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Regulation of the Mouse Medial Prefrontal Cortical Synapses by Endogenous Estradiol

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Recent studies suggest that low endogenous estradiol might be a susceptibility factor for anxiety and trauma-related disorders in women. Consistently, fear extinction, a form of inhibitory learning critical for the management of anxiety symptoms, is positively correlated with endogenous estradiol levels. To understand the synaptic basis of the effect of endogenous estradiol on fear extinction, we studied glutamatergic transmission and plasticity in the infralimbic medial prefrontal cortex (IL-mPFC), a brain region crucial for the regulation of fear extinction. Diestrus mice (low estradiol) exhibited a higher basal glutamatergic transmission compared with proestrus mice (high estradiol). Synaptic plasticity was also regulated by endogenous estradiol, which favored synaptic potentiation in a GluN2B-dependent manner. Activation of estrogen receptor β (ERβ) but not ERα rescued synaptic potentiation in diestrus mice by enhancing GluN2B-mediated NMDA receptor transmission. Our results suggest that both endogenous estradiol and ERβ activation facilitate the ability of the IL-mPFC synapses to undergo potentiation, a mechanism necessary for the regulation of fear extinction.
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INTRODUCTION

The incidence, severity and duration of trauma-related disorders, and anxiety symptoms are significantly higher in women (Holbrook *et al*, 2002). Despite the emergence of this gender difference during puberty, the trauma- and anxiety-related symptoms are negatively correlated with the endogenous estradiol levels (Angold *et al*, 1998; Hankin and Abramson, 1999; Lebron-Milad and Milad, 2012). Congruent with an increased vulnerability to affective disorders during periods of low endogenous estradiol such as menopause, postpartum and following oophorectomy (Altshuler *et al*, 1998; Harsh *et al*, 2009; Parker *et al*, 2009; Schmidt and Rubinow, 2009; Schnatz *et al*, 2010), an impairment in fear extinction, an inhibitory learning process that attenuates anxiety symptoms, has been associated with low endogenous estradiol (Davis, 2011; Milad *et al*, 2010). Although an estradiol-based approach might be beneficial in the management of anxiety symptoms, its multifaceted effects are a limitation for therapeutic utilization (Hall *et al*, 2001). Therefore, an in-depth understanding of the downstream mechanism by which estradiol modulates the neural circuitry pertinent to the regulation of fear behavior is necessary. The infralimbic medial prefrontal cortex (IL-mPFC) is believed to play an important role in the regulation of fear extinction (Quirk *et al*, 2000). It has been suggested that an enhanced glutamatergic input from the IL-mPFC activates amygdala neurons particularly the GABAergic neurons in the intercalated cell masses and suppresses the centromedial amygdala output and fear response (Ninan, 2014; Pare *et al*, 2004). Consistently, the IL-mPFC glutamatergic synapses undergo potentiation during fear extinction (Pattwell *et al*, 2012b; Sepulveda-Orengo *et al*, 2013). To understand whether endogenous estradiol modulates the IL-mPFC synapses, we studied glutamatergic synaptic transmission and plasticity in the IL-mPFC layer 5 pyramidal neurons, the major projection neurons (Gabbott *et al*, 2005), from proestrus (high estradiol) and diestrus (low estradiol) mice (Spencer *et al*, 2010). Our studies revealed an estrous cycle-dependent modulation of the IL-mPFC glutamatergic synapses. Consistent with the high expression of estrogen receptor-β (ERβ) in cortical areas including the mPFC and the beneficial effects of ERβ activation on anxiety behaviors and fear extinction (Kritzer, 2002; Milner *et al*, 2010; Oyola *et al*, 2012; Shughrue *et al*, 1997; Walf *et al*, 2008; Zeidan *et al*, 2011), ERβ agonist restored the ability of the IL-mPFC glutamatergic synapses to undergo potentiation in diestrus mice.

MATERIALS AND METHODS

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the New York University School of Medicine. Three- to five–month-old C57/BL6 mice were maintained on a 12:12 light/dark cycle. Estrous cycle stage was determined by vaginal smear cytology between 0900 and 1100 hours. Hema 3 Stat Pack (Fisher Scientific) was used for processing the samples, and phases of estrous cycle were determined based on the presence of nucleated

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epithelial cells in proestrus, cornified epithelial cells in estrus, leukocytes, cornified epithelial cells, few nucleated epithelial cells, and cell debris in metestrus, and elevated presence of leukocytes and mucus and few nucleated epithelial cells in diestrus (Byers *et al*, 2012) (Figure 1a). Mice were selected for experiments following 8–10 days of regular estrous cycling. Ovariectomy was performed under ketamine/xylazine anesthesia. Ovaries were removed after making incisions of 5 mm on either sides about 10 mm from the midline (Strom *et al*, 2012). A control group was subjected to sham surgeries in which the ovaries were exteriorized but remained intact. Brain slices were prepared from ovariectomized and sham mice 10–14 days after the surgery.

