Estrogen Receptor β Regulates Brain Signaling Involved in Depression

By

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Estrogen Receptor β Regulates Brain Signaling Involved in Depression

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Dedicated to My Dearest Family, For Their Perpetual Love and Unswerving Support.

Abstract

Depression, a common, costly and recurrent disorder is associated with considerable morbidity and excess mortality. Several epidemiological and clinical studies have reported that women exhibit an increased risk for developing depression in comparison to men. This discrepancy has been attributed to the hormonal fluctuations occurring in the perimenopausal phase, however, the underlying molecular mechanism(s) are currently unknown. In addition, depression and depressive symptoms are common in individuals diagnosed with Late-onset Alzheimer disease (LOAD) and it is now well-established that a significant association exists between depression and LOAD. The human apolipoprotein E (APOE) gene exists as three major isoforms (ε2, ε3, and ε4) and the ε4 allele has been independently associated with a greater incidence for both depression and AD. Although mounting evidence has pointed to the potentially complex interaction between these two brain disorders in which APOE may play a role, the underlying mechanisms are largely unknown. Therefore, the purpose of this thesis was to a) characterize the mechanism(s) that underlie the etiology of depression in perimenopausal females and b) determine the impact of APOE status in the well reported interaction of depression and AD, with depression being an independent risk factor of AD.

In the first chapter, we characterized the role of genomic estrogen signaling in the regulation of BDNF and 5HT2A receptor signaling. Our analyses in estrogen receptor (ER) knockout mouse models ($ER\alpha^{-/-}$ and $ER\beta^{-/-}$) demonstrated that BDNF expression was significantly downregulated in $ER\beta^{-/-}$ but not $ER\alpha^{-/-}$ mice, and that the $ER\beta^{-/-}$-mediated effect was brain-region specific. Further analyses in primary hippocampal neurons indicated that ERβ agonism significantly enhanced BDNF-TrkB signaling and the downstream signaling cascades involved in neurogenesis and synaptic plasticity. Our subsequent analysis in the hippocampus of $ER\beta^{-/-}$ rats demonstrated that

ERβ deficiency was associated with significantly elevated expression levels of 5HT2A receptor but not 5HT1A receptor. Moreover, our analyses in primary hippocampal neurons revealed that BDNF/TrkB signaling is likely an upstream regulator of the 5HT2A pathway. Collectively, these findings suggest that ERβ signaling dyshomeostasis during perimenopause results in the dysregulation of the BDNF-5HT2A network. These perturbations along with weakened synaptic plasticity may contribute to the overall female susceptibility for depression. Therefore, we hypothesize that timely intervention with an ERβ-targeted modulator could potentially attenuate this susceptibility and reduce the risk or ameliorate the clinical manifestation of depression.

In the second chapter, we examined the impact of different APOE isoforms on neurotrophic and serotonergic signaling pathways in female brain. We hypothesized that APOE isoforms differentially regulate BDNF and 5HT2A signaling pathways with APOE4 resulting in overall dysregulation and APOE2 conferring neuroprotection. Our analyses in 6-month-old female humanized APOE mice (ApoE2, ApoE3, and ApoE4) demonstrated that BDNF and 5HT2A receptor expression levels were regulated in a genotype-depended manner with ApoE4 brain exhibiting the lowest level of BDNF and the highest level of 5HT2A. Additionally, several pre- and post-synaptic proteins were significantly downregulated in ApoE4 brain indicating a degree of synaptic deficit. Moreover, we find that chronic administration of an ERβ-targeted phytoestrogenic diet induced a number of changes in ApoE2 and ApoE3 brains, including a significant decrease in the expression of 5HT2A receptor and an increase in BDNF/TrkB and synaptic proteins whereas, in contrast, ApoE4 brain was largely unresponsive to the treatment. Collectively, these results indicate that APOE4 negatively impacts BDNF-5HT2A signaling in female brain, which could, in part, underlie the APOE4-mediated increased risk for depression. The data presented here supports our hypotheses that estrogen signaling significantly regulates

BDNF 5HT2A signaling in female brain and thus can provide for a possible mechanistic explanation for depression in perimenopausal females. In addition the data also reveals significant modulation of BDNF 5HT2A signaling pathways and synaptic function in an ApoE genotype dependent manner, thus providing a possible underlying mechanism and therapeutic window for increased risk of AD in depressed patients.

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Thank You

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Introduction

A) Estrogens

Estrogens are a class of steroid hormones which control the development and maintenance of female sexual characteristics. In as early as 1912, Adler [6] and Fellner [7] obtained ovarian extracts that gave evidence of estrogenic activity. Almost a decade later, Fellner published additional experiments in which injectable extracts and an orally active material (named "Feminin") produced sterility in rabbits and mice [8]. By 1927 he concluded that these extracts had different effects at different doses (now a known property of estrogens) and prevented pregnancy by destruction of ova and by inhibition of corpus luteum formation [8]. The breakthrough in the study of estrogenic substances came when Aschheim and Zondek discovered the assay based on an estrus reaction in the immature mouse or rat and concluded that urine was a better source of estrogens than tissue extract. In late 1927, Butenandt [9] and the Allen/Doisy team [10] independently started the work for the isolation of estrogens and in the fall of 1929 the latter team announced the isolation of crystalline estrone [10]. By 1933 estrone had been converted to estradiol, and two years later Doisy showed that this, too, was a physiological substance. Although at least six different natural estrogens have been identified in women, only three, estradiol (E2), estrone (E1), and estriol (E3) are produced in a significant quantity. These estrogenic compounds derive their chemical structure from the parent hydrocarbon estrane. Structurally, the natural estrogens exhibit similar characteristics, having a common steroidal backbone with a phenolic OH group at position 3C of the A-ring and the presence of either a hydroxyl or a ketone functionality at the C17 position. The differences among them are the substituents at 16C and 17C in the D-ring. Estrone has a carbonyl group at 17C, estradiol has an OH group at the β- side of 17C, and estriol has two OH groups, one at the α-side of 16C and the other at β-side of 17C (Figure 1) [11].

A).a) Occurrence

In premenopausal, nonpregnant women, E2 is the most abundant and potent of these; E1 and E3 possess only weak estrogenic properties and must be converted to E2 to have full estrogenic action [12]. E2 is produced at a number of sites throughout the body, including (a) the ovary [13], (b) peripheral tissues (e.g., mesenchymal cells of adipose tissue and skin, osteoblasts, bone, and aortic smooth-muscle cells) [14], and (c) other physiologic and pathologic sites (e.g., hypothalamus, breast cancer cells, and cells of endometriosis) [15].

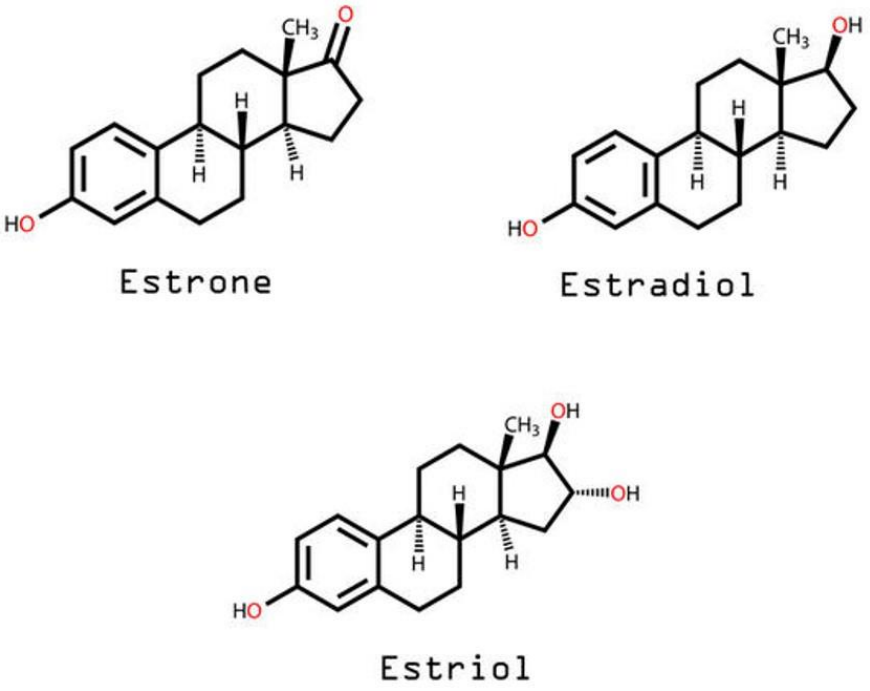

Figure 1 Structure of Endogenous Estrogens. Molecular structure of endogenous estrogens, estrone (3-hydroxyestra-1,3,5(10)-triene-17-one; $C_{18}H_{22}O_2$), estradiol ((17β)-estra-1,3,5(10)-triene-3,17-diol; $C_{18}H_{24}O_2$) and estriol ((16α,17β)-Estra-1,3,5(10)-triene-3,16,17-triol**;** $C_{18}H_{24}O_3$).

Ninety-five percent of E2 is produced by the ovaries premenopausally and only 5% from extra ovarian cells [16]. Although the quantity of E2 produced in each of these extra-ovarian cells is

small, it contributes significantly to net circulating levels by virtue of the relative abundance of these cells. In anovulatory premenopausal and postmenopausal women, extragonadal sites are the sole source of E2 production [17, 18]. On the other hand, although some E1 is produced in the ovaries, the principle source of E1 is through the conversion of androstenedione in the periphery [19]. E3 is formed in both the ovary and the periphery by the C16-hydroxylation of E1 and E2 [20].

A).b) Estrogen synthesis and Metabolism

Steroidogenesis is closely controlled in all three phases of the menstrual cycle (i.e., follicular, periovulatory, and luteal) by the pituitary gland through the actions of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), by the hypothalamus via gonadotropin-releasing hormone (GnRH), and through proximal ovarian factors [21, 22]. Under the positive influence of LH, theca cells produce androgens (i.e., androstenedione and testosterone). After binding to lipo-protein receptors, cholesterol is taken up by steroidogenic cells, stored and moved to the site of steroid synthesis. Androgens are formed by reduction of the number of carbon atoms from 27 to 18. The rate-limiting step in steroid production is the transfer of cholesterol from the cytosol to the inner membrane of the mitochondrion, where the cytochrome P450 enzymes that catalyze the conversion of cholesterol to pregnenolone are located [21, 22]. Androgens produced in theca cells serve as substrates for E2 and E1 production in granulosa cells. Aromatization is the last step in estrogen formation. This reaction is catalyzed by the P450 aromatase monooxygenase enzyme complex that is present in the smooth endoplasmic reticulum and functions as a demethylase. In three consecutive hydroxylating reactions, E1 and E2 are formed from their obligatory precursor

androstenedione and testosterone, respectively [21, 22] (Figure 2). E1 is further converted into estrone sulfate, which remains in the bloodstream and serves as a reservoir of E1.

Estrogens are metabolized by sulfation or glucuronidation, and the conjugates are excreted into the bile or urine [23, 24]. Hydrolysis of these conjugates by the intestinal flora and subsequent reabsorption of the estrogen result in an enterohepatic circulation.

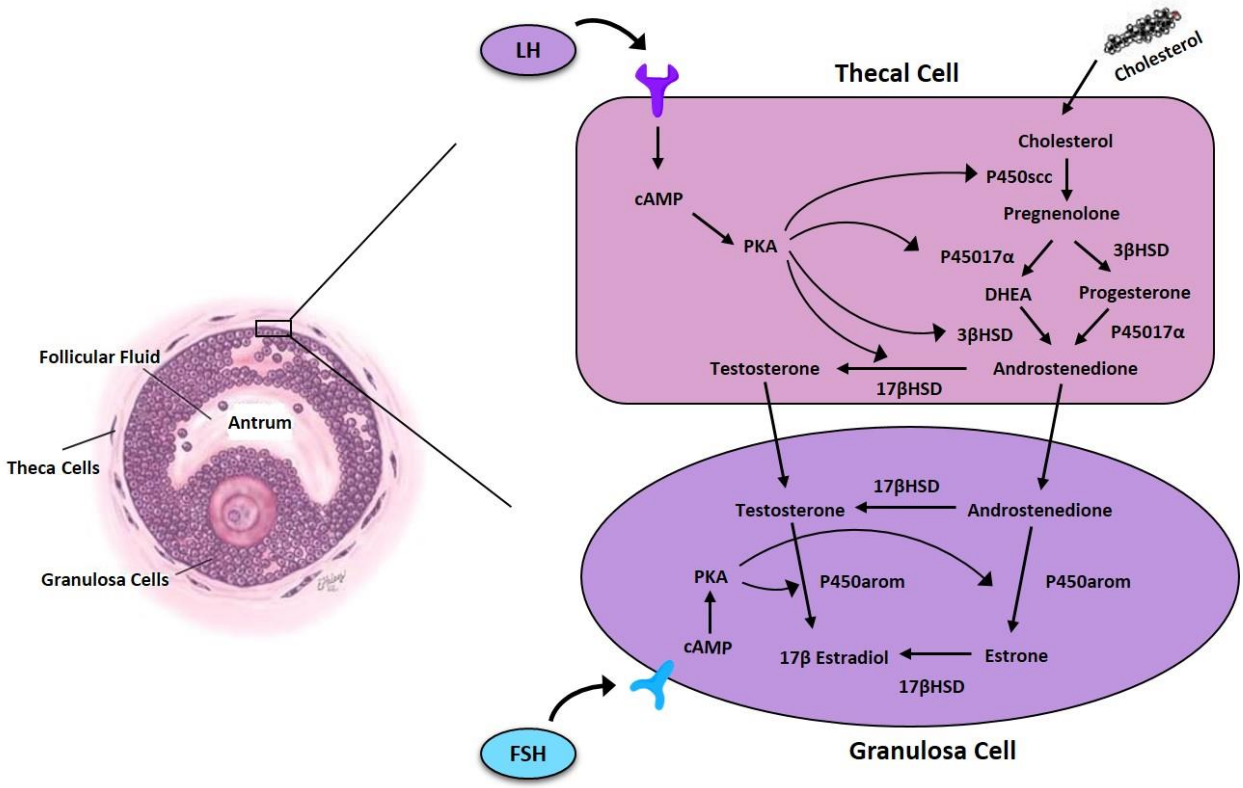

Figure 2. Synthesis of Estrogens. Luteinizing hormone stimulates the production of androstenedione and testosterone from cholesterol in the theca cells. These androgens are then transported to the granulosa cells, where it is converted to 17β-estradiol and estrone respectively.

Estrogens are also metabolized by hydroxylation and subsequent methylation to form catechol and methoxylated estrogens [24]. Hydroxylation of estrogens yields 2-hydroxyestrogens, 4-hydroxyestrogens, and 16a-hydroxyestrogens (catechol estrogens), among which 4-

hydroxyestrone and 16a-hydroxyestradiol are considered carcinogenic. Methylation of the 2- and 4-hydroxyestrogens by catechol O-methyltransferase yields methoxylated estrogen metabolites [25].

A).c) Endogenous Estrogen Fluctuations over Life Span

Fluctuations in estrogen levels are encountered in various phases and interventions related to a woman's reproductive life, including within an ovulatory menstrual cycle, postpartum and periods of unpredictable ovulation (puberty and perimenopause). In prepubertal females the serum E2 levels have been reported to be very low (< 10 pg/ml) which increases with FSH induced growth and proliferation of ovarian follicles [26]. The serum E2 levels increase to 20-60 pg/ml and 25-75 pg/ml in early and late puberty respectively [26]. While cycling, E2 levels have been reported to be low in follicular phase, peak in ovulatory phase, remain high during early luteal phase, and decrease in late luteal phase if pregnancy doesn't occur [1, 2] (Table 1).

Table 1 Production Rate and Serum Concentration of Estrogens in The Menstrual Cycle

Phase	Estrone		17β-Estradiol		Estriol	
	Serum Conc.	Daily Production	Serum Conc.	Daily Production	Serum Conc.	Daily Production
	pg/ml	µg	pg/ml	µg	pg/ml	µg
Follicular	30-100	50-100	40-200	60-150	3-11	6-23
Preovulatory	50-200	200-350	250-500	200-400	-	-
Luteal	50-115	120-250	100-150	150-300	6-16	12-30
Premenstrual	15-40	30-60	40-50	50-70	-	-
Postmenopausal	15-80	30-80	<20	525	311	522

Data taken from [1, 2]

The E2 levels can range from 40- 500 pg/ml depending on the cycling stage of the female. If pregnancy occurs the estradiol levels remain peaked during the entire pregnancy. In the years approaching menopause which have been described as perimenopause and defined as the period, of at least 12 months before cessation of menstrual flow and typically after the age of 45, in which a change in menses (duration, flow rate) occurs with the possible accompaniment of psychological and/or vasomotor symptoms, significant fluctuations in the E2 levels have been reported [27-29].

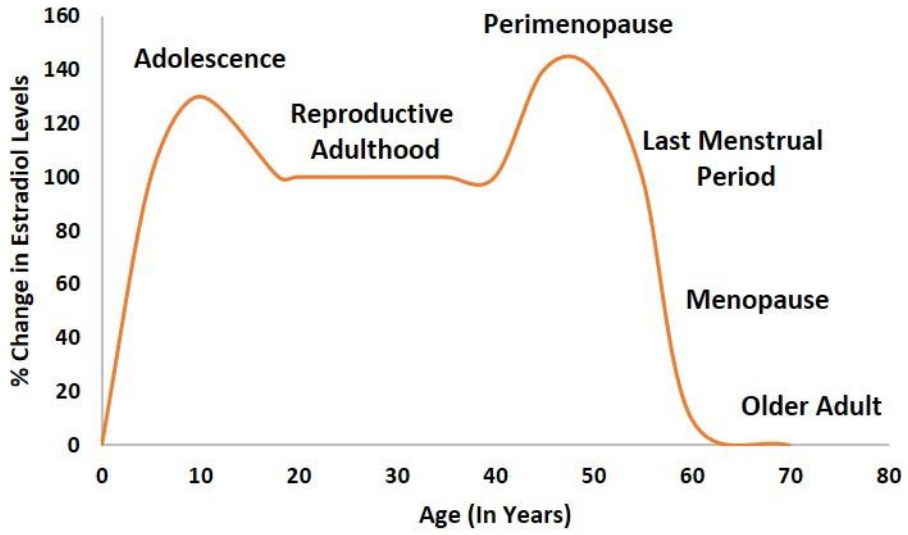

Figure 3. Endogenous Estradiol Levels Fluctuations over Lifetime. Schematic representation of Estradiol levels during the life of an ageing female. Data taken from [5].

Reduced negative feedback and subsequent higher gonadotrophin levels accelerate estrogen production, which then suppresses gonadotrophin and E2 secretion, resulting in the observed oscillations between episodes of hypoestrogenism and hyperestrogenism [30] (Figure 3). The E2 levels tend to decrease significantly when females enter the perimenopausal phase and are reduced by 90% in postmenopausal females [31]. Before menopause, the ratio of E2 to E1 is generally greater than 1. After menopause, this ratio drops as the production of E2 decreases and E1, which is 12 times less potent than estradiol, becomes the predominant estrogen.

