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Estrogen receptor β deficiency impairs BDNF–5-HT_{2A} signaling in the hippocampus of female brain: a possible mechanism for menopausal depression

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Abstract

Depression currently affects 350 million people worldwide and 19 million Americans each year. Women are 2.5 times more likely to experience major depression than men, with some women appearing to be at a heightened risk during the menopausal transition. Estrogen signaling has been implicated in the pathophysiology of mood disorders including depression; however, the underlying mechanisms are poorly understood. In this study, the role of estrogen receptor (ER) subtypes, ER α and ER β , in the regulation of brain-derived neurotrophic factor (BDNF) and serotonin (5-HT) signaling was investigated; two pathways that have been hypothesized to be interrelated in the etiology of depression. The analyses in ER $\alpha^{-/-}$ and ER $\beta^{-/-}$ mouse models demonstrated that BDNF was significantly downregulated in ER $\beta^{-/-}$ but not ER $\alpha^{-/-}$ mice, and the ER $\beta^{-/-}$ -mediated effect was brain-region specific. A 40% reduction in BDNF protein expression was found in the hippocampus of ER $\beta^{-/-}$ mice; in contrast, the changes in BDNF were at a much smaller magnitude and insignificant in the cortex and hypothalamus. Further analyses in primary hippocampal neurons indicated that ER β agonism significantly enhanced BDNF/TrkB signaling and the downstream cascades involved in synaptic plasticity. Subsequent study in ER β mutant rat models demonstrated that disruption of ER β was associated with a significantly elevated level of 5-HT_{2A} but not 5-HT_{1A} in rat hippocampus, indicating ER β negatively regulates 5-HT_{2A}. Additional analyses in primary neuronal cultures revealed a significant association between BDNF and 5-HT_{2A} pathways, and the data showed that TrkB activation downregulated 5-HT_{2A} whereas activation of 5-HT_{2A} had no effect on BDNF, suggesting that BDNF/TrkB is an upstream regulator of the 5-HT_{2A} pathway. Collectively, these findings implicate that the disruption in estrogen

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homeostasis during menopause leads to dysregulation of BDNF–5-HT_{2A} signaling and weakened synaptic plasticity, which together predispose the brain to a vulnerable state for depression. Timely intervention with an ER β -targeted modulator could potentially attenuate this susceptibility and reduce the risk or ameliorate the clinical manifestation of this brain disorder.

Keywords

Menopausal depression; estrogen receptor β (ER β); brain-derived neurotrophic factor (BDNF); 5-HT_{1A}; 5-HT_{2A}; hippocampus

1. Introduction

Depression is a chronic, reoccurring neuropsychiatric disease that currently affects 350 million people worldwide including 6.7% of Americans (World Health Organization, 2012). Although significant progress has been made in the development of antidepressants, current treatments yield a therapeutic efficacy in only 60% of depressed patients (Rush et al., 2009) and are associated with several unfavorable side effects (Feighner, 1999; Holm and Markham, 1999). Moreover, the mechanism of action underlying the therapeutic effect of antidepressants is currently unknown. These facts underscore the importance of understanding the mechanistic etiology of depression and developing novel therapeutic methods based on that understanding.

Depression disproportionately affects men and women. Epidemiological data have revealed that women are 2.5 times more likely to experience major depression than men (Weissman et al., 1984), with some women appearing to be at a heightened risk during the menopausal transition (Campbell et al., 2017; Pratt LA, 2014), a period characterized by irregular cycling coupled with fluctuations in gonadal steroid hormone levels. Moreover, several clinical studies have demonstrated significantly reduced depression and mood-related symptomology in menopausal patients undergoing estrogen therapy (ET) when compared to placebo controls (Schmidt et al., 2000; Soares et al., 2001). It has been suggested that estrogen receptors, ER α and ER β , act as the primary regulators of ET-mediated antidepressant effects, however, their specific roles remain controversial. On one hand, post-partum rats treated with an ER α agonist demonstrated reduced depressive behavior in multiple tests suggesting a primary regulatory role for ER α (Furuta et al., 2013). In contrast, ovariectomized (OVX) rats treated with an ER β agonist (Walf et al., 2009; Weiser et al., 2009) exhibited significantly less depressive behavior than rats treated with vehicle or an ER α agonist (Yang et al., 2014); an effect that was absent in ER β ^{-/-} mice (Walf et al., 2009), indicating that ER β signaling may play a key role in the mechanism of ET in depression. These disparities require further molecular-level investigations.

Serotonin (5-HT) is central to the monoamine hypothesis of depression (Coppen, 1967). Increased 5-HT bioavailability in the synapses and thus increased 5-HT function has been shown to effectively relieve depressive symptoms (Delgado et al., 1990). 5-HT induces its wide range of actions through a myriad of receptors, the expression and function of which has also been heavily studied in an attempt to unravel the pathophysiology of depression. Studies have reported a decrease in the 5-HT_{1A} receptor signaling (Hirvonen et al., 2008)

and an increase in the 5-HT_{2A} receptor signaling (Shelton et al., 2009) in the hippocampus and prefrontal cortex of depressed patients when compared to control subjects, which was reversed in the subjects treated with antidepressants (Celada et al., 2004), implicating the involvement of these receptors in the etiology of depression.