Electrophysiology

Mouse brains were removed between 1000 and 1200 hours following intracardial perfusion with ice-cold artificial cerebrospinal fluid (ACSF) containing NaCl (118 mM), glucose (10 mM), KCl (2.5 mM), NaH_2PO_4 (1 mM), $CaCl_2$ (1 mM) and $MgSO_4$ (2 mM) bubbled with $95\%O_2/5\%CO_2$ (325 mOsm, pH 7.4) under pentobarbital anesthesia. mPFC slices (300 μm) were prepared and allowed to recover for at least 1 h at room temperature before transferring to a recording chamber perfused with aforementioned ACSF (32 °C) containing 2 mM $CaCl_2$. Spontaneous excitatory post-synaptic currents (sEPSCs), excitatory post-synaptic currents (EPSCs), and NMDA EPSCs were evoked in the IL-mPFC layer 5 pyramidal neurons using an electrode solution consisted of CsCl (145 mM), HEPES (10 mM), EGTA (0.5 mM), QX-314 (5 mM), GTP (0.2 mM), and MgATP (5 mM) (osmolarity 290 mOsm, pH 7.4) (Pattwell *et al*, 2012a; Pattwell *et al*, 2012b). NMDA EPSC decay time constant and amplitude were assessed before and after the perfusion of ifenprodil (10 μM) for 15 min. NMDA miniature excitatory post-synaptic currents (mEPSCs) were recorded at −50 mV in the presence of bicuculline (10 μM), NBQX (10 μM), glycine (10 μM), strychnine (1 μM), and tetrodotoxin (1 μM) and in the absence of magnesium. To test the role of pre-synaptic mechanism, we studied paired pulse facilitation

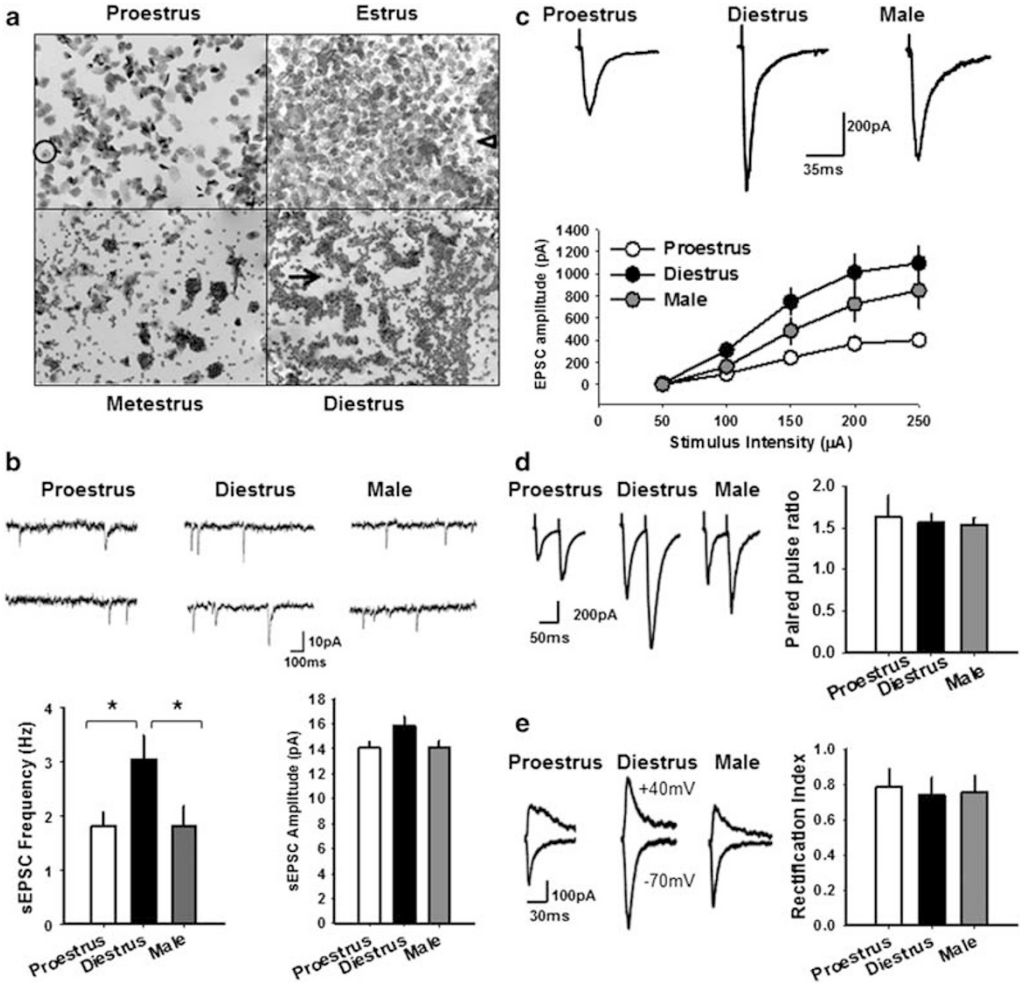

Figure 1 Non-NMDA receptor transmission in the IL-mPFC layer 5 pyramidal neurons. (a) Representative images of vaginal cytology in proestrus, estrus, metestrus, and diestrus mice showing nucleated epithelial cells (circle), cornified epithelial cells (arrow head), and leukocytes (arrow). (b) sEPSC frequency and amplitude in proestrus ($n = 17/6$ mice), diestrus ($n = 16/6$ mice), and male ($n = 15/5$ mice) mice. Upper panel shows examples of sEPSCs. Asterisk indicates a significant difference. (c) EPSC amplitude in proestrus ($n = 15/5$ mice), diestrus ($n = 15/5$ mice), and male mice ($n = 13/5$ mice). Upper panel shows examples of EPSCs evoked by 250 μA stimulation. (d) Paired pulse ratio in proestrus ($n = 9/3$ mice), diestrus ($n = 7/3$ mice), and male mice ($n = 8/3$ mice). Left panel shows examples of EPSCs evoked at 60 ms interval. (e) Rectification index in proestrus ($n = 6/3$ mice), diestrus ($n = 7/3$ mice), and male mice ($n = 7/4$ mice). Left panel shows examples of EPSCs evoked at −70 mV and +40 mV.