A).d) Molecular mechanisms of estrogen action

With the cloning of the first ER cDNA 30 years ago, started the comprehending of the complex molecular mechanisms underlying the diverse physiological actions of estrogens and the multitude of synthetic estrogen ligands. Estrogens induce cellular changes through a number of mechanisms which can be roughly divided into genomic and non-genomic, based on the outcome of cellular events, e.g. modulation of gene expression or activation of signaling cascades, respectively [32] (Figure 4).

A).d).a) Genomic Signaling

A).d).a).a) Direct Genomic Signaling

Direct genomic signaling is considered the classical mechanism of estrogen signaling. The binding of E2 to ERα or ERβ in the cytoplasm of target cells induces a conformational change within the ER that enables receptor homodimerization, translocation to nucleus, and high affinity binding to specific DNA response elements (EREs), which are cis-acting enhancers located within the regulatory regions of target genes [33]. Binding of the ligand to the receptor also triggers recruitment of a variety of coregulators in a complex that alters chromatin structure and facilitates recruitment of the RNA polymerase II transcriptional machinery [34, 35]. In this way, the E2-ER complex acts as a transcriptional activator and, depending on the cell and promoter context, the DNA-bound receptor exerts either a positive or negative effect on expression of the downstream target gene [36].

A).d).a).b) Indirect Genomic Signaling

E2 can also influence expression of genes that do not harbour EREs in their promoter regions [37]. In fact, around one third of the estrogen responsive genes lack ERE-like sequences [38]. In the case of ERE-independent genomic signaling ligand-activated ERs do not bind DNA directly, but rather through protein-protein interactions with other classes of transcription factors at their respective response elements. This mode of action enables activation or repression of target gene expression and significantly broadens estrogen's regulatory influence [36]. For instance, ER activation of IGF-1 and collagenase expression is mediated through the interaction of receptor with Fos and Jun at AP-1 binding sites [39], whereas several genes containing GC-rich promoter sequences are activated via an ER-Sp1 complex [40-42]. Studies show that E2-ER activation of AP-1-responsive elements requires both AF-1 and AF-2 domains of the receptor, which bind and enhance the activity of the p160 components (e.g. SRC-1, GRIP1) of the coactivator complex recruited to the site by Fos/Jun [39]. Other transcription factors that facilitate estrogen signaling also include nuclear factor κB (NFκB) [43, 44], CCAAT/enhancer binding protein β (C/EBPβ) [43], GATA binding protein 1 (GATA1) [45] and signal transducer and activator of transcription 5 (STAT5) [46, 47].

A).d).b) Non Genomic Signaling

The observed rapid biological effects of E2 in the bone, breast, vasculature, and nervous system suggest that estrogens may also elicit nongenomic effects [48-51], possibly through cell-surface ER forms that are linked to intracellular signal transduction proteins. Non-genomic actions are common to steroid hormones and are usually associated with the activation of various protein-

kinase cascades that can eventually lead to indirect changes in gene expression due to phosphorylation of transcription factors.

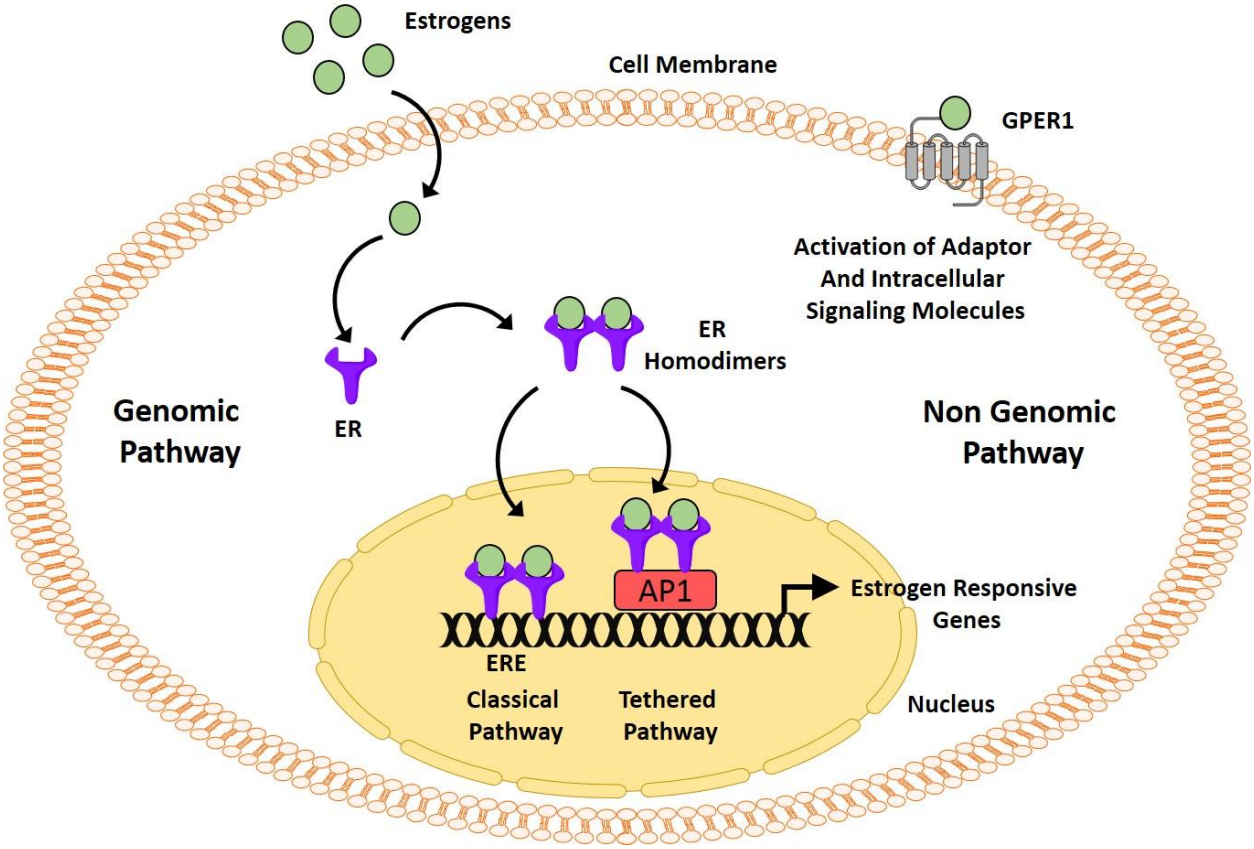

Figure 4. Mechanism of Action of Estrogens. Schematic representation of mechanistically distinct molecular pathways used in the regulatory actions of ERs. **Genomic Pathway**: - The classical (direct) pathway includes ligand activation and a direct DNA binding to estrogen response elements (ERE) before modulation of gene regulation. The tethered pathway includes protein-protein interaction with other transcription factors after ligand activation, and thereby gene regulation is affected by indirect DNA binding**. Non Genomic Pathway**: - In non-genomic pathway, ligand interacts with plasma membrane bound ERs such GPER1, which results in activation of cytoplasmic signaling pathways such as protein kinase C (PKC).

Non-genomic estrogen signaling is most often associated with a subset of membrane bound ER, e.g. GPER1 [52] and certain variants of ERα and ERβ. Binding of estrogens to ERs located at the cell surface can cause mobilization of intracellular calcium [53], stimulation of adenylate cyclase

activity and cyclic adenosine monophosphate (cAMP) production [54, 55], activation of the mitogen-activated protein kinase (MAPK) signaling pathway, activation of the phosphoinositol 3-kinase signaling pathway and activation of membrane tyrosine kinase receptors. The activation of the MAPK signaling pathway by E2 has been extensively studied in several cell types, including breast cancer [56], endothelial [57], bone [58, 59] and neuroblastoma cells [60]. Molecular mechanisms underlying non-genomic estrogen signaling are without a doubt diverse and numerous and may depend on a number of conditions, such as the availability of signal transduction molecules and downstream targets, suggesting a cell type-specific mechanism.

It is highly probable that E2 is able to regulate expression of the same target gene through multiple mechanisms, both genomic and non-genomic. In addition, the same promoter sequence can harbour both ERE as well as response elements associated with other transcription factors. The final gene response therefore depends on multiple factors including a combination of transcription factors present on the gene promoter, expression levels and cellular localization of all three ERs, their numerous coregulators, and signaling components, as well as the nature of the stimuli. Since these variables can differ significantly among various cell types, it is possible that estrogens use distinct signaling pathways depending on the cellular context and in this way ensure very precise and cell-specific regulation of target gene expression.

A).e) Estrogen Receptors

More than 45 years ago, upon observing the specific binding of 17β-estradiol in the uterus, Jensen and Jacobsen concluded that the biological effects of estrogen had to be mediated by a receptor protein [61]. In 1986 the first estrogen receptor, ERα, was cloned [62, 63] and it was assumed that

this receptor was responsible for mediating all of the physiological and pharmacological effects of natural and synthetic estrogens. However, in 1995 and later in early 2000's the discovery of ERβ [64] and GPER1 [65] have forced a re-evaluation of the biology and function of estrogens.

Nuclear signaling of estrogens is mediated through two canonical nuclear estrogen receptors, ERα and ERβ, both belonging to the nuclear receptor (NR) family of transcription factors [66]. Like many other members of the NR family, ERs contain evolutionarily conserved structurally and functionally distinct domains [67] (Figure 5A). The central and most conserved domain, the DNA-binding domain (DBD), is involved in DNA recognition and binding [67], whereas ligand binding occurs in the COOH-terminal multifunctional ligand-binding domain (LBD) [66]. The NH2-terminal domain is not conserved and represents the most variable domain both in sequence and length. Transcriptional activation is facilitated by two distinct activation functions (AF), the constitutively active AF-1 located at the N-terminus of the receptor and the ligand-dependent AF-2 that resides in the C-terminal LBD [68]. Both AF domains recruit a range of coregulatory protein complexes to the DNA-bound receptor. The two ERs share a high degree of sequence homology except in their NH2- terminal domains [69, 70], and they have similar affinities for E2 and bind the same DNA response elements.

A).e).a) ERα

The human ERα gene is located on chromosome 6q25 [71]. It comprises eight exons separated by seven intronic regions and encodes a 595 amino acid protein weighing 66 kDa. Alternative splicing of pre-mRNA results in several splice variants of ERα [72]. For instance ERα46 is encoded by a transcript that lacks the first coding exon (exon 1A) of the ER-α66 gene [73]. In addition ERα36

differs from ER-α66 since it lacks both transcriptional activation domains (AF-1 and AF-2) but it retains the DNA-binding domain, and ligand-binding domains of ER-α66 [74] (Figure 5B).

The ER mRNA has been reported to be highly expressed in epididymis, testis, pituitary, ovary, uterus, mammary gland, placenta, liver, kidney, and adrenal gland [75]. ERα mRNA has been also been detected in central nervous system primarily in the regions of ventromedial thalamic nucleus, ventrolateral thalamic nucleus, lateral and basolateral amygdaloid nucleus, dorsal endopiriform nucleus and throughout lower brainstem and dorsal horn of spinal cord. Lower mRNA expression of ERα has been reported in piriform cortex, cerebral cortex and hippocampus when compared to the expression levels of ERβ [76-78]. Studies have reported a 50% decrease in the spine numbers containing ERα in aged hippocampus when compared to young hippocampus [79] although contradictory reports demonstrating ERα being immune to the effects of age have also been published [80, 81].

ERα signaling has been reported to play a role in physiological functions such as reproduction, bone development and maintenance, cardiovascular tissue and metabolism and mood and behavior [82]. For instance female ERα knockout mice have been shown to be infertile due to a failure to respond to estrogen in the uterus, which is a central organ for reproduction and pregnancy [83]. In addition, bone length and size were found to be significantly decreased in ERα knockout mice when compared with wild type animals. ERα knockout mice have also been found to be diabetic and insulin resistant strengthening the important function of ERα receptors in normal physiological processes.

A).e).b) ERβ

The ERβ gene is located on chromosome 14q23–24.1 [84]. It is composed of eight exons spanning approximately 40 kilobases and encodes a 530 amino acid protein weighing 55 kDa. Several splice variants of ERβ having either extended N-termini or truncations and/or insertions at the C-terminus and in the LBD have been reported. For instance human ERβ cDNA encoding a protein of 530 amino acids was identified in 1998 which was longer than the original rat ERβ clone due to an N-terminal extension, composed of 45 amino acids [85]. In addition to extensions of the N-terminus, three groups have reported cloning of ERβ-503, an isoform with an in-frame insertion of 18 amino acids in the ligand binding domain [86-88]. All ERβ isoforms, 503, 485, and 530, bind to consensus ERE and heterodimerize with each other and with ERα [87, 88].

Transcripts encoding additional ERβ isoforms with variations at the extreme COOH terminus have been found in human testis cDNA libraries [89, 90]. For instance ERβcx is identical to ERβ-530 in exons 1–7, but exon 8 is completely different. Due to the exchange of the last exon, ERβcx lacks amino acid residues important for ligand binding and thus does not bind to E2 and has no capacity to activate transcription of an E2 sensitive reporter gene [90]. Furthermore, ERβcx shows preferential heterodimerization with ERα rather than with ERβ, inhibiting ERα DNA binding. Functionally, the heterodimerization of ERβcx with ERα has a dominant negative effect on ligand-dependent ERα reporter gene transactivation [90]. In total five ERβ isoforms (ERβ 1–5) have been reported [89]. ERβ 1 corresponds to the previously described ERβ-530, and the ERβ 2 variant is most likely identical to ERβcx [90]. However, ERβ 3–5 are novel splice variants with exchanges of the last exon of ERβ-530 for previously unknown exons. As with ERβcx, neither of the novel C-terminal splice variants, ERβ 3–5, can be expected to bind E2 or activate transcription from an ERE-driven reporter, as they all lack amino acids important for ligand binding as well as the core

of AF2. In contrast to what was reported for ERβcx, two of the C-terminal splice variants, ERβ 2 and 3, do bind to a consensus ERE [89] (Figure 5B). ERβ has been reported to be highly expressed in prostate and ovary and moderate expression level of the receptor was revealed in testis, kidney, spleen, uterus, bladder and lungs [75]. In central nervous system ERβ mRNA expression was found in hippocampus, anterior olfactory nuclei, cerebral and piriform cortices, supraoptic and paraventricular nuclei of hypothalamus ventral tegmental area, substantia nigra, pontine nuclei, cerebellum, anterior horn of spinal cord, preoptic area, lower brainstem and pineal gland [76]. ERβ expression has been reported to be modified by age and sex in both rodents and humans [81, 91, 92]. For instance studies have shown a 30% decrease in the ERβ expression levels in female brains while comparing 6 month and 9 month animals [81].

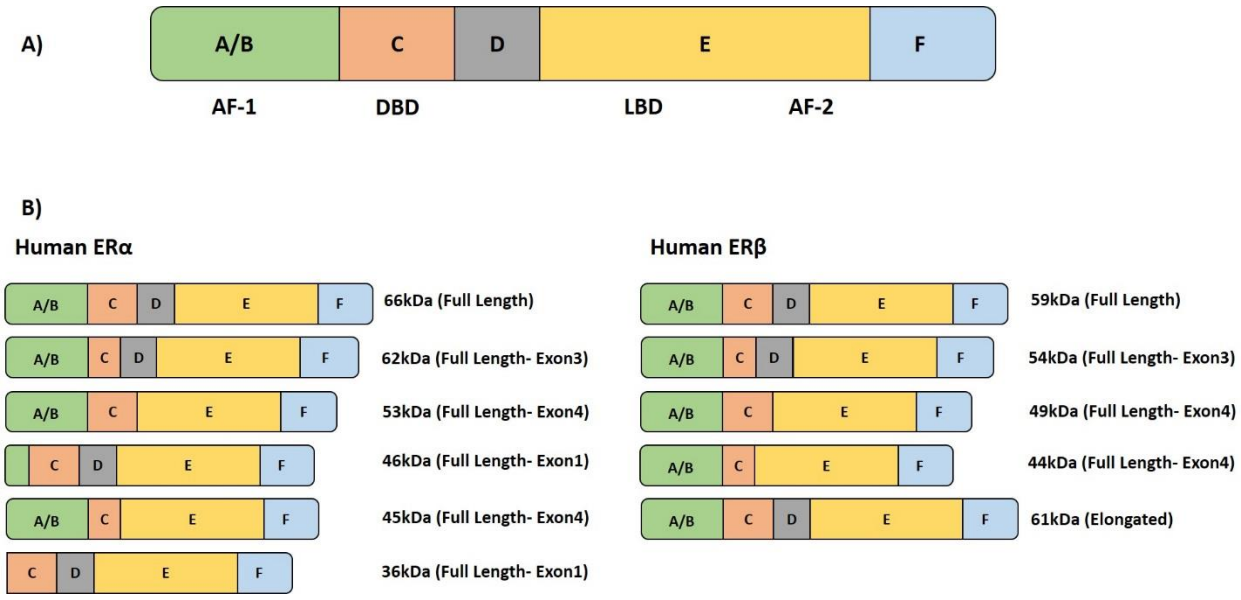

Figure 5. Splice Variants of Estrogen Receptors. A) Schematic representation of domain structure of nuclear receptors. The A/B domain at the NH2 terminus contains the AF-1 site where other transcription factors interact. The C/D domain contains the two-zinc finger structure that binds to DNA, and the C/F domain contains the ligand binding pocket as well as the AF-2 domain that directly contacts coactivator peptides. B) Full length human ERα and ERβ with their different known isoforms.

ERβ signaling has been shown to contribute to reproductive function as ERβ knockout mice have been shown to have increased number of ruptured follicles and reduced number of oocytes[76] resulting in reduced fertility [73]. ERα signaling function has been shown to be important for reproduction whereas ERβ signaling seems to play an important role in brain development. ERβ activation has been shown to increase neuronal survival against neurodegenerative insults in addition to its positive effects on brain development and neural plasticity. For instance ERβ knockout mice have smaller brain sizes at embryonic day 18.5 in addition to the reduced number of migrating neurons and elevated number of apoptotic cells in cerebral cortex [72]. In addition ERβ activation has been demonstrated to increase BDNF protein expression [75, 79, 82] along with a significant increase in the expression levels of synaptic proteins in the hippocampus [77, 78, 83]. Moreover ERβ knockout mice have been reported to exhibit reduced learning acquisition following 17β Estradiol treatment suggesting an important role of ERβ in estrogen induced enhanced learning and memory formation [74].

A).e).c) G-protein coupled estrogen receptor-1 (GPER1)

In addition to canonical ERs, the above mentioned finding of rapid biological effects of E2 in the bone, breast, vasculature, and nervous system suggested that estrogens may also elicit nongenomic effects. The extensive research lead to the discovery that GPR30 can bind with high affinity to E2 and is responsible for the reported effects and thus was renamed as GPER1. GPER-1 is a seven-transmembrane-domain receptor [93] detected broadly in numerous human tissues, such as brain, breast, prostate, ovary, placenta, subcutaneous adipose, visceral adipose, arteries, vessels, heart, liver, lung and intestine tissues [94]. GPER-1 is a member of GPCR superfamily, which is structurally unrelated to the classical ERα and ERβ. There are four transcriptional variants

encoding 375 amino acids composing seven transmembrane proteins [95]. Classical GPCR are cell membrane proteins which bind their ligands at cell surface. But GPER-1 binding domain exists inside the plasma membranes and the endoplasmic reticulum [52, 96, 97]. The biological functions of GPER-1 might be associated with cell types and its location. Estradiol binds to GPER-1 with a high affinity while estrone and estriol have very low binding affinities [98, 99].