Although 5-HT signaling is highly implicated in depression, contradictory reports (for review see (Hinz et al., 2012)) and the inability of the monoamine hypothesis to explain certain clinical findings such as therapeutic lag (Frazer and Benmansour, 2002) have shifted the research focus towards novel alternative mechanisms of depression (Duman and Monteggia, 2006). Brain-derived neurotrophic factor (BDNF) is essential to the more recent and clinically relevant neurotrophic hypothesis of depression. Several clinical and animal studies (Dwivedi et al., 2003; Lippmann et al., 2007) have reported a decrease in the mRNA and protein expression levels of BDNF and TrkB in the hippocampus and prefrontal cortex of depressed subjects when compared with control subjects, which was reversed by chronic but not acute antidepressant treatment (Xu et al., 2003). In addition, mice heterozygous for a BDNF null allele (BDNF^{+/-}) and overexpressing TrkB transgenic gene were found to be resistant to antidepressants (Saarelainen et al., 2003), indicating that BDNF/TrkB signaling is required for the therapeutic effects of antidepressants. Although behavioral and clinical studies strongly support a connection between depression and BDNF signaling, the short half-life of BDNF in systemic circulation (Sakane and Pardridge, 1997) and its blood-brain barrier (BBB) impermeability (Sakane and Pardridge, 1997) hinders its use as a therapeutic agent.

Both the monoaminergic and neurotrophic hypotheses of depression fall short of providing a viable therapeutic window, thus it is highly possible that the key for understanding and successfully treating depression may be found by examining the interaction of these two divergent signaling pathways. 5-HT signaling has been shown to regulate BDNF/TrkB signaling in the hippocampus (Vaidya et al., 1997). Parallel to these studies, BDNF infusion in the midbrain has been reported to increase 5-HT turnover and promote phenotype and function of serotonergic neurons (Siuciak et al., 1996). Additionally, the Val66Met BDNF mouse model exhibits a compromised selective serotonin reuptake inhibitors (SSRIs) response (Yu et al., 2012), while BDNF^{+/-} mice demonstrate reduced 5-HT_{1A} (Hensler et al., 2007) and increased 5-HT_{2A} sensitivity (Trajkovska et al., 2009) in the hippocampus. These observations suggest a possible intertwined interaction of these two different signaling molecules, thus highlighting an important avenue for further investigation.

The goal of this study was to investigate the role of ERs in the regulation of BDNF and 5-HT signaling in the female brain. The analyses in ER α ^{-/-} and ER β ^{-/-} animal models demonstrated brain region-specific regulation of BDNF by ER β but not ER α . Additionally, the data indicate that 5-HT_{2A} and not 5-HT_{1A} is negatively regulated by ER β . Taken together, these results suggest that ER β -mediated regulation of BDNF–5-HT_{2A} signaling could play a major role in both the development and intervention of depressive disorders in menopausal women.

2. Materials and Methods

2.1. Chemicals

4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl) *tris*phenol (PPT; ER α agonist), 2,3-*bis*(4-Hydroxyphenyl)-propionitrile (DPN; ER β agonist), 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol (PHTPP; ER β antagonist), 4-Iodo-2,5-dimethoxy- α -methylbenzeneethanamine hydrochloride (DOI; 5-HT $_{2A/2C}$ agonist), 7,8-Dihydroxy-2-phenyl-4*H*-1-benzopyran-4-one (7,8 DHF; TrkB agonist), and 4-(4-Fluorobenzoyl)-1-(4-phenylbutyl)piperidine oxalate (4F 4PP oxalate; 5-HT $_{2A}$ antagonist) were purchased from Tocris Bioscience (Ellisville, MO). Chemicals were dissolved at 10-50 mM in analytically pure ethanol and further diluted in culture medium to the final working concentrations (PPT: 100 nM, DPN: 100 nM, PHTPP: 1 μ M, DOI: 3 μ M, 7,8 DHF: 10 μ M, and 4F 4PP oxalate: 10 μ M) immediately prior to use. The sources of other materials are indicated in the experimental methods described below.