(PPF) (Hess *et al*, 1987; Zucker, 1989). Paired pulse ratio was measured by 150 μA stimulation at an inter-stimulus interval of 60 ms. Rectification index was assessed by calculating the ratio of the slope of the lines connecting EPSC amplitudes at −70 to 0 and 0 to +40 mV in the presence of bicuculline and D-2-Amino-5-phosphonovaleric acid (APV, 50 μM) in the extracellular solution and spermine (0.1 mM) in the pipette solution (Adesnik and Nicoll, 2007). Excitatory post-synaptic potentials (EPSPs) were recorded at 0.1 Hz and a pre-before-post pairing protocol was applied after a stable baseline recording for 5 min (Froemke and Dan, 2002; Pattwell *et al*, 2012a). Input resistance was monitored throughout the experiment. Recording was acquired using Axopatch 200B amplifier and digitized by Digidata 1322A (Molecular Devices). Data were analyzed using Clampfit (Molecular Devices) and Mini Analysis software (Synaptosoft Inc.).

Drug Administration

Diarylpropionitrile (DPN, a selective ERβ agonist) (Sigma, 1 mg/kg), propyl pyrazole triol (PPT, a selective ERα agonist) (Sigma, 1 mg/kg), or vehicle (sesame oil) were injected subcutaneously 90–120 min before the slice preparation. We used a modified treatment regimen for estradiol and progesterone based on the earlier studies in ovary-intact and ovariectomized rats (Chang *et al*, 2009; Milad *et al*, 2009; Zeidan *et al*, 2011). Ovariectomized and sham mice received two injections of estradiol (Sigma, 15 μg/kg), progesterone (Sigma, 4 mg/kg), or vehicle (sesame oil) at an interval of 16–18 h, and the last dose was injected 90–120 min before the slice preparation.

Statistical Analysis

Data are presented as mean ± SEM. One-way ANOVA followed by least significant difference (LSD) *post hoc* test was used for comparing sEPSCs and NMDA mEPSCs. Repeated measure ANOVA followed by Bonferroni test was used for comparing EPSCs and EPSP slope. Greenhouse–Geisser correction was applied when sphericity was violated. $P<0.05$ was considered statistically significant.

RESULTS

Estrous Cycle-Dependent Modulation of Non-NMDA Receptor Synaptic Transmission in the IL-mPFC

To test whether endogenous estradiol modulates the IL-mPFC glutamatergic synapses, we studied AMPA receptor-mediated non-NMDA receptor transmission in the IL-mPFC layer 5 pyramidal neurons, from proestrus and diestrus mice. Male mice were included for comparison. We observed a significant effect of groups on sEPSC frequency ($F_{(2,45)}=4.02$, $p=0.025$) but not on amplitude ($F_{(2,45)}=2.95$, $p=0.062$) (Figure 1b). sEPSC frequency in diestrus mice was significantly higher than that in proestrus ($p=0.016$) and male mice ($p=0.02$). Comparison of EPSC amplitude in proestrus, diestrus, and male mice revealed a main effect of group ($F_{(2,40)}=7.71$, $p=0.001$), stimulus intensity ($F_{(1.2,47.9)}=80.95$, $p<0.001$), and interaction between factors ($F_{(2.4,47.9)}=5.73$, $p=0.004$), suggesting that non-NMDA receptor transmission is elevated in diestrus compared with proestrus and male mice (Figure 1c).

Modification of synaptic transmission could occur through pre- and/or post-synaptic mechanisms. To test the role of pre-synaptic mechanism, we compared paired pulse ratio in proestrus, diestrus, and male mice. A statistically significant effect of time ($F_{(1,22)}=22.98$, $p<0.001$) but a non-significant effect of groups ($F_{(2,22)}=1.972$, $p=0.163$) and interaction between factors ($F_{(2,22)}=1.269$, $p=0.301$) (Figure 1d) suggested that a modification of glutamate release is unlikely to be responsible for differences in non-NMDA receptor transmission in proestrus, diestrus, and male mice. Another potential mechanism for an increase in the strength of cortical glutamatergic synapses is synaptic insertion of GluA2 subunit-lacking AMPA receptors, which are inwardly rectifying unlike GluA2-containing AMPA receptors (Adesnik and Nicoll, 2007). Comparison of the slope of the lines connecting EPSC amplitudes at −70 to 0 and 0 to +40 mV showed a statistically significant effect of holding potential ($F_{(1,17)}=12.79$, $p=0.002$) but a non-significant effect of groups ($F_{(2,17)}=0.808$, $p=0.462$) and interaction between factors ($F_{(2,17)}=0.266$, $p=0.77$) (Figure 1e), suggesting that the difference in non-NMDA receptor transmission between proestrus, diestrus, and male mice is mediated by GluA2-containing AMPA receptors that are not inwardly rectifying.