Ligands for instance E2 bind to the GPER-1 on endoplasmic reticulum where it activates β and γ subunits of heterotrimeric G proteins and subsequently activate both Src and adenylyl cyclase (AC) leading to the intracellular cAMP production [100]. The phosphorylation of Src induces matrix metalloproteinase (MMP) production, which cleaves pro-heparanbound epidermal growth factor (pro-HB-EGF) releasing free HB-EGF. HB-EGF binds to the EGFR leading to activation of multiple molecules such as Ras, PI3K, AKT, and Erk1/2. GPER-1 also binds to the G couple protein α_s subunit and activates the AC, and CAMP pathways [100].

Having so diverse physiological functions, the dysfunction of estrogenic signaling pathways have been implicated to play a role in the pathophysiology of a number of diseases such as cancer [101, 102], osteoporosis [103], neurodegenerative diseases (stroke [104], Parkinson's disease [105], Alzheimer's disease [106]), cardiovascular diseases [107], mood disorders [108] and autoimmune diseases [109]. In this thesis investigation into the role of estrogens in depression and Alzheimer's disease will be conducted.

Purpose of the Study

Depression is one of the most commonly diagnosed mental disorders and a major leading cause of disability, contributing to an overall 52 million years lived with disability per year across the globe [110]. Epidemiological findings point to female predominance in prevalence, incidence and morbidity risk of depressive disorders. Females have been reported to have twice the risk of developing depression when compared to their counterparts and a lifetime prevalence of 21.3% compared to 12.7% in men [111]. This risk is further compounded in females transitioning into the menopausal phase. Studies have reported that a female entering menopause with no history of depression has four times the risk of developing depression when compared to a female which remains premenopausal [112]. In addition depression has been reported to be a risk factor for developing several diseases such as stroke, ischemia, type 2 diabetes, atherosclerosis and Alzheimer's disease (AD). Depression has been reported to occur in 50% of AD patients [113, 114] and has been reported to be an independent risk factor for developing AD. While several clinical studies have supported these findings, the mechanistic pathway underlying the interaction between depression and AD is unknown.

For the first part of our study we hypothesized that perimenopause-related estrogen signaling perturbation leads to BDNF and 5-HT signaling deficits that result in at-risk phenotype for depression; this signaling dyshomeostasis could be exacerbated by environmental stressors, leading to the onset of depression. In our study we focused on the signaling aspects of this hypothesis and thus aimed to investigate the role of ERα and ERβ in the regulation of BDNF and 5-HT signaling in female brain in the following three aims.

Specific Aim 1:- To determine the role of estrogen signaling in the regulation of BDNF signaling in female brain.

We used 6-month-old, female, ERα$^{-/-}$ and ERβ$^{-/-}$ global knockout mice model to analyze the expression levels of BDNF and TrkB in cortex, hippocampus and hypothalamus. We corroborated our findings using two different ERβ$^{-/-}$ knockout rat models. In addition we agonized ERβ in primary hippocampal neurons in order to analyze BDNF and TrkB expression levels along with expression levels of downstream synaptic and neurogenic proteins.

Specific Aim 2:- To determine the role of estrogen signaling in the regulation of 5HT signaling in female brain.

We used two ERβ-/- knockout rat models, one with a targeted deletion of exon 3 and other with a targeted deletion of exon 4 and looked at the expression levels of 5HT1A and 5HT2A receptor expression.

Specific Aim 3:- To determine possible interaction of BDNF-5HT signaling pathways in female brain.

Hippocampus is the region in which the actions and functions of both 5-HT and BDNF have been shown to converge and overlap. In order to examine the possible interaction between these two pathways, we cultured primary hippocampal neurons and selectively agonized TrkB and 5HT2A receptors in two independent experiments. 5HT2A and BDNF protein expression levels were examined via immunoblot analyses.

For the second part of our study we hypothesized that ApoE isoforms differentially regulate BDNF-5HT2A signaling pathway in female brain, which underlies their discrete role in the etiology of depression. In addition we also hypothesized that enhancing ERβ activity sustains brain

BDNF-5HT2A signaling, which, as a result reduces the risk of developing depression thereby further reducing the risk of developing AD. We examined this hypothesis in the following 2 aims.

Specific Aim 4:- To determine the role of ApoE in the regulation of BDNF 5HT2A signaling and synaptic plasticity in female brain.

The fourth specific aim of the study was to determine the probable role of ApoE in the regulation of BDNF 5HT2A signaling and expression levels of synaptic markers in female brain. We used 6-month-old human ApoE targeted transgenic female mice and analyzed expression levels of BDNF and 5HT2A receptor in cortical region of the brain. In addition we also examined the expression levels of several pre- and post-synaptic proteins in order to elucidate the memory and cognition related regulation in a genotype dependent manner.

Specific Aim 5:- To determine how ERβ agonism affects BDNF 5HT2A signaling and synaptic function in female brains of three APOE genotypes.

The final aim of the study was to determine whether chronic ERβ agonism can positively modulate the probable hypothesized alterations in BDNF-5HT2A signaling and synaptic plasticity in ApoE4 brains. The female transgenic animals were administered with a control diet or a phyto-β-SERM-supplemented diet for 3 months and sacrificed at 6 months of age and expression levels of BDNF, 5HT2A, and pre- and post-synaptic proteins were analyzed and compared between untreated and treated groups.

Chapter 1

Estrogen Receptor β (ERβ) Regulates BDNF-5HT2A Signaling in Female Brain: Implications for Perimenopausal Depression

1.1 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 most cases occurring during the endocrine transition from pre- to perimenopause. Estrogen receptors (ERs) have been implicated in the pathophysiology of mood disorders including depression; however, the underlying mechanisms are poorly understood. In this study, we sought to investigate the role of ERs in the regulation of brain-derived neurotrophic factor (BDNF) and serotonin (5-HT) signaling; two pathways that have been hypothesized to be interrelated in the etiology of depression. Our analyses in ERα and ERβ knockout ($ER\alpha^{-/-}$ 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 not statistically significant in the cortex and hypothalamus. Further analyses in primary hippocampal neurons indicated that ERβ agonism significantly enhanced BDNF-TrkB signaling and the downstream cascades involved in neurogenesis and synaptic plasticity. Our subsequent study in $ER\beta^{-/-}$ rat models demonstrated that ERβ deficiency was associated with a significantly elevated level of 5HT2A receptors but not 5HT1A receptors in rat hippocampus, indicating ERβ negatively regulates 5HT2A receptors. Our additional analyses in primary hippocampal neuronal cultures revealed a significant association between BDNF and 5HT2A pathways, and the data showed that TrkB activation downregulates 5HT2A receptors whereas activation of 5HT2A receptors had no effect on BDNF, suggesting that BDNF/TrkB is an upstream regulator of the 5HT2A receptor signaling pathway. Collectively, these findings implicate that the reduction in ERβ signaling homeostasis during perimenopause leads to dysregulation of BDNF-5HT2A receptor network and

weakened synaptic plasticity, which together predispose the female brain to a susceptible state for depression. Timely intervention with an ERβ-targeted modulator could potentially attenuate this susceptibility and reduce the risk or ameliorate the clinical manifestation of this brain disorder.

1.2 Introduction

1.2.1 Depression

Depression is a chronic, reoccurring neuropsychiatric disease that currently affects 350 million people worldwide and 6.7% of Americans each year [115]. According to the Fifth Edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM V), depression is characterized by the presence of two key symptoms: depressed mood and loss of pleasure. These key symptoms are accompanied by a set of sub-symptoms including, but not limited to, insomnia or hypersomnia, psychomotor agitation or retardation, and diminished concentration which could be present for more than two weeks [116]. The latest Global Burden of Disease Study (GBD), a research study conducted in 2013 by a consortium of seven academic partners (Harvard University; The Institute for Health Metrics and Evaluation at University of Washington, Seattle; John Hopkins University; University of Queensland; Imperial College London; University of Tokyo; and World Health Organization), which systemically quantified the prevalence of 301 acute and chronic diseases across 188 countries, determined that depressive disorders were a major contributor of years lived with disability (YLDs) irrespective of the developmental status of a country. Depression was reported to be the leading cause of YLDs in 56 countries, the second leading cause in 56 countries and the third leading cause in 34 countries contributing to an overall 52 million YLDs per year across the globe [110].

In addition to these data, studies have shown comorbidity between depression and other major diseases such as coronary heart disease (CHD), myocardial infarction (MI), type 2 diabetes (T2DM), stroke, and Alzheimer's disease (AD). For example, studies have indicated that people with a history of depression have a 34% higher risk of developing stroke than non-depressed

patients even after the adjustment of potential confounding factors such as hypertension and diabetes [117, 118]. Moreover, it has been shown that depressed patients are two to three times more likely to develop CHD than non-depressed patients [119] and, while controversies have existed in the past, it has recently been proposed that depression is an independent risk factor for CHD [120]. The HUNT 2 study showed that the two episodes of mixed symptoms of anxiety and depression increased the risk of acute MI by 52% [121]. Another study by Knol et al. indicated that depressed adults have a 37% increased risk of developing T2DM when compared with non-depressed individuals [122]. In addition to disease comorbidity, it has also been shown that depressive patients are noncompliant with medical treatment for other chronic diseases [123]. Furthermore, depression and depression-associated comorbidities are accompanied by an extensive economic burden. A recent research study by Greenberg et al. estimated that the incremental economic burden of depression increased 21.5% from 2005 to 2010 and totaled approximately $210.5 billion [124]. The composition of total costs was attributable to direct costs (48-50%), work related costs (48%-50%) and suicide related costs (5%). The total of the direct costs, which primarily include outpatient and inpatient medical services, was $98.9 billion while depression related suicide cost was estimated to be around $9.7 billion in 2010. Moreover, it was estimated that the average person living with depression lost approximately 32 additional work days per year when compared with non-depressed workers; a deficit that totaled approximately $102 billion in 2010 [124]. With all factors considered, it is speculated that the depression-related world economic burden will increase to around $500 billion in year 2023. With an increase of such magnitude in health and economic burden, there comes a huge need of devising better treatment options for the disorder.

1.2.2 Antidepressants

The epoch of antidepressants started with the discovery of Isoniazid, a hydrazine derivative that was used as an anti-tubercular agent. With the aim of improving efficacy and reducing side effects, scientists at Hoffman La Roche USA made structural modifications to the compound and synthesized iproniazid. An observation by doctors at Staten Island's Seaview hospital reported stimulating side effects in tuberculosis patients after the administration of iproniazid. The patients showed drastic improvement in their social behavior, appetite, sleep behavior and rigor after use of the drug. A subsequent clinical research study by Loomer, Saunder and Kline corroborated these observations and reported an improvement of depressive symptoms in 70% of patients administered iproniazid [125]. In 1952, Zeller discovered that the mood elevation or sometimes "dancing in the hall effect" seen in depressed patients resulted from inhibition of an enzyme known as monoamine oxidase enzyme (MAO) [126]. The mechanistic action of these hydrazine compounds was better understood when it was shown that the inhibition of MAO resulted in an increase of serotonin (5-HT) levels in the brain, thus improving mood symptomatology [127]. The finding was further supported when iproniazid reversed reserpine induced depression [128, 129], as reserpine induces depletion of biogenic amines. Unfortunately, iproniazid and hydrazine agents proved to be hepatotoxic, nephrotoxic and hypertensive, leading to the removal of these compounds from the market. However, these findings paved a new pathway for the development of site-directed drugs specifically aimed at increasing 5-HT levels in the brain. Fluoxetine, the first selective 5-HT re-uptake inhibitor (SSRI) to be developed, potently increased 5-HT levels in the brain without eliciting the side effects caused by MAO inhibitors and tricyclic reagents [130]. Following the trail of SSRIs, several antidepressants have been introduced for the treatment of depression. At present, SSRIs and selective norepinephrine reuptake inhibitors (SNRIs) are the

most commonly prescribed drugs for the treatment of depression. An increase in the knowledge of brain function and the neurobiology of depression has substantially increased the awareness that biogenic amines alone are not responsible for pathophysiology of depression. Thus new targets such as NMDA receptors, GABA and glutamate receptors, and neurotrophic factors have been targeted to improve depressive symptomatology. Although significant progress has been made in development of antidepressants, current antidepressants yield a therapeutic efficacy in only 60% of depressed patients [131]. Moreover, the mechanism of action underlying the therapeutic effect of antidepressants is currently unknown. Taken together, these facts demonstrate the importance of a) understanding the mechanistic etiology of depression and b) developing novel methods to treat depressed patients based on that understanding.

1.2.3 Depression in Females

Sex differences exist in the susceptibility and progression of depression. Research has indicated that females are 2.5 times more likely to experience major depression than men [132] with an estimated life time prevalence of 21.3% [133-136]. This sex-based discrepancy has been linked to several theories or hypotheses. According to one hypothesis, the cyclic fluctuations of gonadal steroid hormone levels occurring in women during the perimenopausal phase exposes females to a higher risk of depression [137]. Another theory, the "estrogen withdrawal theory," proposes that the hypoestrogenic state of women in the perimenopausal phase leads to the onset or worsening of depressive symptoms [137]. These theories have been substantially supported by multiple research studies that deemed women to be most at risk for developing depressive disorders during perimenopausal phase. A study conducted by Bromberger et al. in 2001 reported that early perimenopausal women had higher odds of reporting depression compared with post-menopausal

women [138]. SWAN study reported that the risk for depressive symptoms increased irrespective of demographic and psychosocial factors with onset of perimenopause and remained elevated throughout post-menopause [139]. A study from Freeman and colleagues showed that women were 4 times more likely to develop depression during the transition from pre to perimenopause than women who were premenopausal [112]. The subjects in the study had no previous episodes of any depressive disorder thus abolishing some controversies tied to the issue and providing enough proof to conclude that the endocrine transition, specifically the transition from pre- to perimenopause, increases the risk of depression. A study by Maartens et al. showed that the transition from pre to perimenopause was significantly related to a high increase in Edinburgh Depression Scale (EDS) score and concluded that the transition itself is independently related to an increase in depressive mood symptomatology [140]. The hormonal changes that occur during perimenopausal onset are now well associated with an increased vulnerability for psychiatric disorders, such as depression, however, the mechanistic understanding of the phenomenon is poorly understood.

1.2.4 Role of Estrogens in treatment of Depression

As the loss of ovarian sex hormones appeared to be integrally involved in the onset of depression, hormone supplementation in the form of conjugated estrogens was the obvious choice to treat mood disorders. Estrogen therapy (ET) indeed showed promising effects in the treatment of depression and mood-related symptomatology [141-143]. The first placebo controlled trial of estrogens in the treatment of depression was conducted in 1979 by Klaiber and colleagues, who treated a group of depressed females with increasing doses of oral conjugated estrogens. The study showed a benefit of high-dose estrogen over a 3-month treatment period when compared to vehicle

treated group and concluded that estrogen administration lead to a significant improvement in the depressive phenotype regardless of the diagnosis [144]. In addition Schmidt et al also evaluated beneficial effects of estradiol under placebo-controlled conditions in perimenopausal women and reported a partial or full therapeutic response in 80% of the subjects in the estradiol group but only 22% in the placebo group [145]. The results were replicated in another placebo controlled trial conducted in a mixed sample of 50 perimenopausal depressed women which revealed remission in 68% of women in the estradiol group versus 20% in the placebo group. Moreover, the antidepressant benefit of estradiol was found to be sustained after a 4-week washout period with few to none adverse effects [142]. An open label study from Cohen et al. demonstrated similar results and reported a remission rate of 60% in perimenopausal depressed participants who were administered 17β-estradiol (E2) [146]. In addition it has also been demonstrated that ET is more efficient in perimenopausal women when compared to post-menopausal women [147]. However, despite these findings, ET was shown to increase the reoccurrence of endometrial cancer [148] and venous thromboembolism in women with previous episodes of the disease. Additionally, the Women's Health Initiative (WHI) study demonstrated that ET increased the risk of non-fatal cardiovascular diseases and breast cancer in older women [149]. Thus, due to the substantial risks associated with ET [150, 151], it is imperative that research focuses on the discovery of alternative treatments that provide similar efficacy without the adverse effects [152].

Estrogens have also been shown to modulate the serotonin system which is shown to be involved in the etiology of depression. In general, acute and chronic estradiol administration in ovariectomized rats increases serotonin and its metabolite 5-HIAA in various brain regions, including the dorsal raphe, striatum, medial preoptic nuclei, and ventromedial and cortical amygdaloid nuclei, suggesting increased 5-HT turnover in these areas [31, 144, 153-155]. Estrogen

has also been shown to increase serotonergic postsynaptic responsivity and both the number of serotonergic receptors as well as the transport and uptake of the neurotransmitter. Specifically, studies of 5-HT1A receptor function have suggested that, through unidentified mechanisms, estrogen causes sensitization of postsynaptic 5-HT1A receptors in the hippocampus [156, 157] but desensitization of presynaptic 5-HT1A receptors in the dorsal raphe [158]. Consistent with these findings, behavioral study of chronic estradiol treatment in ovariectomized rats found that estradiol decreased the hyperphagia caused by 8-OH-DPAT (a 5-HT1A agonist), suggesting a decrease in presynaptic receptor activation [159]. Further, treatment with estrogens appear to facilitate down-regulation of 5-HT2 receptors during treatment with antidepressants. For example, Kendall et al. showed that abrupt withdrawal of estrogen by surgical ovariectomy in the rat abolishes antidepressant induced down-regulation of 5-HT2 receptors and that replacement of estrogen reverses the effect [160]. In addition to modulating serotonin synthesis via regulation of tryptophan hydroxylase type 2 gene expression, increasing serotonin 2A receptor binding, and interfering with extracellular serotonin clearance, estradiol diminishes serotonin catabolism by reducing MAO-A levels, messenger RNA expression levels, and enzyme activity [161, 162]. In general, estrogen appears to function as an agonist of the serotonergic system and produces many of the same effects as antidepressants on the serotonergic system [163].