2.2. Animal models

The use of animals was approved by the Institutional Animal Care and Use Committee at the University of Kansas and followed NIH guidelines for the care and use of laboratory animals. Embryonic day-18 fetuses derived from time-pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) were used to obtain primary hippocampal neuronal cultures for *in vitro* experiments. *In vivo* experiments were carried out in ER $\alpha^{-/-}$ and ER $\beta^{-/-}$ mouse and rat models. ER $\alpha^{-/-}$ and ER $\beta^{-/-}$ mouse models (The Jackson Laboratory, Bar Harbor, ME) were created by using a targeting vector containing a neomycin resistance gene driven by the mouse phosphoglycerate kinase promoter to introduce stop codons into exon 2 or exon 3 for ER $\alpha^{-/-}$ and ER $\beta^{-/-}$ respectively. The construct was introduced into 129P2/OlaHsd-derived E14TG2a embryonic stem (ES) cells (BK4 subline). Correctly targeted ES cells were injected into C57BL/6J blastocysts to obtain chimeric animals. These mice were then backcrossed to C57BL/6J for eight generations. The line was then bred to C57BL/6NTac from which homozygotes were generated. The ER β mutant rats were generated using zinc finger nuclease (ZFN) mediated genome editing to target deletion of exon 3 (Ex 3 $^{-/-}$) and exon 4 (Ex 4 $^{-/-}$) in the ER β gene (Cameron et al., 1995) and are available at the Rat Resource and Research Center (University of Missouri, Columbia, MO). The Ex 3 $^{-/-}$ mutation results in a frameshift and a null mutation, whereas the Ex 4 $^{-/-}$ mutation results in a DNA binding deficient ER β variant protein. Animals (mice at 6 months of age and rats at 6 and 10 months of age) were euthanized via thoracotomy and cortical, hippocampal and hypothalamic brain regions were immediately dissected and flash frozen in dry ice (n=5 for each genotype and age group).

2.3. Primary hippocampal neurons

Primary cultures of hippocampal neurons were prepared from embryonic day-18 fetuses derived from time-pregnant Sprague-Dawley rats. Briefly, after dissected from the brains of the rat fetuses, the hippocampi were treated with 0.02% trypsin in Hank's balanced salt solution (137 mM NaCl, 5.4 mM KCl, 0.4 mM KH $_2$ PO $_4$, 0.34 mM Na $_2$ HPO $_4$ ·7H $_2$ O, 10 mM glucose, and 10 mM HEPES) at 37 °C for 5 min and dissociated by repeated passage through a series of fire-polished constricted Pasteur pipettes. Cells were plated at a density

of 5×10^5 on 0.1% polyethylenimine-coated 60 mm petri dishes. Neurons were grown in phenol-red free Neurobasal medium (NBM, Invitrogen, Carlsbad, CA) supplemented with B27, 5 U/ml penicillin, 5 μ g/ml streptomycin, 0.5 mM glutaMAX and 25 μ M glutamate at 37 °C in a humidified 5% CO₂ atmosphere for the first 3 days and NBM without glutamate afterwards. Cultures grown in serum-free NBM yielded approximately 99.5% neurons and 0.5% glial cells.

2.4. Protein extraction

Primary hippocampal neuronal cultures were treated at 5 days *in vitro* (DIV) with respective chemicals for 4 days unless other duration of treatment is indicated. Following the treatment, cultures were washed with cold PBS (pH 7.2), lysed in cold lysis buffer (N-PER, Thermo Scientific, MA) and then harvested with a cell scraper, followed by centrifugation at $10,000 \times g$ for 10 min. Protein concentrations were determined via BCA Assay (Pierce Biotechnology, IL). For tissue protein extraction, 30 mg of hippocampal, cortical and hypothalamic tissue samples were homogenized using a Bullet Blender 24 Homogenizer (Next Advance, NY) in T-PER (Pierce Biotechnology, IL) supplemented with protease and phosphatase inhibitors (Roche Applied Science, IN) and 100 μ L 0.5 mm glass beads (Next Advance, NY) at speed 8 for 3 min at 4°C followed by centrifugation at 12,000 rpm for 8 min at 4°C. Supernatants were transferred to new microcentrifuge tubes and protein concentrations were determined via BCA Assay (Pierce Biotechnology, IL).