Reduced GluN2B-Mediated NMDA Receptor Transmission in Diestrus Mice

We compared NMDA receptor transmission in proestrus, diestrus, and male groups by measuring NMDA mEPSCs, which did not show a statistically significant difference in frequency ($F_{(2,31)}=2.5$, $p=0.098$) or amplitude ($F_{(2,31)}=2.67$, $p=0.085$). However, a significant difference in NMDA mEPSC decay time was observed ($F_{(2,31)}=17.2$, $p<0.001$) (Figure 2a). Decay time was significantly reduced in diestrus mice compared with that in proestrus ($p<0.001$) and male mice ($p<0.001$). Consistent with the NMDA mEPSC decay time, NMDA EPSC decay time also showed a significant effect ($F_{(2,31)}=4.36$, $p=0.022$) with the proestrus group exhibiting a significantly higher decay time compared with the diestrus group ($p=0.006$), an indication of GluN2B-predominant NMDA receptor transmission (Cull-Candy *et al*, 2001). Therefore, we compared NMDA EPSC decay time in proestrus, diestrus, and male groups before and after the perfusion of ifenprodil, a highly selective GluN2B antagonist (Williams, 1993), which showed a main effect of time ($F_{(1,29)}=154.874$, $p<0.001$), a non-significant effect of groups ($F_{(2,29)}=2.357$, $p=0.113$), and a significant interaction between factors ($F_{(2,29)}=7.892$, $p=0.002$) (Figure 2b). Analysis of NMDA EPSC amplitude revealed a main effect of time ($F_{(1,29)}=93.196$, $p<0.001$), a non-significant effect of groups ($F_{(2,29)}=2.728$, $p=0.082$), and a significant interaction between factors ($F_{(2,29)}=5.797$, $p=0.008$) (Figure 2b). These results confirmed a reduced GluN2B-mediated NMDA receptor transmission in diestrus mice compared with proestrus and male mice.

Estrous Cycle-Dependent Regulation of Synaptic Plasticity in the IL-mPFC

Given the estrous cycle-dependent modulation of NMDA receptor transmission, a key determinant of synaptic

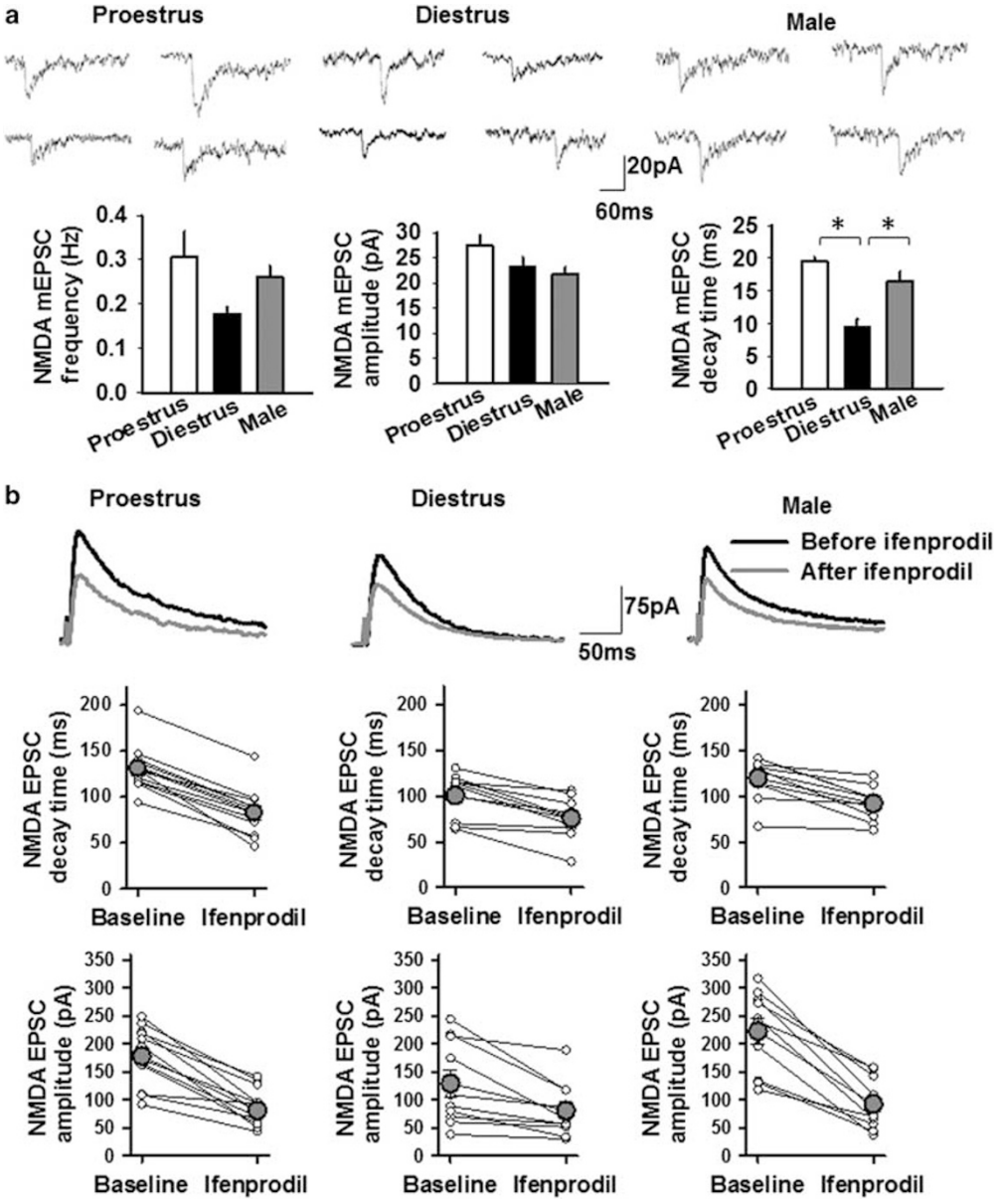

Figure 2 NMDA receptor transmission in the IL-mPFC layer 5 pyramidal neurons. (a) Frequency, amplitude and decay time of NMDA mEPSCs in proestrus ($n = 11/4$ mice), diestrus ($n = 10/4$ mice), and male mice ($n = 13/6$ mice). Upper panel shows examples of NMDA mEPSCs. Asterisk indicates a significant difference. (b) Effect of ifenprodil on NMDA EPSC decay time and amplitude in proestrus ($n = 12/4$ mice), diestrus ($n = 10/4$ mice), and male mice ($n = 10/4$ mice). Small empty and large filled circles represent raw data and mean ± SEM, respectively. Upper panel shows examples of NMDA EPSCs before and 15 min after ifenprodil perfusion.

