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ESTROUS CYCLE REGULATES ACTIVATION OF HIPPOCAMPAL Akt, LIM KINASE, AND NEUROTROPHIN RECEPTORS IN C57BL/6 MICE

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Abstract—Estradiol modulates dendritic spine morphology and synaptic protein expression in the rodent hippocampus, as well as hippocampal-dependent learning and memory. In the rat, these effects may be mediated through nongenomic steroid signaling such as estradiol activation of the Akt and LIM kinase (LIMK) pathways, in addition to genomic signaling involving estradiol upregulation of brain-derived neurotrophic factor expression (BDNF). Due to the many species differences between mice and rats, including differences in the hippocampal response to estradiol, it is unclear whether estradiol modulates these pathways in the mouse hippocampus. Therefore, we investigated whether endogenous fluctuations of gonadal steroids modulate hippocampal activation of the Akt, LIMK, and the BDNF receptor TrkB in conjunction with spatial memory in female C57BL/6 mice. We found that Akt, LIMK, and TrkB were activated throughout the dorsal hippocampal formation during the high-estradiol phase, proestrus. Cycle phase also modulated expression of the pre- and post-synaptic markers synaptophysin and post-synaptic density 95. However, cycle phase did not influence performance on an object placement test of spatial memory, although this task is known to be sensitive to the complete absence of ovarian hormones. The findings suggest that endogenous estradiol and progesterone produced by the ovaries modulate specific signaling pathways governing actin remodeling, cell excitability, and synapse formation.

Key words: estrogen, steroid, spine, plasticity, memory.

The hippocampal formation is a medial temporal lobe structure of the mammalian brain implicated in the formation of episodic and spatial memories and the control of emotion (Kandel et al., 2000). Among the many endogenous regulators of hippocampal function, the ovarian steroids estrogen and progesterone stand out for their relevance to human health and disease. In humans, mood, cognition, and hippocampal activation fluctuate in concert with circulating ovarian steroid levels across the menstrual cycle (Rosenberg and Park, 2002; Halbreich et al., 2003; Protopopescu et al., 2008). Similarly, in laboratory rats and mice, estrous cycle phase modulates hippocampal excitability and some hippocampal-dependent behaviors (Terasawa and Timiras, 1968; Frick and Berger-Sweeney, 2001; Scharfman et al., 2003; Korol et al., 2004). In the rat, spine density on pyramidal cell dendrites in the CA1 stratum radiatum also fluctuates in concert with estradiol levels across the estrous cycle (Woolley et al., 1990).

Studies using the classic endocrine paradigm of ovariectomy and estradiol replacement confirmed that estradiol increases CA1 dendritic spine and synapse density (Gould et al., 1990; Woolley and McEwen, 1992; MacLusky et al., 2005). We now know that estradiol induces maturation of dendritic spines in both rat and mouse CA1, and increases the expression of several synaptic marker proteins in the hippocampus of mouse, rat, and rhesus monkey (Woolley and McEwen, 1992; Brake et al., 2001; Hao et al., 2003; Rapp et al., 2003; Li et al., 2004; Gonzalez-Burgos et al., 2005; Hao et al., 2006; Jelks et al., 2007; Spencer et al., 2008). This estradiol modulation of spines and synapses may have functional consequences, as estradiol also enhances performance on hippocampal-dependent spatial memory tasks in rats and mice (Korol, 2004; Li et al., 2004; Sandstrom and Williams, 2004; Spencer et al., 2008). Currently, our work focuses on identifying the upstream regulators of estradiol's effects on dendritic spine morphology, synapse density, and hippocampal-dependent behaviors.

In the hippocampal formation, estradiol may act through a combination of classical nuclear hormone signaling, and more rapid, "nongenomic" signaling. Rapid activation of cell signaling cascades by estradiol has been described, suggesting that estradiol activates membrane receptors (Levin, 2005; Spencer et al., 2008). One such pathway, the Akt pathway, may be important for the estradiol's neurotrophic actions. In the NG108-15 neuroblastoma cell line, estradiol activation of Akt results in filopodia formation and translation of the post-synaptic density 95 (PSD-95) protein (Akama and McEwen, 2003). In the brain, estradiol treatment increases phosphorylation of Akt in CA1 pyramidal cells and dendrites of ovariectomized female rats and in the striatum of male C57BL/6 mice (Znamensky et al., 2003; D'Astous et al., 2006). Akt may therefore also be an important upstream effector of estradiol actions in the mouse hippocampus.

The rapid, nongenomic actions of estradiol may result from signaling through classical or nonclassical estrogen

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Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; ER, estrogen receptor; ERE, estrogen response element; GPCR, G-protein-coupled receptor; IR, immunoreactivity; LIMK, LIM kinase; PB, phosphate buffer; PMDD, premenstrual dysphoric disorder; PSD-95, post-synaptic density 95; ROD, relative optical density; SGZ, subgranular zone; TS, Tris saline; T1, sample trial; T2, recognition trial.