2.5. Western blot

Equal amounts of total protein (20 μ g/lane) were loaded and separated by 10% Glycine SDS-PAGE/Tricine SDS-PAGE. Resolved proteins were transferred to 0.2 μ m pore-sized PVDF membranes (Bio-Rad, CA) and blocked with 5% Blotting Grade Blocker (BioRad, CA) in TBST (100 mL 10 $\times$ TBS (200 mM Tris, 1.5 mM NaCl, pH 7.6), 10 mL 10% Tween-20, 890 mL ddH₂O) for 1 hr at RT followed by incubation with customized dilutions of primary antibodies at 4°C overnight. Following overnight incubation, membranes were washed 3 times for 10 min in TBST at RT, followed by incubation with the HRP-conjugated secondary antibody (1:5000; Pierce) for 1 hr at RT. Blots were again washed 3 times for 10 min in TBST. Bands were visualized using chemiluminescence with an ECL detection kit (BioRad) and scanned using the C-Digit Blot Scanner (LI-COR, Lincoln, NE). Relative intensities of the immunoreactive bands were quantified using image digitizing software, Image Studio Version 4.0 (LI-COR). Membranes were stripped in 5 mL Restore PLUS Western Blot Stripping Buffer (Thermo Scientific) for 8 min at RT and re-probed with the indicated loading control. The following primary antibodies were used: rabbit polyclonal anti-BDNF (1:500; Santa Cruz Biotechnology, TX), rabbit polyclonal anti-TrkB (1:1000; Abcam, MA), rabbit polyclonal anti-pTrkB (targeting Y515 phosphorylation site; 1:1000; Bioworld Technology Inc., MN), rabbit polyclonal anti-PSD95 (1:500; Alomone Labs, IL), rabbit monoclonal anti-Synaptophysin (1:1000; Abcam, MA), rabbit polyclonal anti-VAMP2 (1:2000; Enzo Life Sciences, NY), rabbit polyclonal anti-5-HT_{1A} (1:1000; Abcam, MA), rabbit polyclonal anti-5-HT_{2A} (1:20,000; a generous gift from Dr. Nancy Muma), rabbit polyclonal anti- β Actin (1:3000; Thermo Scientific, MA), and mouse monoclonal anti- β Tubulin (1:3000; Thermo Scientific, MA).

Validation of specificity of BDNF antibody—For BDNF protein expression, densitometry of the mature form of BDNF (mBDNF) was performed. mBDNF has been shown to exist in monomeric form with a molecular mass of 14 kDa (Koo et al., 2013; Yan et al., 1997) and trimeric form with a molecular mass of 42 kDa (Dunham et al., 2009; Spires et al., 2004; Wessels et al., 2015). The antibody used in the study detected both of these forms (Fig. 1a) and the expression level of each form is presented individually.

2.6. Statistical analysis

Data were presented as mean $\pm$ standard deviation. For comparisons between two groups, Student's *t*-test was used; for comparisons involving multiple groups, one-way or two-way ANOVA with Turkey's or Dunnett's post-hoc test were used. All statistical analyses were performed with GraphPad Prism version 5.0 (GraphPad software Inc., CA). The statistical significance was indicated by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. Results

3.1. ER β , not ER α , knockout downregulates BDNF and TrkB in female mouse hippocampus

Using ER $\alpha^{-/-}$ and ER $\beta^{-/-}$ mouse models, the involvement of ER α and ER β in the regulation of BDNF in different regions of the female brain was examined. Cortical, hypothalamic and hippocampal tissues were harvested from 6-month-old ER $\alpha^{-/-}$ and ER $\beta^{-/-}$ female mice ($n=5$ /group) and probed for both monomeric (14 kDa) and trimeric (42 kDa) BDNF immunoreactivity (Figure 1). The data show no significant difference in BDNF expression levels in cortical tissues derived from either ER $\alpha^{-/-}$ model indicating that BDNF is not regulated by ER signaling in the mouse cortex (Figure 1b, monomeric BDNF: $F(2,6) = 0.4$, $p=0.6870$; trimeric BDNF: $F(2,6) = 2.99$, $p=0.1254$). Similarly, no change in BDNF expression was detected in the hypothalamus of ER $\alpha^{-/-}$ and ER $\beta^{-/-}$ mice (Figure 1c, monomeric BDNF: $F(2,6) = 0.439$, $p=0.6635$; trimeric BDNF: $F(2,6) = 1.33$, $p=0.3320$). In contrast, hippocampal BDNF levels were significantly reduced in ER $\beta^{-/-}$ mice but not ER $\alpha^{-/-}$ mice (Figure 1d, monomeric BDNF: $F(2,6) = 12.76$, $p=0.0075$; trimeric BDNF: $F(2,6) = 7.23$, $p=0.0048$). Further analyses demonstrated a 50% decrease in the expression of the BDNF receptor, TrkB, in ER $\beta^{-/-}$ but not ER $\alpha^{-/-}$ mice (Figure 1e, $F(2,6) = 13.81$, $p=0.0057$). Collectively, these data indicate that ER β , but not ER α , regulates the BDNF and TrkB expression level in the hippocampus of the female mouse brain.

3.2. ER β disruption downregulates BDNF and TrkB in female rat hippocampus

To further validate the findings from ER $\alpha^{-/-}$ mouse models, BDNF and TrkB expression levels were examined in two different ER β mutant rat models in which the ER β gene was disrupted by targeted deletion of exon 3 (Exon 3 $^{-/-}$) or exon 4 (Exon 4 $^{-/-}$). Animals were sacrificed at 6 months and 10 months of age ($n=5$ /group) and hippocampal tissues were collected and probed for BDNF immunoreactivity. Consistent with the findings in ER $\beta^{-/-}$ mice, BDNF expression was significantly reduced in the hippocampus of both ER β mutant rat models at 6 months (Figure 2a, monomeric BDNF: $F(2, 6) = 10.07$, $p=0.0121$; trimeric BDNF: $F(2,6) = 7.67$, $p=0.0222$) and 10 months of age (Figure 2c, monomeric BDNF: $F(2,6) = 9.90$, $p=0.0126$; trimeric BDNF: $F(2,6) = 13.99$, $p=0.005$). Additionally, a similar decrease in expression levels of TrkB was detected in Exon 4 $^{-/-}$ rats at 6 months (Figure 2b,