plasticity, we examined long-lasting synaptic potentiation in proestrus, diestrus, and male mice. The application of a pre-before-post pairing protocol produced a long-term enhancement of EPSP slope in the proestrus group, which was blocked by ifenprodil. A main effect of groups ($F_{(2,21)} = 10.846$, $p = 0.001$), a non-significant effect of time ($F_{(29,609)} = 0.807$, $p = 0.754$), and a significant interaction between factors ($F_{(58,609)} = 4.779$, $p < 0.001$) were observed (Figure 3a). However, the same pairing protocol produced a long-lasting depression of EPSP slope in the diestrus group, which was not sensitive to ifenprodil. A non-significant effect of groups ($F_{(2,23)} = 1.312$, $p = 0.289$), a significant effect of time ($F_{(29,667)} = 3.705$, $p < 0.001$), and a significant interaction between factors ($F_{(58,667)} = 1.525$, $p = 0.009$) were observed (Figure 3b). Similar to the proestrus group, pairing produced a long-lasting increase in EPSP slope in male mice, which was blocked by ifenprodil as shown by a main effect of groups ($F_{(2,22)} = 28.747$, $p < 0.001$), a non-significant effect of time ($F_{(29,638)} = 0.938$, $p = 0.562$), and a significant interaction between factors ($F_{(58,638)} = 2.219$, $p < 0.001$) (Figure 3c). These results suggest that GluN2B-mediated NMDA receptor transmission is critical for synaptic potentiation in the IL-mPFC of proestrus and male mice, and it is impaired in the diestrus mice.

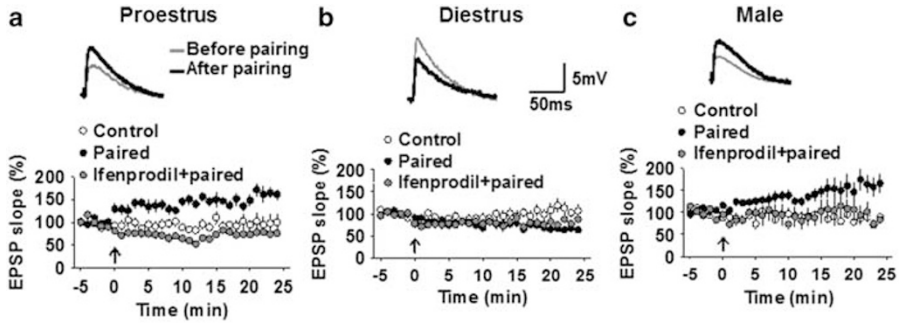

Figure 3 Synaptic plasticity in the IL-mPFC layer 5 pyramidal neurons. (a) Percentage changes in EPSP slope in unpaired proestrus ($n = 7/4$ mice), paired proestrus ($n = 10/5$ mice), and ifenprodil + paired proestrus groups ($n = 7/5$ mice). (b) Percentage changes in EPSP slope in unpaired diestrus ($n = 7/4$ mice), paired diestrus ($n = 11/7$ mice), and ifenprodil + paired diestrus groups ($n = 8/5$ mice). (c) Percentage changes in EPSP slope in unpaired male ($n = 7/4$ mice), paired male ($n = 10/5$ mice), and ifenprodil + paired male groups ($n = 8/4$ mice). Arrow represents the application of the pairing protocol. Upper panel shows examples of EPSPs before and after the pairing protocol.

Differential Effects of ERβ Activation on the IL-mPFC Synapses in Proestrus and Diestrus Mice

To understand whether ERβ activation exerts similar effects as endogenous estradiol on the IL-mPFC glutamatergic synapses, we examined non-NMDA EPSCs in vehicle- and DPN-treated proestrus and diestrus mice. A main effect of groups ($F_{(3,32)} = 7.376$, $p = 0.001$), a significant effect of stimulus intensity ($F_{(1.2,40.9)} = 84.65$, $p < 0.001$), and a significant interaction between factors ($F_{(3.8,40.9)} = 5.05$, $p = 0.002$) were observed (Figure 4a). Whereas DPN treatment increased the EPSC amplitude in proestrus mice ($p = 0.031$), EPSC amplitude was suppressed in diestrus mice following DPN treatment ($p = 0.007$) (Figure 4a). To test whether DPN-induced changes in synaptic transmission involves modulation of glutamate release, we compared PPF in vehicle- and DPN-treated proestrus and diestrus groups, which showed a main effect of groups ($F_{(3,32)} = 6.323$, $p = 0.002$), a significant effect of time ($F_{(1,32)} = 10.493$, $p = 0.003$), and a non-significant interaction between factors ($F_{(3,32)} = 2.358$, $p = 0.09$) (Figure 4b). Thus, the increase in EPSC amplitude in DPN-treated proestrus mice was mediated by an enhanced glutamate release. However, ERβ activation suppressed glutamate release in the diestrus group and prevented any further facilitation in response to the second stimulus.

Next, we asked whether the activation of ERβ has an effect on GluN2B-mediated transmission in diestrus mice. We observed a significantly higher NMDA EPSC decay time in the DPN-treated diestrus group compared with vehicle-treated group ($t_{(16)} = 2.22$, $p = 0.041$). Comparison of the effect of ifenprodil on NMDA EPSC decay time in vehicle- and DPN-treated diestrus mice showed a main effect of groups ($F_{(1,16)} = 5.521$, $p = 0.032$), a significant effect of time ($F_{(1,16)} = 17.548$, $p = 0.001$), and a non-significant interaction between factors ($F_{(1,16)} = 2.998$, $p = 0.103$) (Figure 4c). Comparison of NMDA EPSC amplitude revealed a non-significant effect of groups ($F_{(1,16)} = 0.201$, $p = 0.66$), a significant effect of time ($F_{(1,16)} = 24$, $p < 0.001$), and a non-significant interaction between factors ($F_{(1,16)} = 1.126$, $p = 0.304$) (Figure 4d). The ifenprodil-sensitive increase in decay time by DPN suggests that ERβ activation facilitates GluN2B-mediated transmission in diestrus mice.