A large body of evidence indicates that depression is associated with reduced levels of BDNF and antidepressant treatment alleviates depressive behavior by increasing the expression level of this neurotrophin in hippocampus. Interestingly, estrogens have been shown to regulate the expression of the BDNF mRNA and protein levels especially in hippocampus. In gonadally intact animals, significant fluctuations in the BDNF mRNA levels in CA1 and CA3/4 regions of the hippocampus were detected across the estrous cycle [164]. Moreover ovariectomy has been shown to reduce

BDNF mRNA and protein levels all subregions of the hippocampus except CA1 (CA2 by 38%, CA3 by 44%, CA4 by 39%, and dentate gyrus by 37%) [165]. In addition estrogen replacement in young adult, ovariectomized, female rats has been reported to increase BDNF expression in hippocampus, cortex and olfactory bulb [165]. More specifically significant increase in BDNF mRNA and immunolabeling was detected in the dentate granule cell layer, CA1 and CA3 region of the hippocampus of acutely E2 treated animals when compared with the vehicle treated animals [166]. Similarly administration of 10ug E2 for 14 days lead to 73.4%, 28.1% and 76.9% increase in BDNF mRNA in dentate granule cell layer, CA1 region and CA3/4 region when compared to vehicle treated animals [164]. The results were replicated by a number of studies which reported that estrogen treatment significantly elevated BDNF mRNA and protein levels in the piriform cortex and hippocampus when comparted to vehicle treated group in ovariectomized rats [167] ,the results of which were replicated in a similar study conducted in prairie voles [168]. Similar to results in young female rats, estrogen treatment in ovariectomized aged (23-24 months) female rats, has been found to increase BDNF expression levels in the entorhinal cortex and hippocampus [169].

In vitro studies have further added to the line of evidence. For instance, in dissociated hippocampal cultures, E2 downregulates the expression of BDNF in GABAergic neurons to 40% of control within 24 h of exposure, and the downregulation returns to basal levels within 48 h. This GABAergic dysfunction results in an increase in excitatory tone in pyramidal neurons, and leads to a 2-fold increase in dendritic spine density [170]. Recently, it was reported that E2 increases protein levels of BDNF in hippocampal slice cultures [171], the results of which were replicated in midbrain cultures [172]. Remarkably, E2 induces the release of BDNF in dentate gyrus granule

cells in hippocampal slice cultures, and E2-dependent synaptogenesis was induced via secreted BDNF [173].

The BDNF gene contains a sequence with close homology to the estrogen response element and estrogen-ligand complexes are capable of binding this sequence and protecting it from DNase degradation [174]. The presence of this site in BDNF gene for estrogen receptor complexes to stabilize DNA during transcription, especially in genes with long intronic sequences, such as the one that codes for BDNF suggests potential genomic interaction between estrogen and BDNF, thus explaining the observed regulation.

Although there are many studies addressing the relationship between estrogen and BDNF expression levels and there is indeed an interesting postulated underlying mechanism, future studies should clarify the detailed interactions between estrogen and BDNF-mediated neuronal function in addition to elucidating the molecular mechanisms underlying estrogen controlled BDNF expression.

1.2.5 Estrogen Receptors in Depression

Both ERα and ERβ have been highly implicated to play a role in the underlying effects mediated by ET in the treatment of depression. The studies have shown that three-week-postpartum rats treated with E2 and PPT (ERα agonist) spent significantly longer times in the open arms of the elevated plus maze than vehicle treated 3-week-postpartum rats. In addition, three-week-postpartum rats treated with E2 and PPT displayed a significantly shorter immobility time than control treated animals during the forced swim test. The study also revealed a significant increase in the expression levels of BDNF and TrkB protein levels in preoptic and central and medial

amygdala of PPT treated animals when compared to vehicle treated animals [80]. Parallel to these studies, studies utilizing the forced-swim test, which models anxiety and depression-like behavior, have shown that ERβ is the primary regulator of E2-induced antidepressant effects [175]. Moreover, ovariectomized (OVX) rats administered E2 or the ERβ-specific agonist DPN showed more central entries in the open field test, more open-arm duration in the elevated plus maze, and less immobility duration in the forced-swim test compared with rats administered vehicle or the ERα-specific agonist PPT [176]. Studies have also revealed that administration of ERβ-specific but not ERα-specific selective estrogen receptor modulators (SERMs) to OVX rats decreased anxiety and depressive behavior [177-180]. These findings were further supported by a study which showed that the observed E2-induced antidepressant effect is absent in ERβ knockout mice [175, 181].

In addition to these two canonical estrogen receptors, GPER1, has also been implicated to play a role in the etiology of depression. Studies have shown that G1, GPER1 agonist exhibited antidepressant properties in a mouse model of depression, where it reproduced the effects of 17β-estradiol, which were inhibited by the GPER1-selective antagonist G15 [182]. Although research studies have tried to unravel the underlying molecular mechanism of the antidepressant action of estrogen therapy, the results are contradictory at the best thus asserting the need for further investigation.

1.2.6 Serotonin and Receptors

Serotonin (5-hydroxytryptamine; 5-HT) was named after a Latin word “serum” and Greek word “tonic”. It was first isolated and characterized in 1948 by Maurice Rapport and Irvine Page [183-

185]. 5-HT is a biogenic monoamine similar to dopamine, norepinephrine and histamine. 5-HT is integrally involved in a myriad of physiological processes in the central nervous system (CNS) and periphery such as reward [186], thermoregulation [187], cardiovascular regulation [188], memory [189-192], cognition [192], emotion [193], locomotion [186], reproduction [194] and pain [195]. Thus, the dysfunction of serotonergic systems is implicated in several human pathologies such as depression [196], schizophrenia [197], autism [198], anorexia [199], irritable bowel syndrome [200]. 5-HT is largely synthesized (90%-95%) in gastrointestinal tract mainly in the enterochromafin cells [201]. Within the CNS, 5-HT is produced and stored in presynaptic neurons found in nine clusters located mostly in raphe nuclei of the midbrain, pons, and medulla [202]. The serotonergic axons arborize over motor, somatosensory and limbic areas [202], thus regulating multiple brain functions such as cognition, emotion, and motor functions [203]. 5-HT is produced by a set of hydroxylation reactions followed by a decarboxylation reaction, with the hydroxylation reaction being the rate-limiting step (Figure 5). Tryptophan hydroxylase (TPH) belongs to the family of tyrosine hydroxylase, phenylalanine hydroxylase, and aromatic amino acid hydroxylases which catalyze key steps in important metabolic pathways [204].

TPH exists as two isoforms deemed TPH1 and TPH 2 (sequence identity 70%) encoded by genes located on chromosome 11 and chromosome 12, respectively [204]. TPH1 is primarily expressed in the periphery whereas TPH2 is exclusively expressed in CNS. In the CNS, serotonergic neurons uptake tryptophan which is hydroxylated by TPH2 to 5-hydroxytryprophan. 5-Hydroxytrytophan is further decarboxylated by aromatic L amino acid decarboxylase (AADC) to from 5HT [205]. 5-HT is then stored in presynaptic vesicles and docked at the nerve terminals where it awaits an action potential. Following synaptic transmission, excess 5-HT is taken back into the presynaptic neuron by the 5-HT transporter (SERT) and metabolized by the outer mitochondrial membrane

enzyme MAO-A, which occurs as two molecular subtypes called MAO-A and MAO-B. MAO subtypes differ in substrate specification with MAO-A preferentially oxidizing 5-HT, norepinephrine and dopamine whereas MAO-B oxidizing phenylethylamine and dopamine [206]. MAO-A converts 5-HT to 5-hydroxyindole acetaldehyde (Figure 6).

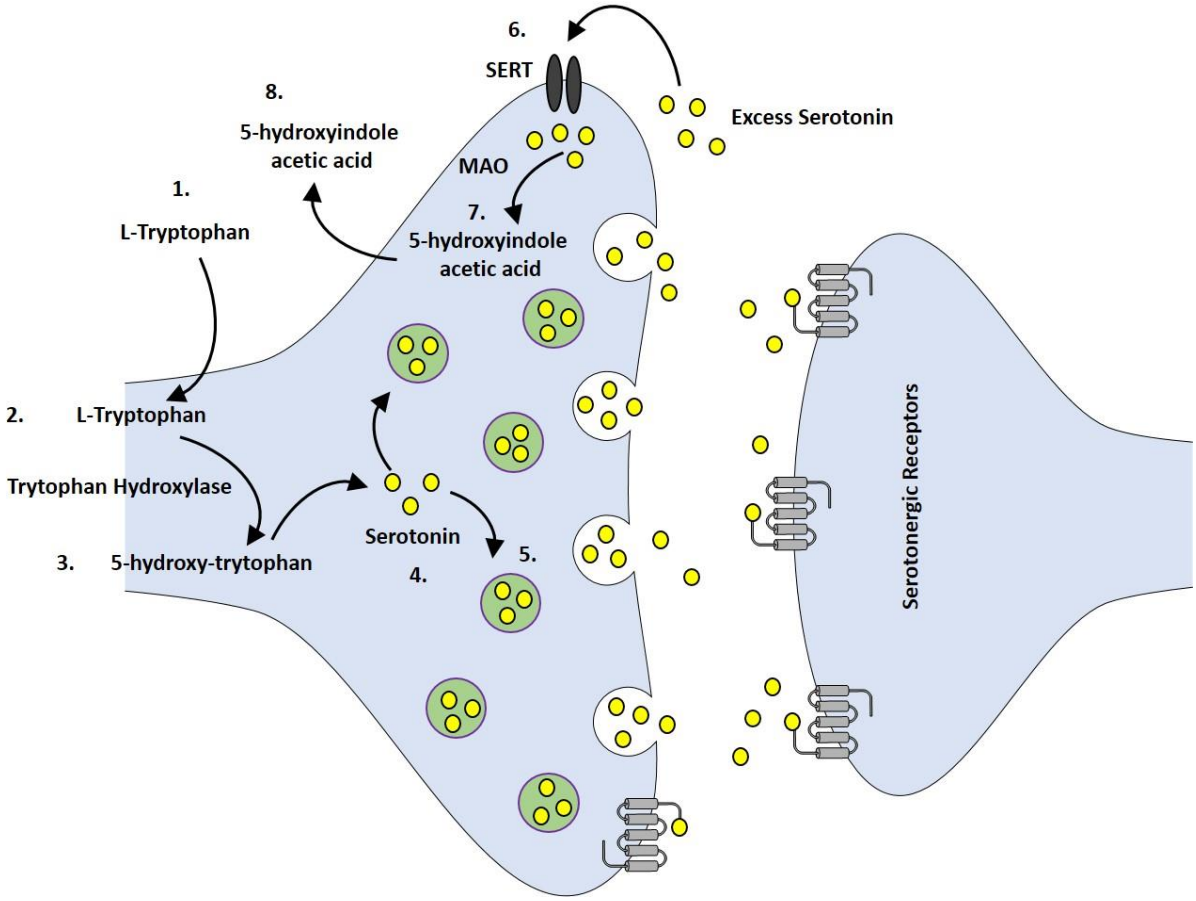

Figure 6. Synthesis and Metabolism of Serotonin. 1) Dietary tryptophan, substrate for serotonin synthesis is actively taken into the neuron. 2) Trytophan is hydroxylated by tryptophan hydroxylase to form 3) 5-hydroxy tryptophan which is immediately decarboxylated by aromatic L amino acid decarboxylase to from 4) serotonin. 5) Newly formed serotonin is then packed into vesicles and transported to synaptic terminal for release. 6) After the release excess serotonin is removed from synaptic cleft by serotonin transporter (SERT). Metabolism of excess serotonin involves deamination by monoamine oxidase enzyme (MAO) to from 7) 5-hydroxyindole acetic acid, which is then 8) excreted out of the cell body.

This metabolite is rapidly oxidized by mitochondrial aldehyde dehydrogenase (ALDH2) to 5-hydroxyindole acetic acid (5HIAA); the major excreted metabolite of 5-HT [207] that is currently used to measure the level/rate of 5-HT turnover [208-210].

5-HT is central to the monoamine hypothesis of depression which states that the pathophysiologic basis of depression is the depletion in the level of key monoamines such as norepinephrine, dopamine, and 5-HT [211, 212]. Several studies have reported that depressed patients have reduced levels of 5HIAA in the cerebral spinal fluid (CSF) indicating a reduced 5-HT turnover in depressed patients. Research from Mann et al. demonstrated that CSF 5HIAA levels were lower in depressed patients with a history of suicides when compared to non-suicidal depressed patients [213]. Moreover, depressed patients administered fenfluramine (a serotonergic stimulator that results in the release of adrenocorticotropic hormone (ACTH) and prolactin from the pituitary gland) generally have reduced prolactin release while depressed patients in remission have a blunted prolactin release comparable to acutely depressed patients [214]. Studies from Heninger et al. and Golden et al. demonstrated similar results using tryptophan and clomipramine to induce release of prolactin [215, 216]. To further corroborate these findings, reduced platelet 5-HT uptake has been reported in medication-free depressed patients compared to normal controls [217, 218]. Furthermore, the development of antidepressants such as monoamine oxidase inhibitors (MAOIs) and SSRIs implicated the undeniable role of 5-HT in depression and showed that increased 5-HT bioavailability in the synapses and hence increased 5-HT function is indeed a starting step to relieve the symptoms of depression [219].

5-HT induces its wide range of actions through a myriad of receptors categorized into seven different classes. Currently 14 different 5-HT receptors have been identified and all but one (5HT3) are members of G Protein Coupled Receptor (GPCR) family. The expression and function of these

receptors have also been heavily studied in an attempt to unravel the etiology of depressive disorders. Post-mortem studies have shown dysfunction of 5HT1A receptor binding in the brains of depressed patients compared to control subjects [220]. One study indicates a significant decrease in the number of 5HT1A receptors and lowered receptor affinity in the hippocampus and amygdala of depressed patients when compared to healthy controls; a finding that was reversed in subjects treated with antidepressants [220]. Numerous additional studies have further corroborated these results by indicating reduced 5HT1A auto-receptor binding in dorsal raphe nuclei [221] and reduced 5HT1A mRNA expression levels in dorsolateral prefrontal cortex and hippocampus [222]. 5HT1A receptor agonist-induced activation of adenylyl cyclase (AC) and phosphoinositide-3-kinase (PI3K) has been also reported to be reduced in depressed suicide patients compared with healthy control subjects [223]. In addition to these results, positron emission tomography (PET) studies by Drevets et al. revealed a reduction in 5HT1A receptor binding in midbrain raphe (41.5%) and medial temporal cortex (26.8%) in depressed patients when compared to healthy controls [224]. Hirvonen et al. reported a similar decrease in 5HT1A receptor availability ranging from 9% to 25% in most of the brain regions in medication naïve patients with major depression disorder [225]. A number of other researchers found a similar decline in binding potential of 5HT1A receptor in midbrain and raphe nuclei region of elderly and female patients [226, 227].

5HT2A receptor, another 5-HT receptor implicated in depression, has been shown to be increased in suicide victims when compared to normal subjects [228]. A radiolabeled binding study from Mann et al., which used [3H]spiperone as a 5HT2A ligand, reported that the binding potential of 5HT2A receptors is increased in post-mortem brains of suicidal depressed patients compared to healthy controls [229]. These results have been replicated in several studies that used different 5HT2A ligands but reported a similar increase in the binding potential of 5HT2A receptors

primarily in the prefrontal cortex of depressed patients compared to healthy control subjects [230]. Additionally, subjects with high scores on the dysfunctional attitude scale exhibit increased 5HT2A binding in frontal cortex; a finding that was independently confirmed in a study in euthymic, medication free, depressed patients [231]. Protein and mRNA expression studies have further corroborated these results. Research studies by Pandey et al., Escriba et al., and Shelton et al. showed increased 5HT2A protein/mRNA expression in subjects suffering from major depressive disorder compared with healthy controls [230, 232, 233]. Chronic administration of antidepressants decreases the increased levels of 5HT2A receptors in frontal cortex implicating a possible role of 5HT2A receptor signaling in antidepressant function [234, 235]. Selective 5HT2A antagonists have been shown to produce antidepressant effects using forced-swim test, sucrose preference test, social interaction and open field test as behavioral responses [236]. Although 5-HT signaling is highly implicated in depression, contradictory reports [237-242], and the inability of the monoamine hypothesis to explain certain clinical findings such as therapeutic lag [243] and intermediate efficacy of antidepressants in patients [244] have shifted the research focus towards new and more relevant hypotheses of depression such as neurotrophic hypothesis [245], neurogenesis hypothesis [246] and glutamate hypothesis of depression [247].

1.2.7 Brain-Derived Neurotrophic Factor

Brain derived neurotrophic factor (BDNF) is a 119-amino-acid basic peptide which was first isolated in 1982 from pig brain [248]. It belongs to the neurotrophin family of growth factors consisting of nerve growth factor (NGF), neutrophin-3, neutrophin-4, neutrophin-6 and neutrophin-7 [249]. BDNF is initially synthesized as a precursor protein (proBDNF) which is first cleaved endoproteolytically by PC/furin (intracellular) or plasmin (extracellular) and then by

exoproteases (carboxypeptidase for COOH-terminal or aminopeptidase for NH_2-terminal) to release the mature form of BDNF (mBDNF) [250] (Figure 7).

mBDNF has been proven to be a central regulator of neuronal survival, development and function [251-254]. It has been shown that mBDNF is essential for neuronal transmission, plasticity [253, 255-258], modulating axon arborization and morphology [259, 260], dendritic arborization and morphology [261, 262] and spine density and morphology [263, 264]. mBDNF exerts its effects by binding to either tropomyosin receptor kinase B (TrkB, high affinity) or p75 receptor, leading to the initiation of different signaling cascades and thus different biological effects (Figure 8).

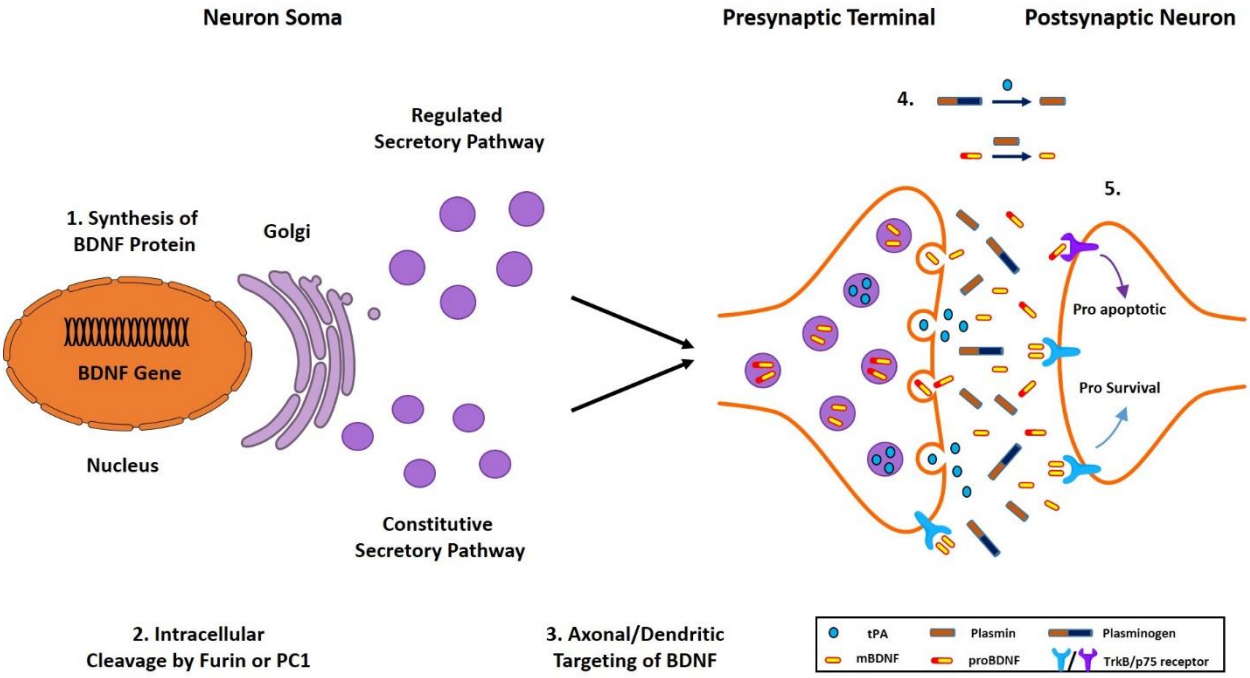

Figure 7. Synthesis and Processing of BDNF Protein. 1) proBDNF, first synthesized in ER is packed into vesicles in Golgi apparatus. 2) proBDNF in trans-Golgi network is sorted in either regulated or constitutive secretory pathway based on the presence or absence of the motif in mature domain of mature BDNF respectively. ProBDNF is either 2) cleaved intracellularly by furin or PC1 to release mature BDNF or 4) cleaved extracellularly by proteases such plasmin to form mature BDNF in which case proBDNF can be released too in synaptic cleft. 5) Mature BDNF and pro BDNF bind with high affinity to TrkB and p75 receptors respectively to elicit biologically opposite responses.