TrkB: $F(2,6) = 25.52$, $p=0.0012$) and both ER β mutant rat models at 10 months of age (Figure 2d, TrkB: $F(2,6) = 13.23$, $p=0.0063$). These findings indicate that ER β regulates the BDNF and TrkB expression level in the rat hippocampus and the importance of the ER β DNA binding domain in mediating estrogen actions.

3.3. BDNF is more significantly impacted by ER β status than aging

To better understand the biological significance of ER β -mediated regulation of BDNF, an age-dependent comparison of hippocampal BDNF immunoreactivity was performed in 6-month and 10-month-old wild type and ER β mutant rats (Figure 3). Consistent with the literature, the data indicated that aging from 6 months to 10 months leads to a significant decrease in BDNF and TrkB expression levels in all three rat models (Figure 3a-c). The comparison of both factors (ER β status and age) on the same western blot revealed that ER β disruption results in a 30%-40% decrease in BDNF expression levels while aging resulted in a 10%-15% reduction. Though both the aging process and disruption of ER β result in a significant decline in BDNF expression levels, the data indicate that ER β disruption more significantly impacts BDNF suggesting that the regulation of BDNF by ER β is more prominent than the regulation of BDNF by aging (Figure 3d, monomeric BDNF: two-way ANOVA, Interaction: $p=0.3062$; column factor (effect of genotype): $p<0.0001$; row factor (effect of aging): $p=0.0040$); trimeric BDNF: two-way ANOVA, Interaction: $p=0.8742$; column factor (effect of genotype): $p<0.0001$; row factor (effect of aging): $p=0.0011$).

3.4. ER β activation upregulates BDNF and TrkB in primary hippocampal neurons

As ER β deficiency was shown to significantly and negatively impact BDNF and TrkB expression, possible effects of ER β activation on BDNF and TrkB expression levels was examined in primary hippocampal neurons treated with the ER subtype-specific agonists PPT (ER α agonist) and DPN (ER β agonist). The data indicate that specific activation of ER β but not ER α resulted in increased expression of BDNF in primary hippocampal neurons (Figure 4a, monomeric BDNF: $F(2,6) = 28.06$, $p=0.0009$; trimeric BDNF: $F(2,6) = 16.07$, $p=0.0039$). The data also revealed that ER β activation resulted in increased phosphorylation of the TrkB receptor (Figure 4b, $F(2,6) = 14.76$, $p=0.0048$). The ER β -mediated upregulation of BDNF and TrkB was abolished in neurons pre-treated with the ER β -specific antagonist PHTPP (Figure 4b, monomeric BDNF: $F(2,6) = 8.9$, $p=0.0159$; trimeric BDNF: $F(2,6) = 69.51$, $p<0.001$; TrkB: $F(2,6) = 14.76$, $p=0.0048$).

3.5. ER β activation upregulates the expression levels of synaptic proteins in primary hippocampal neurons

Antidepressants have been reported to increase the expression and function of proteins related to synaptic plasticity (O'Leary et al., 2009; Sairanen et al., 2007). Therefore, we assessed whether ER β activation in primary hippocampal neurons would mimic the effects of antidepressant treatment. DPN and DPN plus PHTPP-treated neurons were harvested and probed for expression of the synaptic plasticity markers synaptophysin (major synaptic vesicle protein p38), VAMP2 (vesicle-associated membrane protein 2) and PSD95 (postsynaptic density protein 95) (Figure 4c). The data indicate that ER β activation significantly upregulated the expression levels of synaptophysin, VAMP2, and PSD95 (Figure 4c, Synaptophysin: $F(2,6) = 13.34$, $p=0.0062$; VAMP2: $F(2,6) = 14.30$, $p=0.0052$;

PSD95: $F(2,6) = 28.6$, $p=0.0009$). The DPN-mediated increased protein expression of all three markers was attenuated or abolished in neurons pre-treated with the ER β -specific antagonist PHTPP, indicating that ER β is a potential regulator of synaptic plasticity in hippocampal neurons that is likely mediated by BDNF signaling.