DPN-treated diestrus mice showed a significant synaptic potentiation in an ifenprodil-sensitive manner after the pre-before-post pairing as shown by a main effect of groups ($F_{(2,23)} = 21.426$, $p < 0.001$), a significant effect of time ($F_{(29,667)} = 2.385$, $p < 0.001$), and a significant interaction between factors ($F_{(58,667)} = 4.123$, $p < 0.001$) (Figure 4e). Thus, ERβ activation restores synaptic potentiation in diestrus mice by a GluN2B-dependent mechanism. The rescue of synaptic potentiation in diestrus mice is selective to ERβ but not ERα activation as PPT, an ERα agonist, failed to affect EPSP slope in diestrus mice as shown by a non-significant effect of groups ($F_{(1,16)} = 1.334$, $p = 0.265$), a significant effect of time ($F_{(29,464)} = 4.029$, $p < 0.001$), and a non-significant interaction between factors ($F_{(29,464)} = 1.254$, $p = 0.173$) (Figure 4e). Surprisingly, DPN blocked synaptic potentiation in the proestrus group as shown by a main effect of groups ($F_{(1,11)} = 9.789$, $p = 0.007$), a non-significant effect of time ($F_{(29,406)} = 0.923$, $p = 0.583$), and a significant interaction between factors ($F_{(29,406)} = 1.873$, $p = 0.005$) (Figure 4f). Thus, ERβ activation exerts opposite effect on synaptic potentiation in proestrus and diestrus mice.

Exogenous Estradiol Enhances Non-NMDA Receptor Transmission in the IL-mPFC

As both estradiol and progesterone are elevated during proestrus (DeLeon *et al*, 1990), we examined which of these hormones mimic glutamatergic transmission during proestrus and whether ovariectomy exerts a similar effect as low endogenous estradiol on glutamatergic transmission. Although ovariectomy did not affect EPSC amplitude, administration of estradiol enhanced the EPSC amplitude in both ovariectomized and sham mice as revealed by a main effect of group ($F_{(3,30)} = 19.16$, $p < 0.001$), stimulus intensity ($F_{(1.5,44.6)} = 67.73$, $p < 0.001$), and a significant interaction between factors ($F_{(4.5,44.6)} = 2.561$, $p = 0.046$) (Figure 5a). Comparison of PPF in estradiol- and vehicle-treated ovariectomized and sham mice showed a significant effect of groups ($F_{(3,30)} = 10.35$, $p < 0.001$), a significant effect of time ($F_{(1,30)} = 18.75$, $p < 0.001$), and a non-significant interaction between factors ($F_{(3,30)} = 1.232$, $p = 0.315$) (Figure 5b), suggesting that estradiol enhances EPSC amplitude by a