Binding of BDNF to TrkB receptor elicits various intracellular pathways including mitogen activated protein kinase/extracellular signal regulated protein kinase (MAPK/ERK), phospholipaseCγ (PLCγ), and PI3K pathways [265]. It has recently been shown that proBDNF can be secreted and is a signaling protein in its own [266]. However, unlike mBDNF signaling, proBDNF binds to p75 receptors with high affinity and leads to activation of proapoptotic mediators leading to neuronal cell death [265]. Moreover, proBDNF acts as a neurite extension suppressor and synapse eliminator in developed brain [267, 268].

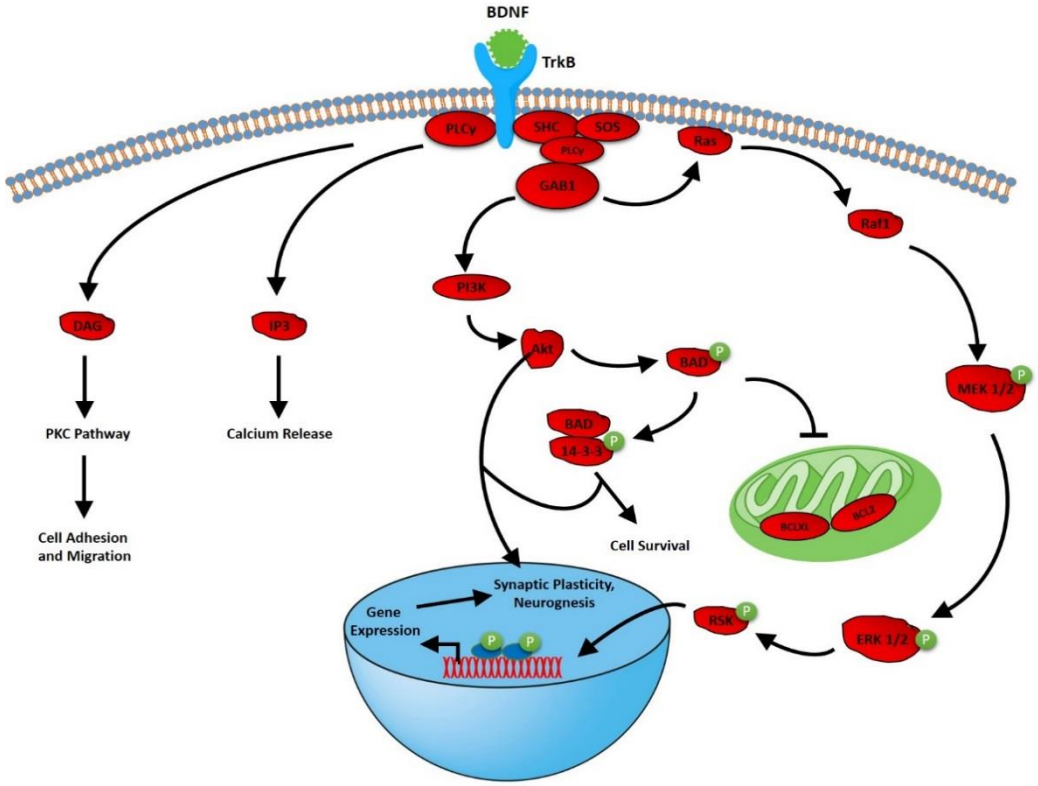

Figure 8. BDNF/TrkB Signaling Cascade. Activation of TrkB by BDNF results in auto phosphorylation of tyrosine residues leading to the onset of the signaling cascades Major downstream pathways include PLCγ, MEK and PI3K which have been implicated in the biological end points of increased cell adhesion/migration, cell survival, synaptic plasticity and neurogenesis.

BDNF is central to the more recent and more clinically relevant neurotrophic hypothesis of depression which states that depression arises as a result of decreased neurotrophic support which can be blocked or reversed by antidepressant treatment. This hypothesis is supported by several clinical and preclinical studies. For example, a study by Diwedi and colleagues reported a 40-50% decrease in the mRNA and protein expression levels of BDNF and TrkB in prefrontal cortex and hippocampus of suicidal patients when compared with control subjects. The observed changes were reported across all suicide patients irrespective of postmortem interval, age and sex of the patients [269]. Further supporting the notion of BDNF involvement in depression, clinical studies have also reported increased BDNF/TrkB mRNA/protein expression levels in the dentate gyrus and hilus regions of patients treated with antidepressants when compared with untreated patients [270]. These clinical findings are supported by multiple animal studies which demonstrate a significant decrease in BDNF protein levels in the hippocampi of animals exposed to multiple depression and/or stress-inducing paradigms. Berry and colleagues reported that socially deprived mice have decreased BDNF levels (50%) in frontal cortex, hippocampus and cerebellum [271]. Another study by Barrientos et al. confirmed these findings in rat and reported that social isolation greatly reduced BDNF mRNA in dorsal dentate gyrus, ventral dentate gyrus, dorsal CA3 region of hippocampus and parietal cortex regions compared with non-isolated animals [272]. Additional studies strengthened the notion of involvement of BDNF in depression by reporting similar results using various depression/stress inducing paradigms such as maternal separation [273, 274], restraint stress [275], foot shock stress [276] and the social defeat paradigm [271]. In support of these findings, several animal studies have demonstrated that treatment with multiple types of antidepressants result in a significant increase in the expression levels of BDNF in a brain region specific manner [277, 278]. A study from Thome et al. reported a significant increase in cAMP

response element (CRE)-mediated gene transcription as well as phospho-CREB, a major regulator of BDNF transcription, in animals treated with antidepressants compared to control animals [279, 280]. The study indicates that chronic but not acute antidepressant treatment induces CRE-mediated gene transcription in cerebral cortex, hippocampus and hypothalamus [281]. Another study from Saarelainen and colleagues showed that heterozygous BDNF null and TrkB transgenic mice were resistant to effects of antidepressants in forced-swim test indicating that BDNF/TrkB signaling is required for behavioral effects of antidepressants [282]. In addition to effects of antidepressants on BDNF expression profile and subsequent decrease in depressive behavior in animals, BDNF protein injection into the midbrain itself alleviated depressive symptoms as early as 3 days post-injection and these effects have been shown to last for at least 10 days post-injection [283]. Though behavioral and clinical studies strongly support a connection between the effects of antidepressants and BDNF signaling, the short half-life of BDNF in systemic circulation [284] and its blood-brain barrier (BBB) impermeability [285] hinders its use as a therapeutic agent.

1.2.8 Serotonin-BDNF Duo in Depression

While the monoaminergic and neurotrophic hypotheses of depression attempt to address the etiology of depression, both fail to fully explain this complex disorder. Therefore, it is possible that the answer for understanding and successfully treating depression may be found by examining the interaction of these two different, yet very similar, signaling molecules. The hippocampus is the region in which the actions and functions of both 5-HT and BDNF converge and overlap. BDNF protein expression has been shown to be considerably high in hippocampus, where most mossy fibers of the dentate granule cells display high BDNF immunoreactivity [286]. BDNF has also been shown to play a role in several functions such as modulation of long term potentiation

[287], neurogenesis [288] and mood regulation in mature hippocampus. 5-HT receptors are also diversely expressed in hippocampus [289] and 5-HT signaling has been shown to be involved in somewhat similar functions in the region, particularly in mood and cognition. The notion of their probable interaction is supported by several independent studies that demonstrate synergism between these two pathways in hippocampus. Studies have shown that antidepressant treatment yields an up-regulation of both 5-HT and BDNF; most notably in dentate gyrus granular cell layer of hippocampus [281, 290]. This increase has been reported to promote neuroplasticity and neurogenesis which counters the effects of stress and depression [291, 292]. Pharmacological studies have shown that chronic SSRI treatment increases BDNF transcription in hippocampus depending on different variables associated with drug treatment and time period of treatment [293, 294]. A study from Rebeca et al. reported a 60% increase in BDNF mRNA expression in the CA1, CA3 and dentate gyrus regions of the hippocampus of Wistar rats following fluoxetine treatment [295]. Additionally, activation of 5HT4 receptors has been shown to increase BDNF levels in the hippocampus as early as 3 days post-treatment suggesting very rapid modulation of neuroplasticity by 5HT4 activation [296]. 5HT2A receptors, which are increased in depression, have been shown to mediate the stress-induced downregulation of BDNF in rat hippocampus [297]. Studies using transgenic animal models have indicated that 5HT1A knockout mice have decreased levels of BDNF and TrkB phosphorylation [298]. Moreover, 5-HT knockout mice demonstrate significantly altered BDNF expression levels in the hippocampus [299]. Parallel to these studies, BDNF signaling has also been shown to regulate 5-HT signaling. One study showed that BNDF infusion in midbrain increases turnover for 5-HT, thus promoting the function of serotonergic signaling pathways [300]. Chronic treatment with BDNF has been shown to downregulate 5HT2A protein expression in hippocampal neuronal and mature hippocampal organotypic cultures [301].

Additionally, the variant BDNF mouse model (Val66Met), which exhibits impaired BDNF transport also exhibits a compromised SSRI response [302] while heterozygous BDNF knockout mice demonstrate reduced 5HT1A sensitivity in hippocampus [303]. These results were supported by another study using acute mild handling stress which revealed that 5HT1A sensitivity was decreased in BDNF deficient mice and fluoxetine reversed this effect [304]. These observations point towards a possible intertwined interaction of these two different, yet very similar, signaling molecules.

The goals of the first part of my thesis are to investigate the role of ERα and ERβ in the regulation of BDNF and 5-HT signaling in female brain. Our analyses in ERα and ERβ knockout animal models demonstrated that BDNF is upregulated by ERβ but not ERα and this regulation is specific to hippocampus. In addition, our data indicate that 5HT2A and not 5HT1A is negatively regulated by ERβ in hippocampus. Taken together, these results suggest that ERβ-mediated regulation of BDNF-5HT2A signaling could play a major role in both the development and intervention of depressive disorders in perimenopausal women.

1.3 Materials and Methods

1.3.1 Animal Models

ERα$^{-/-}$ and ERβ$^{-/-}$ Mouse Models. The model was created by using a targeting vector containing a neomycin resistance gene driven by the mouse phosphoglycerate kinase promoter to introduce stop codons into exon 3. 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 mice used in these studies were purchased at 6 month of age from The Jackson Laboratories, sacrificed and tissue samples were collected.

ERβ$^{-/-}$ Rat Models. Two estrogen receptor knockout rat models were provided by Dr. Karim Rumi (Institute for Reproductive Health and Regenerative Medicine, Kansas University Medical Center) generated by using zinc finger nuclease (ZFN) mediated genome editing to target deletion of exon 3(ΔE3) and exon 4(ΔE4) in ERβ gene. The ΔE3 results in frameshift and null mutation whereas ΔE4 leads generates nonfunctional form of ERβ. Tissue samples were collected from animals at 6 months and 10 months of age (n=5 for each age group).

1.3.2 Tissue Protein Extraction. Tissue samples were homogenized using the Bullet Blender 24 Homogenizer (Next Advance, NY, USA) in T-PER reagent (Pierce Biotechnology, IL, USA) supplemented with protease and phosphatase inhibitors (Roche Applied Science, IN, USA) and 100 µL 0.5 mm glass beads (Next Advance, NY, USA) at speed 8 for 3 min at 4°C followed by centrifugation at 12,000 rpm for 8 min at 4°C. Supernatant was transferred to a new micro centrifuge tube and protein concentration was determined via BCA Assay (Pierce Biotechnology, IL, USA).

1.3.3 Isolation of Primary Cortical and Hippocampal Neurons. Primary cultures of rat hippocampal and cortical neurons were isolated from Day 18 embryonic rat pups as previously described [111]. Briefly, hippocampal and cortical tissues were harvested from embryonic day 18 (E18d) rat fetuses. Following dissection, tissues were treated with 0.02% trypsin in Hank's balanced salt solution (137 mM NaCl, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.34 mM $Na_2HPO_4 \cdot 7H2O$, 10 mM glucose, and 10 mM HEPES) for 5 min at 37 °C. Trypsinized tissues were dissociated by repeated passage through a series of fire-polished constricted Pasteur pipettes. For morphological analyses, 2×10^5 cells were plated on poly-d-lysine double-coated 25 mm coverslips in covered 35 mm petri dishes. For biochemical analyses, 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 μg/ml streptomycin, 0.5 mM glutamine and 25 μM glutamate at 37 °C in a humidified 5% CO2 atmosphere for 3 days. At the 72 hour mark, media was replaced with NBM in the absence of exogenously added glutamate. Cultures were further grown at 37 °C in a humidified 5% CO2 atmosphere. Cultures grown in serum-free NBM yield approximately 99.5% neurons and 0.5% glial cells.

1.3.4 Protein Extraction from Primary Cell Culture. Primary hippocampal cells used for immunoblot analysis were treated at DIV 5 with respective pharmacological agents for 5 days as indicated. At DIV 10 cells were washed with cold PBS (pH 7.2), lysed in cold lysis buffer (N-PER, Thermo Scientific, MA, USA) and then harvested with a cell scraper, followed by centrifugation at 10,000*g for 10 min. Protein concentrations were determined via BCA Assay (Pierce Biotechnology).

1.3.5 Drug Treatment. 2,3-*bis*(4-Hydroxyphenyl)-propionitrile (DPN; ERβ agonist); 4,4',4"-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl) *tris*phenol (PPT; ERαagonist); 4-[2-Phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol (PHTPP; ERβ antagonist); 4-Iodo-2,5-dimethoxy-α-methylbenzeneethanamine hydrochloride (DOI; 5HT2A/2C agonist); 4-(4-Fluorobenzoyl)-1-(4-phenylbutyl)piperidine oxalate (4F4PP Oxalate; 5HT2A antagonist); 7,8-Dihydroxy-2-phenyl-4*H*-1-benzopyran-4-one (7,8 DHF; TrkB agonist) were purchased from Tocris Bioscience (MO, USA). Stock solutions of PPT, DPN, PHTPP, DOI, 4F4PP oxalate and 7,8 DHF (10 mM-50mM) were prepared in 99% ethanol and further diluted to final working concentrations of 100nM, 100nM, 1μM, 3μM, 10μM and 10μM respectively immediately prior to use in phenol-red free NBM supplemented with B27, 5 U/ml penicillin, 5 μg/ml streptomycin, and 0.5 mM glutamine.

1.3.6. Western blotting. Equal amounts of total protein (20 μg/lane) were loaded and separated by 10% SDS-PAGE. Resolved proteins were transferred to 0.2 μm pore-sized PVDF membranes (Bio-Rad, CA, USA) and blocked with 5% Blotting Grade Blocker (BioRad, Hercules, CA) in TBST (100 mL 10X 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, USA); rabbit polyclonal anti-β Actin (1:3000; Thermo Scientific, MA, USA); rabbit polyclonal anti-TrkB (1:1000; Abcam, MA, USA); mouse monoclonal anti-β Tubulin (1:3000; Thermo Scientific, MA, USA); rabbit polyclonal pTrkB (1:1000; Bioworld Technology Inc., MN, USA); mouse monoclonal anti-MAP2 (1:750; Thermo Scientific, MA, USA); rabbit monoclonal anti-NeuN (1:3000; Abcam, MA, USA); rabbit polyclonal anti- PSD95 (1:500; Alomone Labs, JRS, IL); rabbit monoclonal anti-Syanptophysin (1:1000; Abcam, MA, USA), rabbit polyclonal 5HT2A (1:20,000; A generous gift by Dr. Nancy Muma).

1.3.7. Immunocytochemistry and Confocal Imaging. Primary hippocampal cells were grown on precoated Poly-D-lysine 25mm #1 thickness coverslips (Neuvitro Corporation, WA, USA) till DIV 15. Seeded cells on cover slips were then fixed in 4% paraformaldehyde (Electron Microscopy Sciences; PA, USA) for 15 min at RT, and washed 2 times in PBS for storage at 4°C prior to staining. Cells were permeabilized in PBS containing 0.5% Triton-X-100 (PBST) for 5 min at RT and blocked in 5% goat serum (Vector Laboratories) in PBST for 30 min at RT. Coverslips were then incubated with primary antibodies at the appropriate dilutions in PBST supplemented with 1% goat serum for 2 hrs at RT, washed 3 times for 10 min with PBS, and incubated with fluorescently-labeled secondary antibodies diluted in PBST for 1h at RT. Cover slips were washed 3×10 min with PBS and mounted on glass slides with Vectashield Mounting Medium containing DAPI (Vector Laboratories) and sealed with clear nail polish. The following primary antibodies were used: mouse monoclonal anti-MAP2 (1:750; Thermo Scientific, MA, USA); rabbit

polyclonal anti-BDNF (1:500; Santa Cruz Biotechnology, TX, USA); mouse monoclonal anti-Tau (1:500; Thermo Scientific, MA, USA). Goat anti-mouse and anti-rabbit IgGs conjugated with FITC (1:1000, Abcam, MA, USA) and Cy3 (1:1000, Abcam, MA, USA) respectively were used. Mounted cover slips were examined using a customized Olympus IX81/spinning disk confocal inverted microscope (Olympus, Yokogawa) equipped with an Olympus 60X 1.45 NA Oil Immersion objective (Olympus). Images were collected and analyzed using the Slidebook Software Version 6.0 (Intelligent Imaging Innovations) with 15-20 image stacks with a 0.5 μm step size through the cells.

1.3.8. Statistical Analysis

The statistical analyses were conducted using GraphPad Prism version 5.0 (Graph Pad software Inc., CA, USA) and is presented as mean±standard deviation. For data comparisons between two groups, Student's *t*-test and for analysis involving multiple groups, one-way ANOVA/two way ANOVA with *post* hoc Bonferroni's test was used. For all purposes, $p<0.05$ was considered as statistically significant.