3.6. ER β disruption upregulates 5-HT $_{2A}$ but has no effect on 5-HT $_{1A}$ in female rat hippocampus

After determining that ER β does regulate BDNF expression in the hippocampus, we further examined the role of ER β in the regulation of 5-HT signaling in the female rat hippocampus. As 5-HT $_{1A}$ and 5-HT $_{2A}$ have been implicated in the etiology of depression, our investigation was focused on these two 5-HT receptors. The data indicate that ER β disruption had no effect on 5-HT $_{1A}$ expression levels in female rat hippocampus at either 6 months or 10 months of age (Fig. 5a and 5b, 6 month animals (5a): $F(2,6) = 1.579$, $p=0.2812$; 10 month animals (5b): $F(2,6) = 0.9962$, $p=0.4231$). However, ER β disruption resulted in a nearly 15% increase in expression levels of 5-HT $_{2A}$ in the hippocampus of 6-month-old animals. In addition, this dysregulation was enhanced in 10-month-old ER β mutant rats which showed nearly 40% increase in the expression level of 5-HT $_{2A}$ receptors (Fig. 5a and 5b, 6 month animals (5a): $F(2,6) = 9.16$, $p=0.0150$; 10 month animals (5b): $F(2,6) = 34.63$, $p=0.0005$). These results indicate that ER β regulates 5-HT $_{2A}$ but not 5-HT $_{1A}$ in the female rat hippocampus, which may be enhanced by aging.

3.7. Activation of TrkB downregulates 5-HT $_{2A}$ in primary hippocampal neurons

In order to determine the possible interaction between the BDNF/TrkB and 5-HT $_{2A}$ -mediated signaling pathways, primary hippocampal neurons were treated with agonists of TrkB (7,8 DHF) or 5-HT $_{2A}$ receptor (DOI) and 5-HT $_{2A}$ and BDNF protein expression were examined via immunoblot analyses. The data demonstrate a 30-35% decrease in the levels of 5-HT $_{2A}$ after 7 days of treatment with 7, 8 DHF (Figure 5c, 4-day treatment: $t=1.128$, $p=0.3580$; 7-day treatment: $t=7.547$, $p=0.0080$) while treatment with DOI did not induce a significant change in the expression levels of either BDNF or pTrkB (Figure 5d, monomeric BDNF: $F(3,8) = 1.914$, $p=0.2059$; trimeric BDNF: $F(3,8) = 0.9540$, $p=0.4596$; pTrkB: $F(3,8) = 0.6413$, $p=0.6096$).

4. Discussion

This study sought to elucidate the role of ERs in the regulation of BDNF and 5-HT signaling, both of which have been highly implicated in the etiology of depression (Coppen, 1967; Duman and Monteggia, 2006). Analyses in ER $\alpha^{-/-}$ and ER $\beta^{-/-}$ mouse models demonstrated that BDNF was significantly downregulated in ER $\beta^{-/-}$ but not ER $\alpha^{-/-}$ mice, and the ER β -mediated response was limited to the hippocampus. This brain region-specific regulation of BDNF is intriguing as it parallels clinical (Dwivedi et al., 2003) and preclinical observations (Duman and Monteggia, 2006; Lippmann et al., 2007) reporting decreased mRNA and protein levels of BDNF and TrkB in the hippocampus of depressed subjects, an observation which was reversed by chronic antidepressant treatment (Balu et al., 2008; Xu et al., 2003). These regional specificities could be related to the most robust expression of ER β (Osterlund et al., 2000; Osterlund and Hurd, 2001) and BDNF (Conner et al., 1997; Ernfors

et al., 1990; Hohn et al., 1990; Kawamoto et al., 1996; Yan et al., 1997) in hippocampal formation in comparison to other brain regions.

A number of studies have reported decreased BDNF mRNA in ovariectomized animals which was reversed by estradiol treatment (Cavus and Duman, 2003; Singh et al., 1995). Although this estrogen-mediated action has been extensively studied in the past, the underlying mechanism is still not clearly understood. Some studies have implicated the role of ER α (Calabrese et al., 2013) whereas other reports have supported a role for ER β in mediating this effect (Li et al., 2010; Yang et al., 2014). The results of this study showed that BDNF and its receptor TrkB are regulated specifically by ER β and not ER α as only ER β ^{-/-} animals showed decreased BDNF and TrkB expression in specific brain regions. This finding indicates that estrogen exerts its effects via ER β , at least in the hippocampus, suggesting that the decreased estrogen signaling in menopause can lead to a subsequent disruption of ER β signaling creating a deficit in BDNF signaling. The demonstrated regulation of BDNF by ER β could possibly be occurring through the regulation of gene expression of BDNF by ER β as an estrogen response element (ERE) has been reported in the BDNF gene (Sohrabji et al., 1995).

Antidepressants have been reported to increase the expression and function of proteins related to synaptic plasticity (O'Leary et al., 2009; Sairanen et al., 2007). Our data demonstrates that activation of ER β results in a two-fold increase in the synaptic vesicle structural protein, Synaptophysin, 50% increase in VAMP2, a SNARE protein involved in the docking of vesicles to the presynaptic membrane, and a 50% increase in the expression of the postsynaptic density protein PSD95 when compared to vehicle treated neurons. These findings are in line with the outcomes of research studies which reported a similar increase in levels of synaptic proteins in the hippocampus of mice following treatment with ER β but not ER α specific agonists (Liu et al., 2008), although contradictory results have also been reported (Jelks et al., 2007). Hence our data lend further support that ER β agonism is indeed capable of inducing positive changes in synaptic proteins in hippocampal neurons. Accordingly, it is logical to postulate that the decreased ER β signaling during the menopausal transition can lead to a downregulation of these synaptic proteins via decreased BDNF signaling, thus resulting in a weakened ability to adapt to environmental stressors, which have been associated with the onset of depressive episodes.