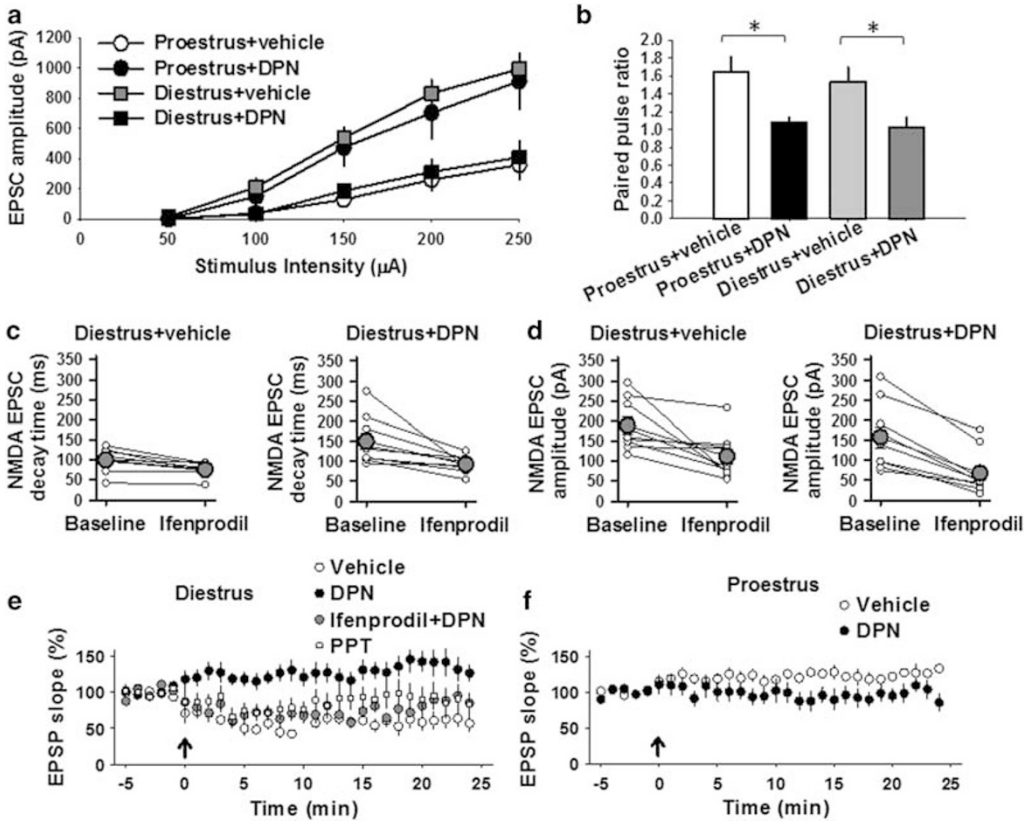

Figure 4 Effect of ERβ activation on the IL-mPFC glutamatergic synapses. Non-NMDA EPSC amplitude (a) and paired pulse ratio (b) in proestrus + vehicle ($n = 8/4$ mice), proestrus + DPN ($n = 9/4$ mice), diestrus + vehicle ($n = 9/4$ mice), and diestrus + DPN groups ($n = 10/4$ mice). Asterisk indicates a significant difference. Effect of ifenprodil on NMDA EPSC decay time (c) and amplitude (d) in vehicle- ($n = 9/4$ mice) and DPN-treated diestrus mice ($n = 9/4$ mice). Small empty and large filled circles represent raw data and mean ± SEM, respectively. (e) Percentage changes in EPSP slope in paired vehicle-treated ($n = 10/5$ mice), paired DPN-treated ($n = 9/5$ mice), paired ifenprodil + DPN-treated ($n = 7/4$ mice), and paired PPT-treated diestrus mice ($n = 8/4$ mice). (f) Percentage changes in EPSP slope in paired vehicle-treated ($n = 8/5$ mice) and paired DPN-treated proestrus mice ($n = 8/4$ mice). Arrow represents the application of the pairing protocol.

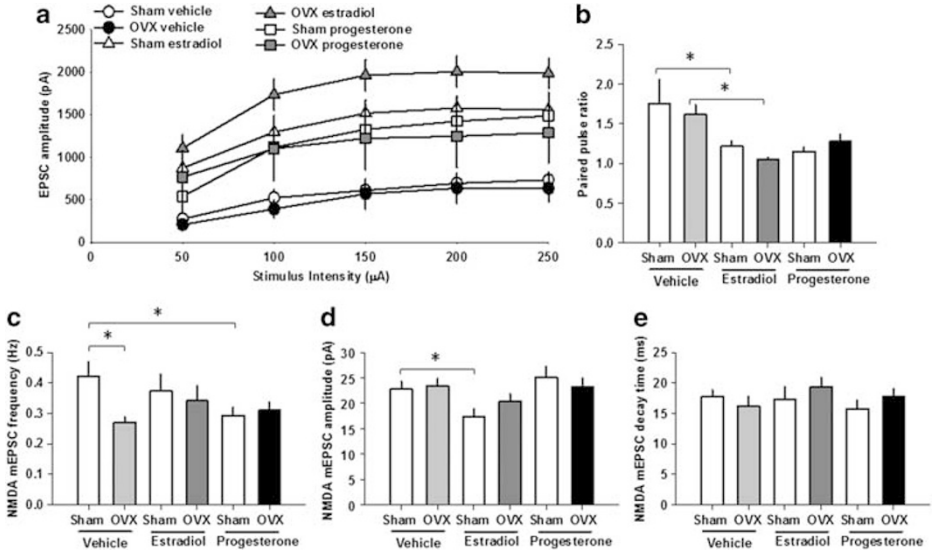

Figure 5 Effect of exogenous estradiol and progesterone on the IL-mPFC glutamatergic synapses. EPSC amplitude (a) and PPF (b) in vehicle-treated sham ($n = 8/3$ mice), vehicle-treated ovariectomized (OVX) ($n = 9/3$ mice), estradiol-treated sham ($n = 9/3$ mice), estradiol-treated ovariectomized mice ($n = 8/3$ mice), progesterone-treated sham ($n = 9/3$ mice), and progesterone-treated ovariectomized mice ($n = 9/3$ mice). NMDA mEPSC frequency (c), amplitude (d), and decay time (e) in vehicle-treated sham ($n = 9/3$ mice), vehicle-treated ovariectomized ($n = 9/3$ mice), estradiol-treated sham ($n = 9/3$ mice), estradiol-treated ovariectomized ($n = 9/3$ mice), progesterone-treated sham ($n = 9/3$ mice), and progesterone-treated ovariectomized mice ($n = 9/3$ mice). Asterisk indicates a significant difference.

pre-synaptic mechanism. EPSC amplitude in vehicle- and progesterone-treated ovariectomized and sham mice showed a non-significant effect of group ($F_{(3,31)} = 2.404$, $p = 0.086$), a significant effect of stimulus intensity ($F_{(1.6,49.7)} = 49$, $p < 0.001$), and a non-significant interaction between factors ($F_{(4.8,49.7)} = 2.153$, $p = 0.077$) (Figure 5a). Consistently, we observed a non-significant effect of groups ($F_{(3,31)} = 1.32$, $p = 0.286$), a significant effect of time ($F_{(1,31)} = 16$, $p < 0.001$), and a non-significant interaction between factors ($F_{(3,31)} = 1.852$, $p = 0.158$) when PPF was compared between progesterone- and vehicle-treated ovariectomized and sham mice (Figure 5b). Thus, in contrast to the decreased non-NMDA receptor transmission in proestrus, exogenous estradiol enhanced EPSC amplitude, whereas neither ovariectomy nor progesterone produced a significant effect.