1.4. Results

1.4.1. Validation of Specificity of BDNF antibody. For BDNF protein expression, densitometry of mature form of BDNF (mBDNF) was performed. mBDNF has been shown to exist in monomeric form, having molecular weight of 14kDa [305, 306] and trimeric form, having molecular weight of 42kDa [307-309]. The antibody used in our studies detected both of these forms (Figure 9) and expression level of each form is presented in the study individually as measure of BDNF expression level.

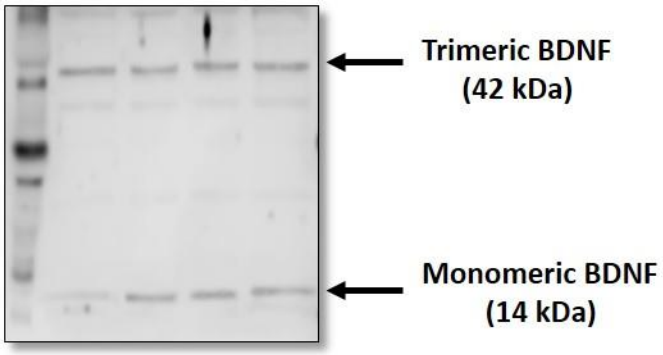

Figure 9. Validation of specificity of BDNF antibody. Western blot shows both the monomeric (14 kDa) and trimeric (42 kDa) forms of BDNF in mouse hippocampal lysates. Briefly, 15μg of protein lysate was separated via 10% SDS-PAGE and transferred onto a PVDF membrane for 1 hour. The membrane was blocked with 5% blotting grade blocker and incubated with anti-BDNF overnight at 4°C. The membrane was then incubated with HRP-conjugated goat-anti-rabbit for 1 hour at RT. The immunoreactive protein bands were visualized with enhanced chemiluminescence (ECL) reagent and scanned using the C-Digit Blot Scanner.

1.4.2. ERβ, not ERα, knockout downregulates BDNF/TrkB in female mouse hippocampus. Using ERα and ERβ-knockout mouse models, we first examined the involvement of estrogen receptors, ERα and ERβ, in the regulation of BDNF in different regions of female brain. Cortical, hypothalamic and hippocampal tissues were harvested from 6-month-old ERα- and ERβ-knockout

female mice (n=5/group) and probed for both monomeric (14 kDa) and trimeric (42 kDa) BDNF immunoreactivity (Figure 10).

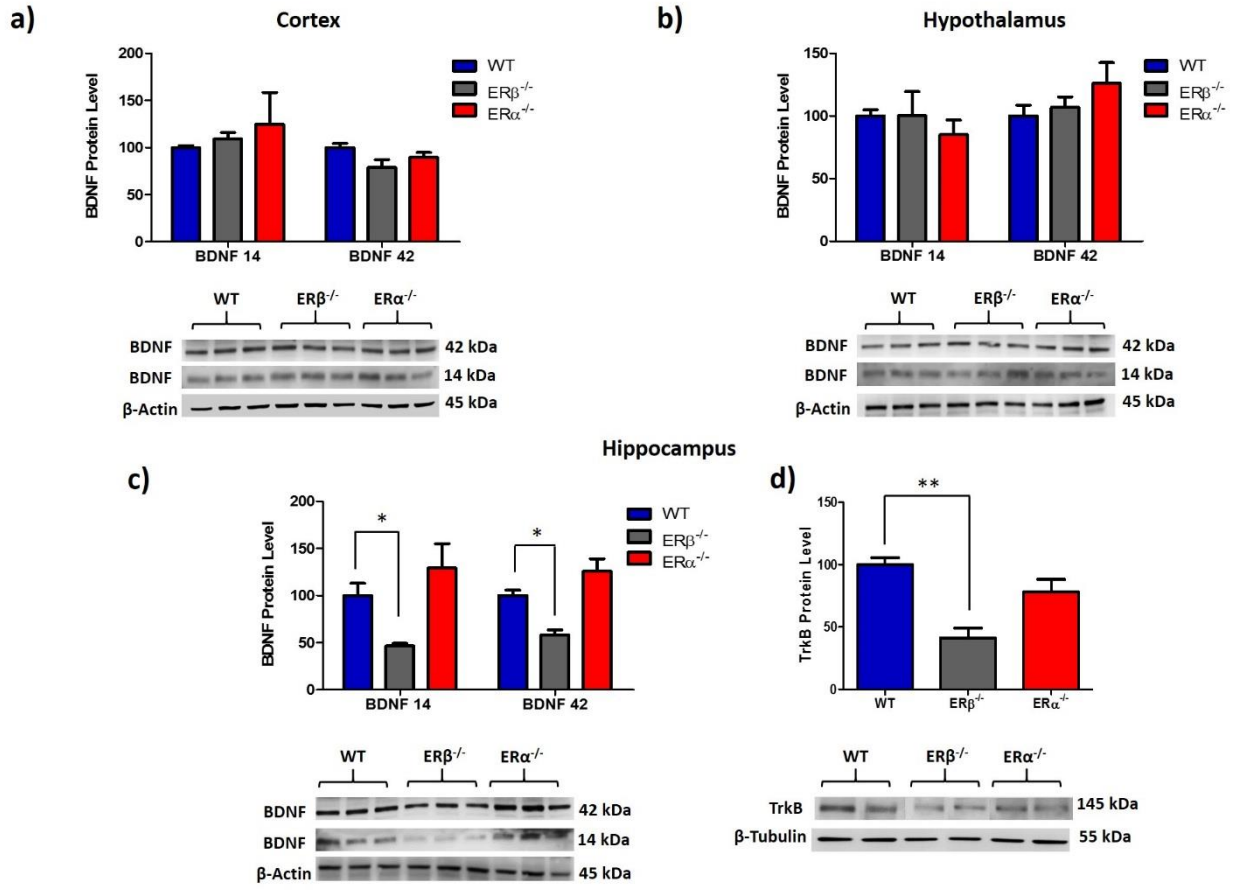

Figure 10. ERβ, not ERα, knockout downregulates BDNF/TrkB in female mouse hippocampus. BDNF regulation by ERs was examined in three brain regions of ER knockout mice by probing for monomeric (14kDA) and trimeric (42kDA) forms of BDNF using western blots. Data indicate: a-b) BDNF is not regulated by either ER in mouse cortex and hypothalamus; c-d) ERβ but not ERα regulates BDNF and TrkB protein expression in mouse hippocampus Data were normalized to an internal loading control (β-actin or β-tubulin) and to wild type group. Data are shown as mean ± standard deviation, n=5. 10% SDS-PAGE gels. One-way ANOVA with Bonferroni's post hoc test *p<0.05, **p<0.01.

The data show no significant difference in BDNF expression levels in cortical tissues derived from either ER-knockout model indicating that BDNF is not regulated by ER signaling in mouse cortex (Figure 10a, monomeric BDNF: $F(2,6) = 0.4$, NS; trimeric BDNF: $F(2,6) = 2.99$, NS). Similarly,

no change in BDNF expression was detected in the hypothalamus of ERα- and ERβ-knockout mice (Figure 10b, monomeric BDNF: $F(2,6) = 0.439$, NS; trimeric BDNF: $F(2,6) = 1.33$, NS). In contrast, hippocampal BDNF levels were significantly reduced in ERβ-knockout mice but not ERα-knockout mice (Figure 10c, monomeric BDNF: $F(2,6) = 12.76$, $p=0.0069$; trimeric BDNF: $F(2,6) = 7.23$, $p=0.025$). Our further analyses expression levels of, demonstrate a 50% decrease in the BDNF receptor, TrkB expression levels in ERβ but not ERα-knockout mice (Figure 10d, $F(2,6) = 13.81$, $p=0.0057$). Collectively, these data indicate that ERβ, but not ERα, regulates BDNF signaling in the hippocampus of female brain.

1.4.3. ERβ knockout downregulates BDNF/TrkB in female rat hippocampus. To further validate our findings from ER-knockout mouse models, we examined BDNF/TrKB in two different ERβ-knockout rat models in which ERβ gene was disrupted by targeted deletion of different exons (Exon3/4). The animals were sacrificed at 6 months of age (n= 5/group) and hippocampal tissues were collected and probed for BDNF immunoreactivity. Consistent with our findings in ERβ-knockout mice, BDNF expression was significantly reduced in the hippocampus of both models (Figure 11, monomeric BDNF: $F(2, 6) = 10.07$, $p=0.012$; trimeric BDNF: $F(2,6) = 7.67$, $p=0.022$). Additionally, a similar decrease in expression levels of TrkB was detected in $Ex4^{-/-}$ knockout rats (Figure 11, TrkB: $F(2,6) = 25.52$, $p=0.0012$), however, the decrease in TrkB levels in $Ex3^{-/-}$ rats did not reach statistical significance.

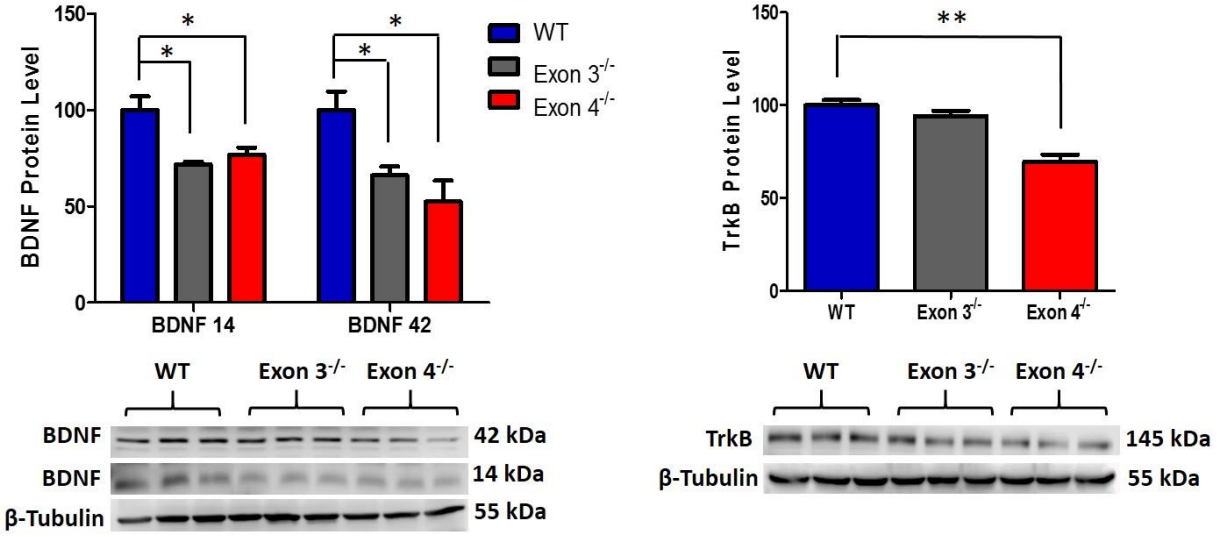

Figure 11. ERβ knockout downregulates BDNF/TrkB in 6-month-old female rat hippocampus. BDNF/TrkB regulation by ERβ was further examined in hippocampus of 6-month-old ERβ knockout rats. ERβ knockout either by targeted exon 3 deletion or targeted exon 4 deletion lead to a significant reduction in the levels of BDNF and TrkB protein expression in rat hippocampus. Data was normalized to an internal loading control (β Tubulin) and to wild type group. Data is shown as mean ± standard deviation, n=5. 10% SDS-PAGE gels. One-way ANOVA with Bonferroni's post hoc test *p<0.05, **p<0.01.

Thereafter, we performed a similar analysis in ERβ-knockout rats at a different age and found that our results were replicated (Figure 12). Specifically, our data indicate an ~45% and ~40% reduction in the expression of both monomeric and trimeric BDNF in both rat models at 10 months of age (Figure 12, monomeric BDNF: $F(2,6) = 9.90$, $p=0.012$; trimeric BDNF: $F(2,6) = 13.99$, $p=0.005$). Expression levels of TrkB protein were also reduced in both models at 10 months of age (Figure 12, TrkB: $F(2,6) = 13.23$, $p=0.0063$). These findings indicate that ERβ regulate BDNF signaling in rat hippocampus, irrespective of the age of the animal.

1.4.4. BDNF is more significantly impacted by ERβ signaling status than aging. To better understand the biological significance of ERβ-mediated regulation of BDNF, we performed an age-dependent comparison in 6-month and 10-month-old wild type and ERβ knockout rats by probing for hippocampal BDNF immunoreactivity (Figure 13).

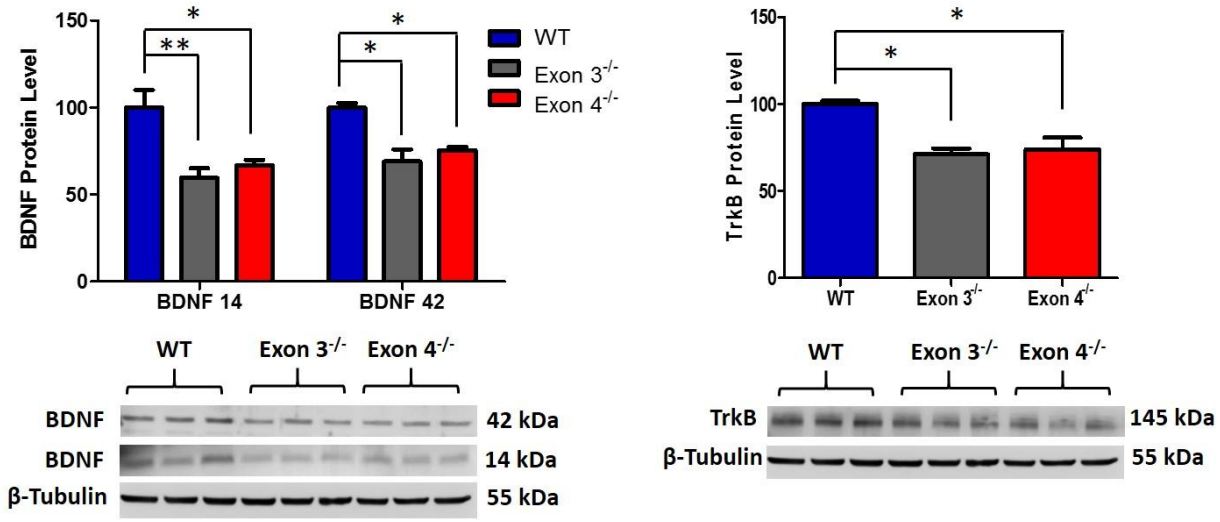

Figure 12. ERβ knockout downregulates BDNF/TrkB in 10-month=old female rat hippocampus. BDNF regulation by ERβ was tested in hippocampus of 10-month-old ERβ knockout rats via immunoblot analyses. ERβ knockout either by targeted exon 3 deletion or targeted exon 4 deletion resulted in a significant decrease in both (a) BDNF and (b) TrkB expression. The integrated density value of the bands in western blots was determined using densitometry and data was normalized to an internal loading control (β Tubulin) and to wild type group. Data is shown as mean ± standard deviation, n=5. 10% SDS-PAGE gels. One-way ANOVA with Bonferroni's post hoc test *p<0.05, **p<0.01, ***p<0.001.

Consistent with the literature [310], our data indicate that aging from 6-month to 10-month leads to a significant decrease in BDNF and TrkB expression levels in all three rat models (Figure 13a-c). The comparison of both factors (ERβ status and age) on same western blot revealed that ERβ knockout results in a 30% - 40% decrease in BDNF expression levels while aging resulted in a 10% -15% reduction. Though both the aging process and removal of ERβ result in a significant

decline in BDNF expression levels, our data indicate that ERβ depletion more significantly impacts BDNF indicating that the regulation of BDNF by ERβ is more prominent than the regulation of BDNF by aging (Figure 13d) (Two Way ANOVA; Interaction; p =0.8742, NS; Column Factor (Effect of Genotype); p=<0.0001; Row Factor (Effect of Aging); p=0.0011).

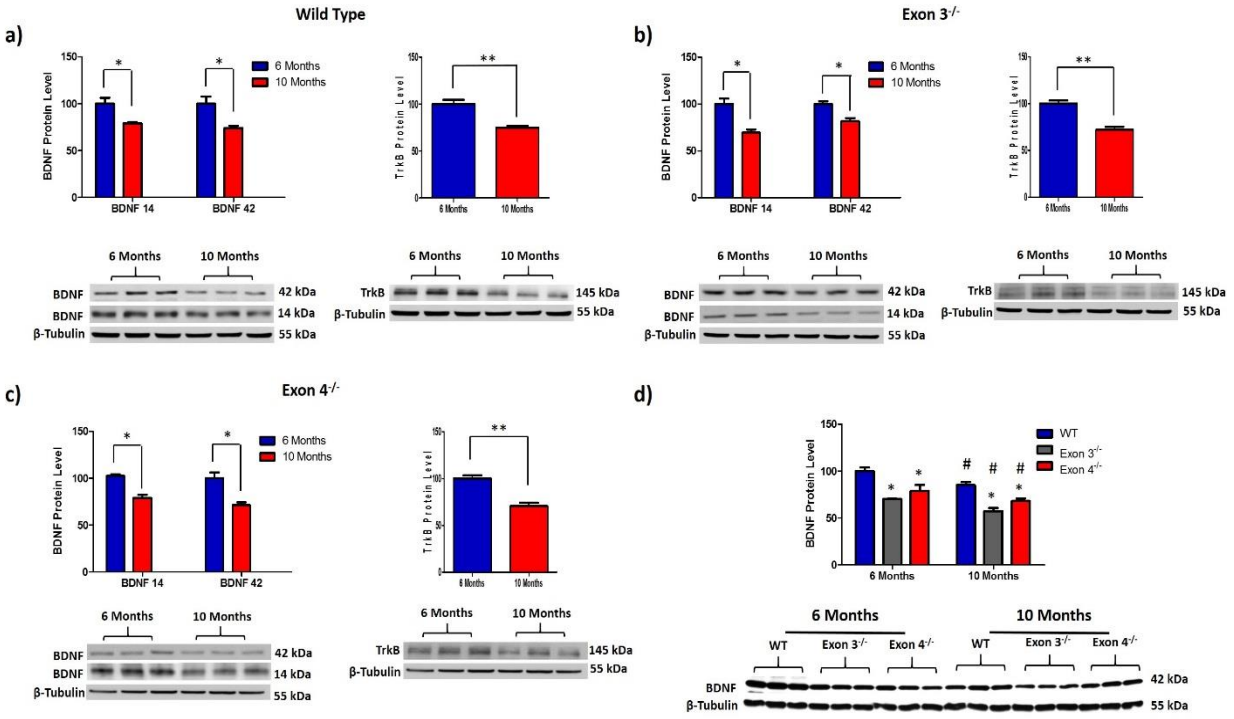

Figure 13. BDNF is more significantly impacted by ERβ signaling status than aging. BDNF regulation by ERβ and aging was tested in the hippocampus of 6- and 10-month-old ERβ knockout. Representative western blots indicate that aging decreases BDNF and TrkB protein expression in (a) WT animals, (b) Ex3$^{-/-}$ animals, and (c) Ex4$^{-/-}$ animals. (d)A comparison of the impact of both age and ERβ status on BDNF expression levels revealed more prominent regulation of BDNF by ERβ in comparison to aging. The integrated density value of the bands in western blots was determined using densitometry and data was normalized to an internal loading control (β Tubulin) and to wild type group. Data is shown as mean ± standard deviation, n=5. 10% SDS-PAGE gels. T test/Two-way ANOVA/post hoc test *p<0.05, **p<0.01. $^{\#}$ Comparison between 6 Month and 10 Month group.