The monoamine theory is the oldest hypothesis proposed for understanding the etiology of depression (Schildkraut, 1965) and 5-HT is an important player in this hypothesis (Coppen, 1967). There are very few studies that have analyzed potential molecular mechanisms underlying the interactions of estrogen, BDNF, and 5-HT signaling, three signaling pathways that appear to converge in the hippocampus (for review see (Scharfman and MacLusky, 2006)). In the present study, expression levels of 5-HT_{1A} and 5-HT_{2A} were analyzed, as these serotonergic receptors have been implicated in depressive disorders (for review see (Naughton et al, 2000)). Analyses in both 6 and 10-month-old ER β mutant rats revealed an significant increase in expression levels of 5-HT_{2A}, but there were no significant changes in the expression of the 5-HT_{1A} receptor. These results correspond with clinical findings that have reported an increase in 5-HT_{2A} receptor expression in depressed patients (Shelton et al., 2009) and antidepressant treatments ranging from SSRIs to monoamine

oxidase inhibitors induce a downregulation of 5-HT_{2A} receptor in postsynaptic regions in the brain (for review see (Gray and Roth, 2001)). Thus there appears to be a possibility that decreased ER β signaling in menopause leads to a dysfunction in both BDNF and 5-HT_{2A} signaling, which precipitates the onset of depression.

In order to determine whether the ER β -regulated BDNF and 5-HT_{2A} signaling pathways interact with one another, a direct approach of stimulating either 5-HT_{2A} or TrkB signaling in primary hippocampal neurons followed by analyses of the expression levels of other signaling molecules was performed. There have been previous reports indicating that BDNF can regulate 5-HT_{2A} (Trajkovska et al., 2009) in the hippocampus and vice versa (Vaidya et al., 1997). Our data revealed that 5-HT_{2A} activation by DOI didn't lead to a significant change in the expression level of BDNF and pTrkB; in contrast, treatment with 7, 8 DHF significantly downregulated 5-HT_{2A} in hippocampal neurons. These results indicate that BDNF is not being regulated by 5-HT_{2A}, at least at the level of protein, but 5-HT_{2A} is being regulated by BDNF in the hippocampus. Thus it is possible that during menopause decreased BDNF signaling leads to increased 5-HT_{2A} activity, which heightens susceptibility for the development of mood disorders. Moreover, antidepressants have been shown to induce their effects after two to three weeks of time and the regulation we saw in our study mimics that time frame strengthening the possibility of antidepressants working via 5-HT_{2A} receptor signaling.

Overall, the findings of this study are insightful but there remain issues that need to be further addressed. Pro-BDNF is not only a precursor of mature BDNF but has been shown to act as a signaling molecule on its own. It has been proposed that mature and pro-BDNF exist in an equilibrium (Lu et al., 2005); interruption of the equilibrium potentially contributes to the development of depression. One of the weaknesses of this study is that the levels of pro-BDNF were not measured; however, the absence of these data does not reduce the significance of the findings. Changes in the mature form of BDNF were evident indicating a dyshomeostasis in the equilibrium, which would impact susceptibility to depression. Secondly, the use of neuronal cultures derived from both male and female fetuses represents another limitation of the study and the results obtained from these cultures need to be further examined in sex-specific neuronal populations. Lastly, it has been reported that ER β knockout animals do not develop depression-like phenotype (Walf et al., 2009), suggesting that the loss of ER β function alone is not sufficient to trigger a depressive episode, but instead requires interactions with environmental stressors to reach the threshold to cause depression.

5. Conclusions

The findings of this study provide evidence for a possible mechanistic cascade underlying the increased susceptibility of depression in menopausal women (Figure 6). Based on these results, we hypothesize that menopause disrupts estrogen homeostasis which decreases the stimulation of ER β -mediated estrogen signaling in the brain. The attenuated ER β signaling leads to reduced transcription of the BDNF gene, thus decreasing the BDNF protein level in the hippocampal region of the brain. Decreased level of BDNF and reduced BDNF/TrkB signaling weakens synaptic strength thus compromising the brain's ability to adapt and

increasing the risk of depression. Attenuated BDNF/TrkB signaling also increases the activity of 5-HT_{2A}, which could contribute to the increased susceptibility to depression. These molecular changes are escalated in the presence of environmental stressors and lead to the development of depression in menopausal women. Further research is highly warranted to examine the pathophysiological and pharmacological significance of these mechanistic findings in the context of menopausal depression.