Next, we compared the effect of exogenous estradiol and progesterone on NMDA receptor transmission. Although the ovariectomized mice showed a decrease in NMDA mEPSC frequency compared with sham group ($t_{(16)} = 2.93$, $p = 0.009$), both the amplitude and decay time remained unaffected. Exogenous estradiol did not affect frequency ($F_{(3,32)} = 1.965$, $p = 0.139$) or decay time ($F_{(3,32)} = 0.671$, $p = 0.576$) (Figure 5c and e). However, a main effect on NMDA mEPSC amplitude was observed ($F_{(3,32)} = 3.257$, $p = 0.034$) with a significant suppression of NMDA mEPSC amplitude in estradiol-treated sham mice ($p = 0.018$) (Figure 5d). Unlike estradiol, progesterone did not affect the amplitude ($F_{(3,32)} = 0.37$, $p = 0.775$) but showed an effect on NMDA mEPSC frequency ($F_{(3,32)} = 4.332$, $p = 0.011$) with a significant suppression in sham mice ($p = 0.008$) (Figure 5c and d). Similar to estradiol, progesterone did not affect decay time of NMDA mEPSCs ($F_{(3,32)} = 0.608$, $p = 0.615$) (Figure 5e). Thus, exogenous administration of neither estradiol nor progesterone mimics NMDA receptor transmission in proestrus mice but exerts a suppression of NMDA receptor transmission in ovary-intact mice.

DISCUSSION

Here we demonstrate an estrous cycle-dependent modulation of glutamatergic synaptic transmission and plasticity in the IL-mPFC. In contrast to the effect of exogenous estradiol, which showed an enhancement of non-NMDA receptor transmission, proestrus mice exhibited a reduced non-NMDA receptor transmission compared with diestrus and male mice. Also, the activation of ERβ exerted an opposite effect on non-NMDA receptor transmission in diestrus and proestrus mice, a suppression and potentiation, respectively. While the exogenous activation of estradiol signaling modulated glutamate release, synaptic GluA2-containing AMPA receptors might be responsible for the differences in non-NMDA receptor transmission in proestrus, diestrus, and male mice as we did not observe any modification of PPF or inward rectification of EPSCs, a hallmark of synaptic GluA2-lacking AMPA receptors (Adesnik and Nicoll, 2007). Circulating estradiol level might have a role in the differential effects of endogenous and exogenous estradiol on non-NMDA receptor transmission.

The metaplasticity induced by the endogenous estradiol appears to define a temporal window for enhanced synaptic potentiation in a GluN2B-dependent manner. The enhancement of GluN2B NMDA receptor transmission by estradiol signaling might involve synaptic recruitment of GluN2B-containing NMDA receptors (Snyder *et al*, 2011). Estradiol-mediated enhancement of GluN2B function and synaptic plasticity have been reported in other brain areas (Smith and McMahon, 2006; Snyder *et al*, 2011; Xiao *et al*, 2013). Given the role of brain-derived neurotrophic factor (BDNF) downstream of estradiol signaling and its effect on GluN2B-mediated transmission, BDNF might be involved in estradiol-induced augmentation of GluN2B transmission (Levine and Kolb, 2000; Miranda *et al*, 1994). The attenuation of NMDA receptor transmission by exogenous estradiol and progesterone suggests the possibility that the beneficial effects of exogenous estradiol and progesterone in fear extinction might be mediated by its effect on brain areas other than the IL-mPFC, which might include the hippocampus and amygdala (Milad *et al*, 2009). Consistently, exogenous estradiol was shown to exert a selective effect on BDNF signaling in the hippocampus compared with the cortex (Singh *et al*, 1995).

The aforementioned metaplasticity causes enhanced glutamate release by ERβ activation during periods of high endogenous estradiol, which occludes a subsequent activity-dependent synaptic potentiation. However, during periods of low endogenous estradiol, ERβ activation suppresses basal transmission but facilitates activity-dependent potentiation by enhancing GluN2B transmission. A recent study showed that dopamine D_1 receptor activation impaired fear extinction during periods of high endogenous estradiol but reversed fear extinction deficits during periods of low endogenous estradiol (Rey *et al*, 2013). Consistently, estradiol was reported to suppress and enhance hippocampal long-term potentiation in proestrus and diestrus rats, respectively (Foy *et al*, 2008). Therefore, endogenous estradiol level might have a critical role in determining how the IL-mPFC synapses respond to not only exogenous estradiol and progesterone but also neuromodulators such as dopamine. It is unclear whether local synthesis of estradiol has a role in the IL-mPFC. Sex difference in the expression of aromatase, the biosynthetic enzyme for estradiol, and its effect on prefrontal cortical functions warrant future studies to understand the regulation and function of local synthesis of estradiol in the IL-mPFC (Wei *et al*, 2013; Xiao *et al*, 2013).

Comparable fear extinction and the IL-mPFC synaptic plasticity in male and high estradiol females suggest that endogenous estradiol level might be a key to sex difference in IL-mPFC function and fear extinction (Milad *et al*, 2010). The IL-mPFC-mediated top-down regulation of the amygdala, a mechanism believed to control fear extinction, involves synaptic potentiation in the IL-mPFC (Pare *et al*, 2004; Pattwell *et al*, 2012b; Sepulveda-Orengo *et al*, 2013). Therefore, the estradiol-dependent facilitation of synaptic potentiation in the IL-mPFC might contribute significantly to the enhancement of fear extinction (Chang *et al*, 2009; Zeidan *et al*, 2011). Also, GluN2B-mediated synaptic transmission might be an effective alternative to augment the IL-mPFC plasticity and fear extinction, which is consistent with the unique role of GluN2B-mediated NMDA receptor transmission in the mPFC (Flores-Barrera *et al*, 2013; Wang *et al*, 2008). Thus, our findings provide important insights into not only the synaptic mechanism by which estradiol

signaling could modulate the IL-mPFC synapses but also potential molecular targets for fear-related psychiatric disorders.

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