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receptors (ERs) localized on or near the membrane (Pedram et al., 2006). The extranuclear localization of immunoreactivity (IR) for ERs alpha (ERα) and beta (ERβ) in the CA1 region of the rat hippocampus (Milner et al., 2001, 2005), the area most sensitive to estradiol-induced spine and synapse formation (Woolley and McEwen, 1992), suggests that some rapid actions of estradiol in the hippocampus may occur via signaling through a membrane-localized classical ER (Milner et al., 2001, 2005). Finally, although most studies have investigated the effects of several days of estradiol treatment on spine density and behavior, two studies demonstrated that estrogenic compounds can enhance hippocampal-dependent behavior and increase CA1 spine density within hours (Luine et al., 2003; MacLusky et al., 2005), a temporal course consistent with the role of rapid actions of estradiol in downstream morphological and behavioral effects *in vivo*.

Our laboratory has recently provided evidence that nongenomic estradiol signaling in neurons may regulate the cellular machinery for actin remodeling, which governs the formation and turnover of estrogen-sensitive dendritic spines and synapses in hippocampal pyramidal cells (Yuen, 2004). In hippocampal cells, the actin depolymerizing factor, cofilin, and its regulatory kinase, LIM kinase (LIMK), may be particularly important modulators of dendritic spine dynamics implicated in lasting cellular changes such as long-term potentiation (Meng et al., 2003). Studies of a LIMK knockout mouse have implicated LIMK in spine morphology and hippocampal function *in vivo* (Meng et al., 2002). Estradiol activates LIMK in the NG108-15 cell line via PI3 kinase-dependent activation of Rac1, leading to deactivation of cofilin and filopodia formation (Yuen, 2004). This suggests that estradiol may regulate dendritic spine density and maturation through the LIMK/cofilin pathway. Indeed, estradiol also increases pLIMK-IR in the CA1 stratum radiatum of ovariectomized rats (Yildirim et al., 2008).

Although estradiol's ability to regulate dendritic spine dynamics, spine formation, and synaptic protein expression depend on signaling through ERs (McEwen et al., 1999; Jelks et al., 2007; Spencer et al., 2008), it is likely that other membrane proteins, such as neurotrophin receptors, participate in these effects. Substantial evidence indicates that brain-derived neurotrophic factor (BDNF) may be important for estradiol effects in the hippocampal formation. Exogenous estradiol administration to ovariectomized rats increases BDNF mRNA and protein expression in the hippocampal formation (Gibbs, 1998; Jezierski and Sohrabji, 2001; Scharfman et al., 2003; Zhou et al., 2005). Activation of the BDNF receptor TrkB in the hippocampal formation leads to LIMK and Akt phosphorylation, dendritic spine regulation, and enhancement of long-term potentiation, similar to the effects of estradiol (Chao, 2003; Scharfman and Maclusky, 2005, 2006a; Spencer et al., 2008).

In sum, emerging evidence suggests that estradiol modulates several overlapping cellular pathways in the hippocampal formation leading to enhancement of hippocampal function. The current model suggests that in rats, estradiol activates the PI3K pathway, resulting in Akt activation, LIMK activation, and increased PSD-95 expression, leading to actin remodeling and maturation of dendritic spines. Additionally, estradiol induces BDNF expression, followed by BDNF release and TrkB activation, leading to increased pyramidal cell excitability, enhanced long-term potentiation, and Akt/LIMK activation. Despite this extensive progress in research using rats, very few studies have investigated the mechanism of estradiol modulation of synaptic protein expression, dendritic spine density, and hippocampal function in female mice. Previous work demonstrated differences in the estradiol sensitivity of the mouse and rat hippocampal formation that suggest possible differences in the mechanism of steroid actions in these two species. For example, while estradiol increases synaptic protein expression mainly 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, while estradiol increases the total CA1 dendritic spine density as well as the density of mature "mushroom" shaped spines in the rat, only mushroom spines increase in the mouse (Gould et al., 1990; 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). Because of these differences and the preponderance of genetic tools available in mice, it is necessary to determine whether endpoints known to be sensitive to circulating ovarian steroids in the female rat hippocampus are similarly sensitive in mice.

The choice of a cycling mouse model provides two advantages over the popular ovariectomy and estrogen replacement model for the initial verification of steroid-sensitive endpoints in the mouse hippocampal formation. First, it is difficult to establish a regimen of hormone replacement in ovariectomized mice that is "equivalent" to those regimens commonly used in rats. In rats, estradiol effects on CA1 dendritic spine density, hippocampal Akt activation and BDNF expression, and spatial memory have been demonstrated in both ovariectomized animals replaced with estradiol and in naturally cycling rats during the high-estradiol phase of proestrus (Woolley et al., 1990; Scharfman et al., 2003; Znamensky et al., 2003; Korol et al., 2004; Gonzalez-Burgos et al., 2005). Therefore, investigating cyclic changes in several of these endpoints is a reasonable starting point to establish which pathways are sensitive to physiological concentrations of circulating estrogens in mice. Second, studies in young adult cycling animals are more immediately relevant to women than studies of young adult ovariectomized animals. Investigations of hippocampal sensitivity to the estrous cycle may be relevant to neurologic and psychiatric disorders related to the human menstrual cycle, such as catamenial epilepsy and premenstrual dysphoric disorder (PMDD) (Halbreich et al., 2003; Scharfman and MacLusky, 2006b; Huo et al., 2007).

Based on studies of estradiol and estrous cycle sensitivity of the rat hippocampus, we chose several endpoints and examined their regulation by the estrous cycle in female mice. Activation of Akt, LIMK, and TrkB, and the