the labeling intensity was linear, and antibody dilutions were chosen that produced slightly less than half-maximal labeling intensity to optimize the detection of intensity variations (Chang et al., 2000).

Polyclonal rabbit anti-ER alpha (1:20,000) was a gift from the S. Hayashi laboratory. This antibody has been shown to recognize both ligand-bound and unbound receptors (Okamura et al., 1992; Alves et

al., 1998). Specificity of this antibody was verified by Western blotting, peptide pre-adsorption, and pre-immune serum (Milner et al., 2001).

Polyclonal rabbit anti-ER beta (1:10,000) was obtained from Merck Research Laboratories (Rahway, NJ). The specificity of this antibody was verified using secondary-only immunocytochemistry and peptide pre-adsorption (Milner et al., 2005).

Monoclonal mouse anti-PSD-95 (1:20,000) was purchased from Sigma (St. Louis, MO).

Polyclonal rabbit anti-phosphothreonine 308 Akt (1:1500) was purchased from Cell Signaling Technology (Danvers, MA). Specificity of the antibody was confirmed by Western blot and peptide preadsorption in acrolein/paraformaldehyde-fixed tissue (Znamensky et al., 2003).

Polyclonal rabbit anti-phosphotyrosine 816 TrkB (1:1000-5000) was a gift from Moses Chao at New York University. Specificity of this

antibody was confirmed by Western Blot (Arevalo et al., 2006) and peptide pre-adsorption (Chapter 5).

Polyclonal guinea pig anti-dynorphin (1:2000) was purchased from Bachem AG (Torrance, CA). Specificity of this antibody was verified by peptide pre-adsorption (Svingos et al., 1999).

Polyclonal rabbit anti-progesterone receptor (1:500) was purchased from Dako North America, Inc. (Carpinteria, CA). Specificity of this antibody was demonstrated by using knockout-mice and peptide preadsorption (Waters et al., 2008).

Pre-embedding immuno-electron microscopy

Immunocytochemistry and tissue preparation. Labeling was conducted as described above for acrolein-fixed tissue. To verify the localization and more discretely visualize the immunoreactivity, a few sections were labeled using gold particles conjugated to secondary antibody (Chan et al., 1990). Instead of the secondary antisera described above, these sections were incubated in colloidal gold-labeled (1 nM) goat anti-rabbit IgG

(1:50; Electron Microscopy Sciences, Fort Washington, PA) in 0.08% BSA, 0.01% gelatin in PB with 0.9% saline (PBS) for two hours at room temperature. These sections were post-fixed in 2% glutaraldehyde in PBS for 10 minutes followed by 0.2 M sodium citrate buffer (pH 7.4). Gold labeling was enhanced using a silver solution (IntenSE; Amersham, Piscataway, NJ).

To determine whether pTrkB labeled mossy fiber axon bundles and terminals in CA3, a few sections were double-labeled for pTrkB and dynorphin, an opioid peptide contained exclusively in mossy fiber axons and terminals in the CA3 (Drake et al., 2007). First, tissue was labeled with pTrkB primary antiserum (1:2000) as described above. Tissue was then incubated in dynorphin primary antiserum (1:2000) with 0.025% Triton for 48 hours at 4°C. pTrkB labeling was then completed using the ABC protocol as described above. Dynorphin labeling was completed using colloidal gold-labeled anti-guinea pig IgG (1:50) for the secondary antiserum as described above for pTrkB (Chan et al., 1990).

Sections for electron microscopy were incubated in 2% osmium tetroxide (one hour) and dehydrated in an alcohol series and propylene oxide. They then were then embedded in EMBed 812 (Electron Microscopy Sciences, Hatfield, PA) between two sheets of plastic (Chan et al., 1990).

One embedded section from 3 animals in each group (proestrus, estrus, diestrus, and male) was selected and mounted on Epon chucks. Ultrathin 70-nm sections within 1.5 mm of the tissue surface were cut on a Leica UTC Ultratome (Leica Microsystems, Bannockburn, IL), collected on grids, and counterstained with uranyl acetate and Reynold's lead citrate. Sections were examined on a Tecnai Biotwin transmission electron microscope (FEI Company, Hillsboro, OR) and images acquired using Advanced Microscopy Techniques software (v. 3.2). Digital images for figures were cropped and adjusted for levels, brightness, contrast, and sharpness in Adobe Photoshop 7.0, and final figures were assembled in Photoshop and Powerpoint.

Quantitative Analysis. From each block, a 220.5 μm^2 of neuropil was examined from each of the following hippocampal laminae: stratum oriens, stratum radiatum (proximal to the pyramidal cell layer), stratum radiatum (distal from the pyramidal cell layer), and lacunosum moleculare of CA1; stratum oriens, stratum lucidum, and stratum radiatum of CA3; outer, middle, and inner thirds of the molecular layer, subgranular zone, and central hilus of the dentate gyrus. The distribution of labeled profiles was determined by counting immunolabeled profiles, which were classified using accepted nomenclature (Peters et al., 1991). Dendrites contained

microtubular arrays and synaptic contacts with adjacent axon terminals. Spines had a smaller diameter than dendrites (usually less than 0.2 μm) and contacted neighboring axon terminals. Unmyelinated axons had a small diameter (less than 0.2 μm), few vesicles, and no synaptic contacts in the plane of section. Axon terminals had a larger diameter than axons, contained numerous vesicles, and made synaptic contacts with neighboring dendritic spines or shafts. Glial profiles contained no microtubules and conformed to the shape of surrounding structures. Each hippocampal lamina contained a similar distribution of profile types, limiting the possibility of sampling error when comparing the distribution of profiles between animals. In addition to the labeled profiles, all unlabeled axon terminals and dendritic spines were counted in the CA1 stratum radiatum (distal) from pTrkB-labeled sections to determine the percentage of axon terminals and dendritic spines containing pTrkB-ir.

Statistical Analysis. For each block (animal), the number of pTrkB-labeled profiles in each cell compartment within one lamina was expressed as a percentage of the total counted profiles in the whole hippocampal formation, and as a percentage of the total counted profiles and in that particular hippocampal subregion (CA1, CA3, or dentate gyrus). Means for

each estrous cycle group and males were compared using two-way ANOVA and Bonferroni post-hoc tests in Prism 5.0 software. The number of labeled axon terminals and dendritic spines in distal CA1 stratum radiatum was expressed as a percentage of the total terminals or spines in this lamina. The proportion of labeled axons and terminals were compared among groups using one-way ANOVA and Bonferroni post-hoc tests.

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