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Highlights

- ER β but not ER α regulates BDNF/TrkB signaling in the hippocampus of female brain.
- ER β agonism positively regulates synaptic proteins in hippocampal neurons.
- ER β disruption upregulates 5-HT_{2A} but not 5-HT_{1A} protein expression in the female hippocampus.
- TrkB signaling negatively regulates 5HT_{2A} protein expression in hippocampal neurons.

Figure 1. ERβ, not ERα, knockout downregulates BDNF and TrkB in female mouse hippocampus

a) Validation of specificity of BDNF antibody. Western blots show both the monomeric (14 kDa) and trimeric (42 kDa) forms of mature BDNF in mouse hippocampal protein lysates.

b) BDNF regulation by ERs was examined in three brain regions of ERα^{-/-} and ERβ^{-/-} female mice. Data indicate that: b-c) BDNF is not regulated by either ER in mouse cortex and hypothalamus; d-e) ERβ but not ERα regulates BDNF and TrkB protein expression in mouse hippocampus. Data are shown as mean ± standard deviation, n=5. *p<0.05, **p<0.01.

Figure 2. ER β disruption downregulates BDNF and TrkB in female rat hippocampus
 BDNF and TrkB regulation by ER β was further examined in the hippocampus of a-b) 6-month-old and c-d) 10-month-old ER β mutant female rats. ER β mutant rats with deletions of exon 3 or exon 4 leads to a significant reduction in the expression levels of BDNF and TrkB in rat hippocampus. Data are shown as mean $\pm$ standard deviation, $n=5$. * $p<0.05$, ** $p<0.01$.

Figure 3. BDNF is more significantly impacted by ER β status than aging
 BDNF regulation by ER β and aging was comparatively examined in the hippocampus of 6 and 10-month-old ER β mutant female rats. Data indicate that: a-c) Aging decreases BDNF and TrkB protein expression in rat hippocampus irrespective of ER β status; d) ER β disruption exerts a more prominent impact on BDNF in comparison to aging. Data are shown as mean $\pm$ standard deviation, n=5. * p <0.05, ** p <0.01. # p <0.05 comparison between 6 Months and 10 Months group.

Figure 4. ERβ activation upregulates BDNF/TrkB signaling and synaptic markers in primary hippocampal neurons

Primary hippocampal neurons were treated with PPT (100 nM), DPN (100 nM), or DPN (100 nM) in combination with PHTPP (1 μM). Data indicate that: a) Activation of ERβ but not ERα results in a significant increase in BDNF protein expression in primary hippocampal neurons; b-c) Activation of ERβ also leads to an increase in the expression levels of pTrkB and synaptic proteins synaptophysin, VAMP2, and PSD95; ERβ agonism-mediated increase was reverted back to basal levels in neurons co-treated with ERβ antagonist PHTPP. Data are shown as mean ± standard deviation, n=3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5. a-b) ER β disruption upregulates 5-HT_{2A} but has no effect on 5-HT_{1A} in female rat hippocampus

ER β regulation of serotonergic receptors, 5-HT_{1A} and 5-HT_{2A}, was examined in the hippocampus of ER β mutant female rats. Data indicate that ER β mutant rats with deletions of exon 3 or exon 4 leads to an increase in 5-HT_{2A} protein expression but has no effect on 5-HT_{1A} in the hippocampus of both 6 and 10-month-old rats. **c-d) TrkB activation**

downregulates 5-HT_{2A} in primary hippocampal neurons. Interaction between BDNF/TrkB and 5-HT_{2A} signaling was examined in primary hippocampal neurons. Data indicate that: c) Activation of BDNF/TrkB negatively regulates 5-HT_{2A}; d) Activation of 5-HT_{2A} does not regulate BDNF/TrkB. Data are shown as mean $\pm$ standard deviation, $n=3$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 6. ERβ regulation of BDNF–5-HT_{2A} signaling in the hippocampus of female brain
 Based on the above findings, we hypothesize that: (1) the onset of menopause results in (2) an irregular profile of estrogen in the brain. Disruption of estrogen homeostasis (3) decreases the activation of ERβ signaling in the brain. Decreased ERβ signaling results in reduced transcription of the BDNF gene, (4) decreasing BDNF protein levels in the hippocampus. Decreased BDNF protein levels leads to (5) decreased TrkB signaling, which through unknown mechanisms (6) increases 5-HT_{2A} protein expression and (7) 5-HT_{2A}-mediated signaling. The decrease in BDNF/TrkB signaling also leads to (8) downregulation of synaptic proteins, thus weakening the synaptic strength. Decreased BDNF/TrkB signaling and increased 5-HT_{2A} signaling along with decreased neuroplasticity predispose the brain to (9) an increased risk of developing depression, which could be exacerbated by environmental stressors leading to (10) the onset of depression.