1.4.5. ERβ activation upregulates BDNF/TrkB in primary hippocampal neurons. As ERβ deficiency was shown to significantly and negatively impact BDNF and TrkB expression, we hypothesized that ERβ activation would positively regulate BDNF and TrkB. We examined this hypothesis *in* primary hippocampal neurons treated with the ER-specific agonists PPT (ERα agonist) and DPN (ERβ agonist).

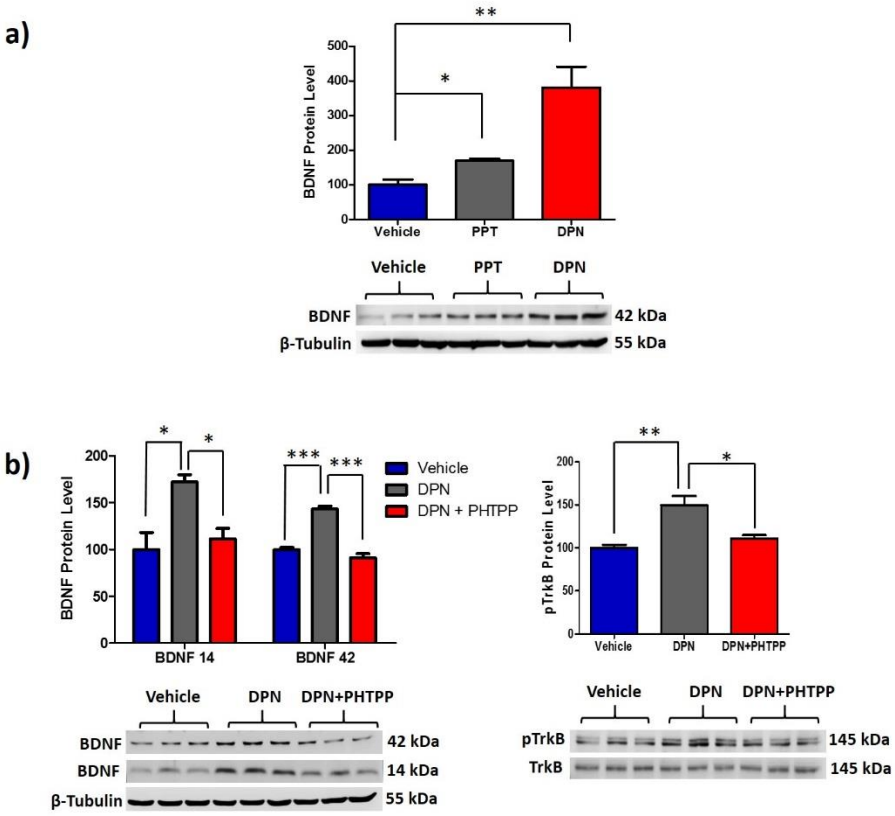

Figure 14. ERβ activation upregulates BDNF and pTrkB expression in primary hippocampal neurons. Primary hippocampal neurons were isolated and treated with DPN (100 nM) or PPT (100 nM) alone (**a**) or in combination with PHTPP (1 μM, **b**). The integrated density value of the bands in western blots was determined using densitometry and data was normalized to an internal loading control (β Tubulin) and to vehicle treated group. Data is shown as mean ± standard deviation, n=3. 10% SDS-PAGE gels. One-way ANOVA with Bonferroni's post hoc test **p*<0.05, ***p*<0.01, ****p*<0.001.

We found that activation of both ERα and ERβ resulted in increased expression of BDNF in primary hippocampal neurons. However, the magnitude of regulation by ERβ was much greater than the magnitude of regulation by ERα (Figure 14a, $F(2,6) = 16.07$, $p=0.0039$). Our data also

indicate that ERβ activation results in the increased phosphorylation of TrkB receptor. The ERβ-mediated upregulation of BDNF/TrkB was abolished in neurons pre-treated with the ERβ-specific antagonist PHTPP (Figure 14b, monomeric BDNF: $F(2,6) = 8.9$, $p=0.015$; trimeric BDNF: $F(2,6) = 69.51$, $p<0.001$; TrkB: $F(2,6) = 14.76$, $p=0.0048$).

1.4.6. ERβ activation upregulates synaptic and neurogenic markers in primary hippocampal neurons. Antidepressants have been reported to increase the expression and function of proteins related to synaptic plasticity and neurogenesis [291, 311-314]. Therefore, we assessed whether ERβ activation in primary hippocampal neurons would mimic the effects of antidepressant treatment. DPN/PHTPP-treated neurons were harvested and probed for expression of the synaptic plasticity markers synaptophysin (synaptic vesicle protein) and PSD95 (post synaptic density protein) and the neurogenic markers MAP2 (marker for post mitotic neurons) and NeuN (marker for matured neurons) (Figure 15). The data indicate that ERβ activation significantly upregulated the expression levels of synaptophysin and PSD95 (Figure 15a, Synaptophysin: $F(2,6) = 13.34$, $p=0.006$; PSD95: $F(2,6) = 28.6$, $p=0.0009$) and induced a similar increase in the expression of MAP2 and NeuN (Figure 15b, MAP2: $F(2,6) = 26.6$, $p=0.001$; NeuN: $F(2,6) = 8.77$, $p=0.016$). The DPN-mediated increased protein expression of all four markers was attenuated or abolished in neurons pre-treated with the ERβ-specific antagonist PHTPP, indicating that ERβ is a potential regulator of synaptic plasticity and neurogenesis in hippocampal neurons that is likely mediated by BDNF signaling.

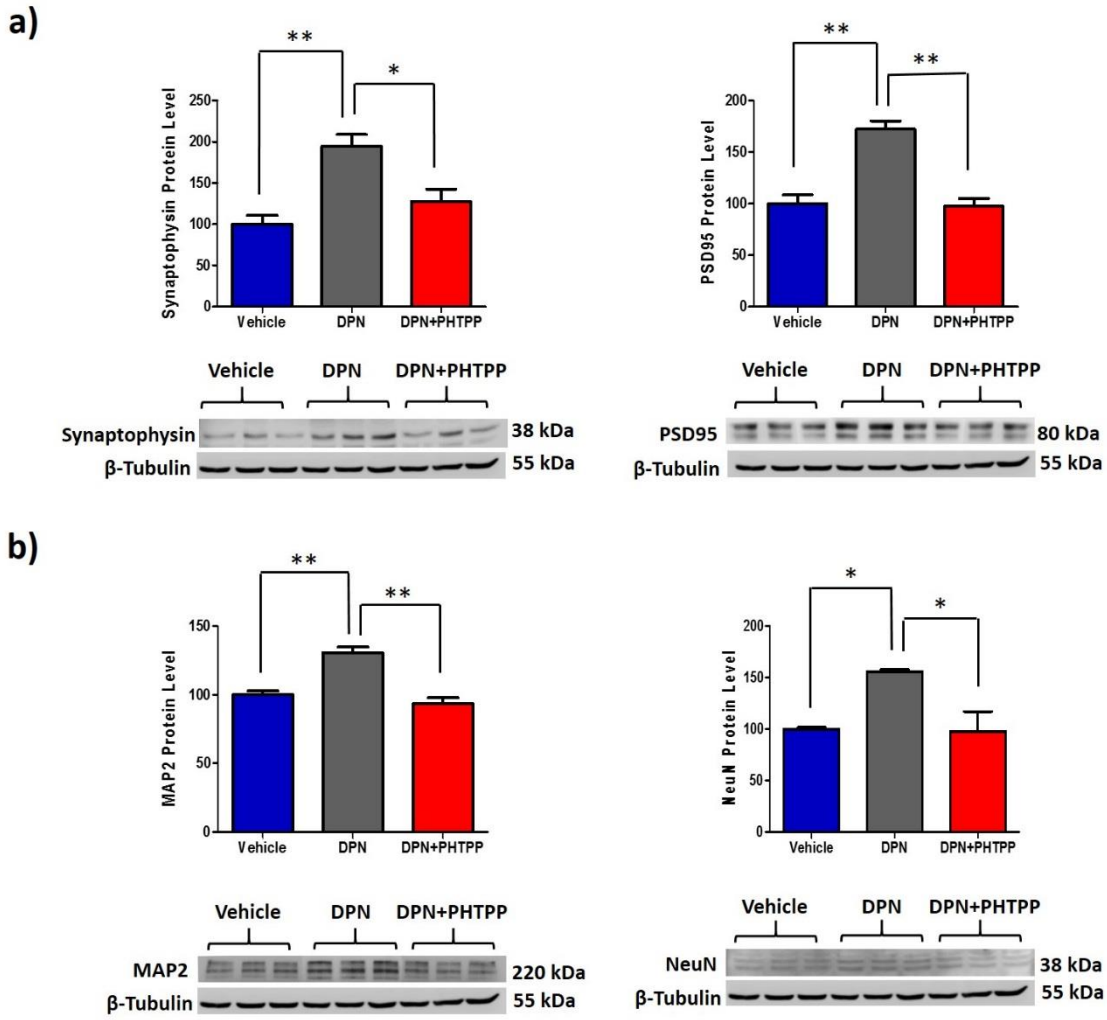

Figure 15. ERβ activation upregulates synaptic and neurogenic markers in primary hippocampal neurons. ERβ regulation of synaptic and neurogenic markers was tested in E18 primary hippocampal cell culture. Neurons were treated on DIV 5 for 5 days with Veh, DPN (100 nM), or pre-treated with PHTPP (1 μM) followed by combination DPN + PHTPP. Expression levels of (a) synaptic plasticity and (b) neurogenic markers were assessed via immunoblot analyses. The integrated density value of the bands in western blots was determined using densitometry and data was normalized to an internal loading control (β Tubulin) and to vehicle treated group. Data is shown as mean ± standard deviation, n=3. 10% SDS-PAGE gels. One-way ANOVA with Bonferroni's post hoc test *p<0.05, **p<0.01.

1.4.7. ERβ knockout upregulates 5HT2A, but not 5HT1A, in rat hippocampus. After determining that ERβ does regulate BDNF signaling in hippocampus, we shifted our focus to 5-HT; specifically, we assessed whether or not ERβ regulates 5-HT signaling. As 5HT1A and 5HT2A receptors are currently the most heavily studied 5-HT receptors in depression, we focused

our investigations on these two receptors. Our data indicate that ERβ knockout resulted in no significant change in 5HT1A expression levels in female rat hippocampus at either 6 months or 10 months.

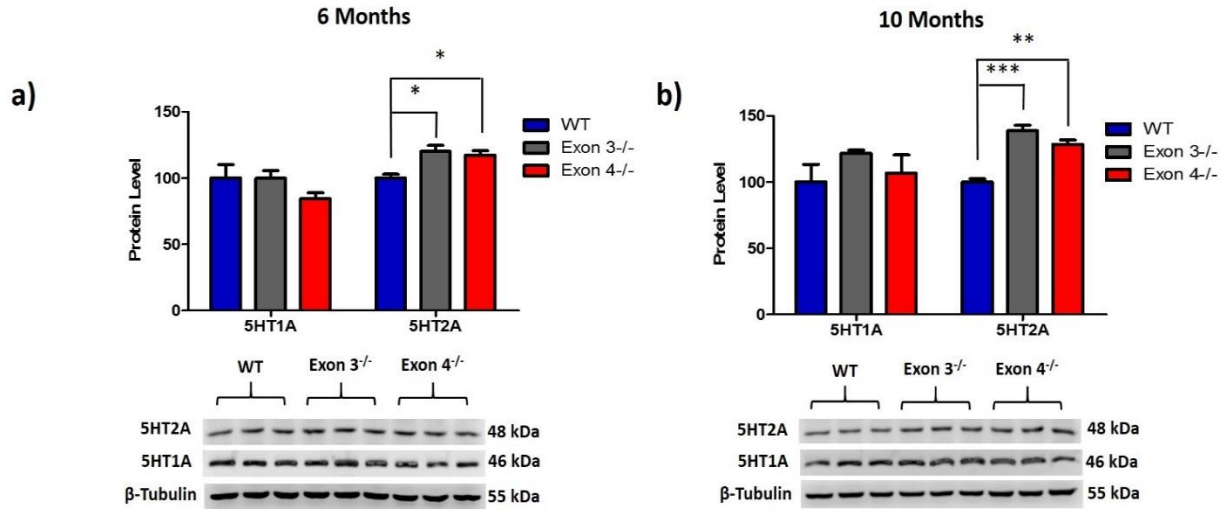

Figure 16. ERβ knockout upregulates 5HT2A, but not 5HT1A, in 6-month and 10-month-old rat hippocampus. ERβ regulation of serotonergic receptors, 5HT1A and 5HT2A was tested in hippocampus of ERβ knockout rats of age 6 month and 10 with the help of western blots. Representative blots show that ERβ knockout either by targeted exon 3 deletion or targeted exon 4 deletion lead to an increase in 5HT2A expression levels in both a) 6 months and b) 10 months old animals. The expression level of 5HT1A receptor was not affected by this deletion irrespective of the age of the animals. The expression of 5HT2A was not effected by age progression as seen in c). The integrated density value of the bands in western blots was determined using densitometry and data was normalized to an internal loading control (β Tubulin) and to vehicle treated group. Data is shown as mean ± standard deviation, n=3. 10% SDS-PAGE gels. One-way ANOVA with Bonferroni's post hoc test *p<0.05, **p<0.01, ***p<0.001.

However, we found that ERβ knockout resulted in a nearly 20% increase in expression levels of 5HT2A in hippocampus of 6-month-old animals. In addition, we found that this regulation is enhanced in 10-month-old ERβ knockout rats which show nearly 40% increase in the expression level of 5HT2A receptors (Fig. 16a and 16b, 6 month animals (16a): $F(2,6) = 9.16$, $p=0.015$; 10

month animals (16b): $F(2,6) = 34.63$, $p=0.0005$). These results indicate that ERβ regulates 5HT2A but not 5HT1A in female rat hippocampus in which age may also play a role.

1.4.8. Activation of TrkB downregulates 5HT2A in primary hippocampal neurons. In order to determine the possible interaction between the BDNF/TrkB and 5HT2A-mediated signaling pathways, we treated primary hippocampal neurons with either TrkB agonist (7, 8 dihydroxy flavone; 7,8 DHF)) or 5HT2A/2C agonist (DOI) combined 5HT2A specific antagonist (4F4PP Oxalate). We analyzed the protein expression levels of 5HT2A receptors in 7,8 DHF-treated neurons and BDNF/pTrkB in DOI-treated neurons -via immunoblot analyses. Our data demonstrate a 30-35% decrease in the levels of 5HT2A receptor after 7, but not 4, days of treatment with 7, 8 DHF (Figure 17a, 4-day treatment: $F(1,4) = 8.892$, $p=0.3226$, NS; 7-day treatment: $F(1,4) = 6.806$, $p=0.0017$). Treatment with either DOI or DOI+4F4PP Oxalate did not induce a significant change in the expression levels of either BDNF or pTrkB (Figure 17b; One Way ANOVA, BDNF: $F(3,8) = 0.9540$, $p=0.4596$, NS; pTrkb: $F(3,8) = 0.6413$, $p=0.6096$, NS).

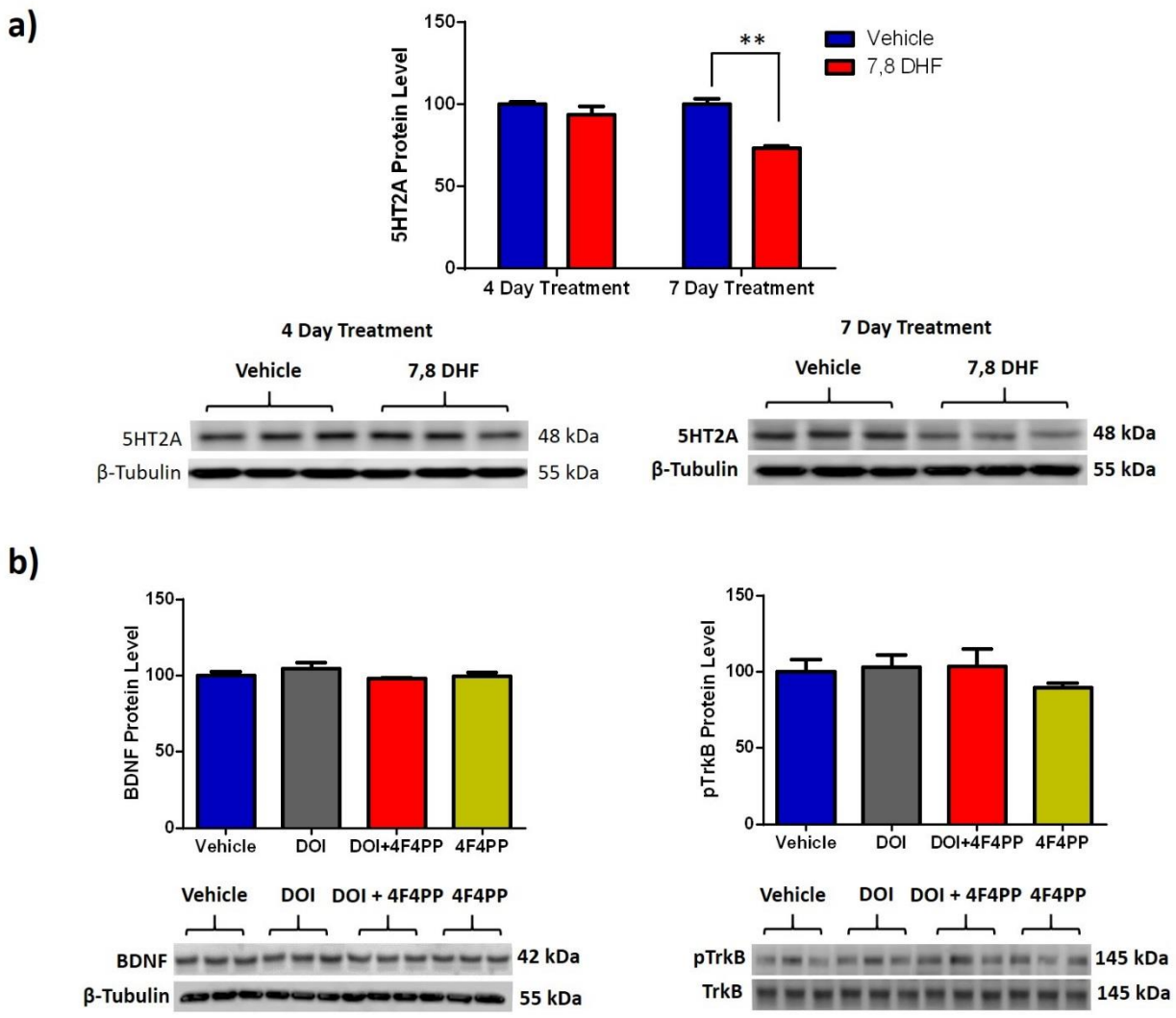

Figure 17. TrkB activation downregulates 5HT2A in primary hippocampal neurons. BDNF regulation of 5HT2A receptor expression and 5HT2A regulation of BDNF expression level was studied. Primary hippocampal neurons were treated with either DOI or 7, 8 DHF and expression level of other signaling molecule was measured. Representative western blots indicate that a) BDNF negatively regulates 5HT2A expression level as chronic treatment of neurons with 7, 8 DHF downregulated 5HT2A expression level by at least 25%. B) 5HT2A receptor signaling doesn't regulate either BDNF or pTrkB expression levels as treatment with either DOI or DOI+4F4PP Oxalate didn't induce any signaificant changes in the expression level of both proteins. The integrated density value of the bands in western blots was determined using densitometry and data was normalized to an internal loading control (β Tubulin) and to vehicle treated group. Data is shown as mean ± standard deviation, n=3. 10% SDS-PAGE gels. t Test/One-way ANOVA **p<0.01.

1.5. Discussion

We have a general hypothesis that perturbation of ovarian hormone homeostasis during perimenopause/menopause leads to dysregulation of BDNF and 5-HT signaling in female brain, thus rendering females susceptible to increased risk of depression. In this study, we attempted to elucidate the role of estrogen signaling in the regulation of BDNF and 5-HT signaling, both of which have been highly implicated to play a role in the etiology of mood disorders [315, 316].

Our analyses in ERα and ERβ knockout ($ER\alpha^{-/-}$ and $ER\beta^{-/-}$) mouse models demonstrated that BDNF was significantly downregulated in $ER\beta^{-/-}$ but not $ER\alpha^{-/-}$ mice, and the $ER\beta^{-/-}$-mediated response was brain region-specific. A nearly 45% reduction in BDNF expression levels was found in the hippocampal region of $ER\beta^{-/-}$ mice; in contrast, the changes in BDNF were not significantly altered in the cortex and hypothalamus of these mice (Figure 10). The brain region-specific regulation of BDNF is intriguing as it is parallel to the clinical observations in depressed subjects. Evaluation of changes in hippocampal volume revealed that depressed patients have a 4%-5% smaller hippocampus compared to normal control subjects indicating hippocampal atrophy associated with depression. [317]. A post mortem study by Diwedi et al. reported a significant decrease in mRNA and protein levels of BDNF and TrkB in the hippocampus of depressed subjects compared to normal subjects [269]. A recent study by Ray et al. corroborated these results and reported a 30% decrease in BDNF mRNA in hippocampus of depressed patients compared to normal subjects [318]. Along with BDNF and TrkB, CREB, a major transcriptional regulator of BDNF, has also been reported to decrease in depressed suicidal subjects compared to normal subjects [319]. Furthermore, antidepressant-treated depressed patients have been reported to show an increased hippocampal BDNF immunoreactivity compared with untreated depressed patients [270]. The findings of these studies are supported by several animal studies that have reported

decreased BDNF levels in hippocampus after stress/depression [245, 271, 274]. A study by Lippmann et al. reported a significant decrease in BDNF levels in hippocampus, but not in cortex of Long-Evans hooded rats exposed to maternal separation as a paradigm of stress induction [274]. Antidepressant treatment in animal studies has also been shown to increase BDNF levels in hippocampus compared to non-treated animals [320-323]. A study by Dias et al. reported an increase in BDNF mRNA levels in hippocampus and cortex of Sprague-Dawley rats but the increase was much more significant in hippocampus compared to the change in cortex [324]. Thus our results in mice animal model demonstrate brain region specific regulation of BDNF by ERβ, pointing towards a probable pathway for the phenomenon seen in clinical and basic science research studies.

The signaling [170, 325-332] and function [332-336] of estrogens and BDNF have been shown to somewhat converge in hippocampus, implying a possible interaction in this brain region. In support of this notion, a number of studies have reported decreased BDNF mRNA in ovariectomized animals which was reversed by estradiol treatment [165, 337-340]. Although this regulation has been extensively studied in the past, the underlying mechanism is not clearly understood. Some studies have implicated the role of ERα in the regulation [310] whereas other reports have supported the role of ERβ in mediating this effect [176, 311], thus reaching contradictory results at the best. Our study showed that BDNF is regulated specifically by ERβ, and not ERα as only ERβ knockout animals showed decreased BDNF expression in specific brain regions (Figure 10). This finding indicates that estrogen exerts its effects via ERβ at least in the hippocampus, hence implicating that the irregularity or diminished levels of estrogens in perimenopause/menopause can lead to a subsequent reduction of ERβ signaling creating a deficit in BDNF signaling, thus rendering females susceptible to an increased risk of depression. Adding

onto the regulation of BDNF by ERβ, expression levels of the BDNF receptor, tyrosine kinase B (TrkB) were also downregulated to a similar extent (~50%) after ERβ knockout, but not after ERα knockout. These data were further validated in two different ERβ$^{-/-}$ rat models; one with targeted deletion of exon 3 and the other with a targeted deletion of exon 4 in the ERβ gene. As expected, ERβ deficiency in both Exon 3$^{-/-}$ and Exon 4$^{-/-}$ models resulted in a significant reduction in the levels of both monomeric and trimeric forms of BDNF. Moreover, TrkB expression was reduced by 30% in the hippocampus of Ex4$^{-/-}$ animals; however, downregulation of the protein did not reach statistical significance in Ex3$^{-/-}$ animals (Figure 11). We further analyzed BDNF expression levels in aged female rats (10 months) and observed a significant decrease in the levels of both BDNF and TrkB in both Ex3$^{-/-}$ and Ex4$^{-/-}$ models (Figure 12). The demonstrated regulation of BDNF by ERβ is possibly occurring through the regulation of gene expression of BDNF by ERβ as an estrogen response element (ERE) has been reported in the BDNF gene [174].

The BDNF system including transcription, protein synthesis and activation of TrkB receptors has been shown to be impaired in aging animals. A study by Calabrese et al. reported that BDNF expression is reduced by 20% in hippocampus of aged male Wistar rats compared with 3-month-old rats [310]. This study also reported an aging-mediated decrease of up to 30%-40% in transcription factors associated with BDNF such as Npas4, Creb and Carf [310]. In light of these findings, we compared the effect of aging and ERβ status on BDNF expression in our ERβ knockout rat models. We found that aging does significantly decrease the BDNF protein expression in rat hippocampus; however, ERβ-mediated regulation of BDNF was found to be much more significant (Figure 13).

Our *in vivo* findings were further validated in primary hippocampal neurons. Analyses in primary hippocampal neurons indicated that ERβ activation by DPN significantly enhanced BDNF-TrkB

signaling. We found that BDNF is also regulated by ERα, but the magnitude of regulation by ERβ is much greater than that of regulation by ERα. The activation of ERβ signaling also significantly increased levels of pTrkB in primary hippocampal neurons (Figure 14).

Antidepressants have been reported to increase the expression and function of proteins related to synaptic plasticity [311-313]. A study by Li et al. reported a startling increase in the expression levels of Synapsin1, PSD95 and GluR1 in synaptoneurosomes from prefrontal cortex (PFC) after ketamine administration [311]. Two independent studies by Leary et al. [312] and Sairanen et al. [313] reported that chronic imipramine and fluoxetine treatment induced an increase of synaptic proteins in the hippocampus and PFC of rats respectively. In order to determine whether specific activation of ERβ can similarly increase and/or modulate the expression levels of synaptic proteins, we treated primary hippocampal neurons with DPN. Our data indicate that activation of ERβ results in a two-fold increase in the pre-synaptic vesicle protein Synaptophysin and a 50% increase in the expression of post density protein, PSD95 when compared to vehicle treated neurons (Figure 15). These findings are in line with the research outcomes of a study from Liu et al. which reported a similar increase in levels of synaptic proteins in the hippocampus of mice following treatment with ERβ, but not ERα specific agonists [341]. Interestingly, a contradictory report from Jelks et al. showed that ERβ activation by DPN does not induce any change in synapse number in hippocampal neurons [342]. Hence our study added a significant piece of evidence to the existing small contradictory set of studies and supports that ERβ agonism is capable of inducing plasticity changes in hippocampal neurons. Thus it is highly probable that the perturbation of estrogen signaling in the perimenopausal/menopausal phase might lead to a downregulation of these synaptic proteins via decreased BDNF signaling and thus may predispose the individual to an increased risk of depressive and cognitive disorders.

Soon after the discovery of adult hippocampal neurogenesis in humans [343], it was theorized that basal deficits in hippocampal neurogenesis may underlie symptoms of psychiatric disorders, particularly depression [344]. This theory was largely based on the findings indicating decreased hippocampal volume in depressed patients [345, 346] and increased neurogenesis with the administration of antidepressant medication and therapies [344, 347]. For instance research studies have demonstrated that antidepressants can increase the number of neural progenitor cells in humans [348], adult born hippocampal granule cells [349] and expression of neurogenic markers such MAP2 and Dcx [350]. These findings have been largely supported by recent studies indicating decreased numbers of granule cells and decreased granule cell layer volume in the anterior and mid-dentate gyrus (DG) in unmedicated depressed patients in comparison to controls [292, 351, 352], as well as increased hippocampal neurogenesis and increased granule cell layer volume in treated depressed patients relative to the unmedicated patients [348, 353, 354]. So in the light of these findings, we decided to determine whether specific activation of ERβ can similarly increase and/or modulate the expression levels of neurogenic proteins. Our analysis revealed nearly 50% increase in molecular markers pertaining to neurogenesis in DPN-treated neurons compared to vehicle treated neurons. These findings point towards a therapeutic possibility that agonism of ERβ can be used to alleviate the depression symptomatology, as very recently adult hippocampal neurogenesis has been shown to be sufficient enough to relive depressive phenotype in mice [355].

Our finding of ERβ regulation of BDNF in hippocampus prompted us to further examine signaling pathways related to mood disorders. The monoamine hypothesis is the oldest hypothesis postulated for depression [211] and 5-HT is an important player in this hypothesis. Moreover, there are not many studies that have analyzed the probable molecular mechanism underlying the interaction of

estrogen signaling, BDNF signaling and 5-HT signaling; three signaling pathways thought to converge in the hippocampus (for review see [332, 356]). We chose to analyze the expression levels of 5HT1A and 5HT2A receptors as these are most heavily studied serotonergic receptors in depressive disorders (for review see [357, 358]). Our analyses in 6-month-old ERβ knockout rats revealed a 20% increase in expression levels of 5HT2A, but there was no significant change in the expression level of 5HT1A receptor (Figure 16). These findings were further validated in 10-month-old ERβ knockout rats. Specifically, we observed a 40% and 20% increase in hippocampal expression level of 5HT2A receptor in $Ex3^{-/-}$ and $Ex4^{-/-}$ animals with no changes in 5HT1A receptor expression (Figure 16). These results correspond with clinical findings that have reported an increase in 5HT2A receptor expression in depressed patients [228-230] and antidepressants ranging from SSRIs to MAOIs have been reported to induce a downregulation of binding sites of these receptors in post synaptic regions in brain (for review see [234]). Thus there appears to be a possibility that perturbed ERβ signaling in perimenopause/menopause leads to a dysfunction in both BDNF and 5HT2A signaling which in turn increases the vulnerability for the onset of depression.

In order to determine whether the ERβ-regulated BDNF and 5HT2A signaling pathways interact with one another, we decided to follow a simple approach of agonizing either 5HT2A or TrkB signaling in primary hippocampal neuronal cell culture and analyzing the expression level of other signaling molecule. In literature, there have been reports indicating that BDNF can regulate 5HT2A in hippocampus and vice versa. A research report by Vaidya et al reported that 5HT2A/2C signaling activation by DOI can lead to a downregulation in BDNF mRNA levels in rat hippocampus and specific blockage of 5HT2A receptor signaling can reverse this effect [297]. The author followed up on the study by using more specific antagonists of 5HT2A and 5HT2C

signaling and replicated the results [359]. Evidence for the regulation of 5HT2A receptor expression by BDNF comes from a study by Trajkovska et al who reported that chronic but not acute treatment of primary hippocampal cells with BDNF leads to a downregulation of 5HT2A receptor protein level possibly by modulating the gene expression of the receptor through unknown signaling pathways [301]. In our study we found that 5HT2A receptor activation by DOI did not lead to a significant change in the expression level of BDNF and pTrkB (Figure 17). Interestingly we found that 7 day, but not 4 day treatment with 7, 8 Dihydroxyflavone (7, 8 DHF) significantly downregulates expression level of 5HT2A receptor in primary hippocampal cell culture (Figure 16). The antithetical results from the study of Vaidya et al [360]may result as the author of the study was looking at the mRNA levels of the BDNF whereas our study looked at the protein levels after the agonism of 5HT2A receptors. In addition, the serotonin in the FBS serum was not removed when the study was conducted. Thus the experimental conditions are not mimicking the endogenous environment and the presence of serotonin in FBS serum could have also modulated the activation of 5HT2A receptors thus leading to the varying results. The results of our study filled the gap in the literature as no known study has studied the interaction of these two signaling pathways in the same research model. Our study demonstrated that BDNF is not being regulated by 5HT2A at least at the levels of protein regulation but 5HT2A is being regulated by BDNF in hippocampus probably via signaling pathways that regulate the gene expression of the receptor as there was no evident regulation of 5HT2A receptor expression level with acute agonism of TrkB signaling. Thus it is possible that during perimenopause/menopause declined BDNF signaling leads to an increased 5HT2A expression level which increases the susceptibility for mood disorders. Moreover the 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 5HT2A receptor signaling.

1.6. Limitations of the Study

Overall, the findings of our study are very informative and enlightening but there remains a number of key issues that need to be kept in mind and addressed.

- **Pro BDNF Levels**

Pro-BDNF is not only a precursor of mature BDNF but has been shown to act as a signaling molecule on its own. It's been suggested that mature and pro BDNF elicit their respective biological actions maintaining a possible equilibrium [361]. The phenomenon has been termed as yin and yang hypothesis of neurotrophin action, mature BDNF being yang and proBDNF being the yin [361]. Any possible dyshomeostasis of the equilibrium can result possibly in mood disorders. One of the weaknesses of our research study model is that we did not measure the levels of pro BDNF at any point of time in our study. However, although the measurement of proBDNF in the study model is important, the absence of these data does not diminish the significance of the findings as we saw significant change in the expression of mature form of BDNF. The decline or rise in mature form of BDNF in our animal or cell culture models directly demonstrates a dyshomeostasis in the equilibrium and thus would elicit deleterious or beneficial effects with respect to mood disorders.

- **ERβ Regulation of BDNF**

Another probable weakness is that we did not study the mechanism of ERβ regulation of BDNF. A study by Sohrabji et al. reported the presence of an ERE at the junction of intron IV and exon V of the then known BDNF gene [174]. Although the results are informative we do not know for sure whether BDNF regulation by ERβ is indeed occurring at the level of gene regulation or mediated by secondary signaling pathways downstream of ERβ.

- **ERβ Regulation of 5HT Signaling**

The study focused on the two 5HT receptors, 5HT1A and 5HT2A receptors thus leaving aside 12 other serotonin receptors that could have also been studied, therefore limiting the translational ability of the study. Although 5Ht1A and 5HT2A receptors have been heavily characterized in the field of depression, inclusion of some more receptors into the study would have made the results more translational.

- **Lack of Depressive Phenotype in ERβ$^{-/-}$ Animals**

Lastly, but most importantly, we found that ERβ$^{-/-}$ leads to a significant decrease in BDNF and an increase in 5HT2A receptor expression levels but very interestingly it has been well-established that ERβ knockout animals (βERKO) do not have a depressive phenotype. For instance βERKO mice did not show any significant alteration in immobility time in forced swimming test paradigm when compared with wild type animals [362]. The results were corroborated by another study which replicated the results and reported a non-significant change in immobility time when comparing βERKO and wild type animals [363].

The reported observations lead us to hypothesize that the molecular alterations in the βERKO animals itself are not sufficient enough to lead to a depressive phenotype but must interact with stressors to reach the threshold to cause depression. Supporting this notion, stress and adrenal corticosterone secretion have been reported to increase 5-HT_{2A} expression and activity. For instance subordinate rats subjected to chronic social stress [125] and rats subjected to inescapable shock [65] have significantly increased serum corticosterone levels and increased 5-HT_{2A} receptors in the parietal cortex, and these changes in 5-HT_{2A} receptor number are proportional to the extent of HPA axis activation and corticosterone secretion. Consistent with

these findings, chronic administration of corticosterone significantly increases 5-HT_{2A} receptor density in the neocortex of the rat forebrain as measured by radioligand binding using ^{3}H-ketanserin [93], and behaviorally by increased wet dog shakes [364-366]. Moreover, corticosterone restores selective brain site decreases in 5-HT_{2A} receptor density following adrenalectomy [367]. These studies strongly show that stressors can trigger 5-HT_{2A} receptor upregulation via increased adrenal secretion of corticosterone. In addition both acute and chronic stress has been shown to decrease BDNF expression levels in hippocampus. For instance studies have reported that single or repeated immobilization stress markedly reduces BDNF mRNA levels in hippocampus especially in dentate gyrus [368, 369]. In addition, stress induced by 6 hours of restraining lead to similar results and induced a 70% down regulation in BDNF mRNA content when compared to non-stressed control animals [370]. Similar to these findings, results from a number of other studies have established that both acute and chronic stress can significantly down regulate BDNF expression levels in the hippocampus [371] [372]. So when this observed modulation of BDNF and 5HT2A levels by stress is considered in the context of our data, our hypothesis seems to hold a very probable ground.

1.7. Future Directions

Based on the interesting findings of the study, considerable amount of future research is needed to strengthen and make the findings translational. Specifically, as above mentioned further investigation to unravel the underlying molecular mechanism by which ERβ regulates BDNF is needed. Although an ERE has been reported in the BDNF gene, future studies should be focused at finding the pathway resulting in the observed regulation, be it either direct genomic, tethered genomic or non-genomic pathway. In addition broader investigation of the regulation of 5HT signaling by ERβ should be carried out. Future studies should be aimed at looking at any probable regulation of other serotonin receptors, thus expanding the significance of these findings. Lastly and most importantly, translational investigation in animal behavior model for perimenopausal depression should be conducted. Specifically, experiments should be carried out to determine whether $ER\beta^{-/-}$ female animals when exposed to environmental stressors can develop depressive symptoms or not as proposed by our hypothesis. Secondly, determining whether ERβ agonism at the onset of perimenopause in normal stressed female animals can sustain brain BDNF and 5-HT signaling and thus reduce depressive symptoms would provide significant insights.

1.8. Conclusion

Our findings illustrate a possible mechanism underlying the increased susceptibility for depression associated with perimenopause and menopause in women (Figure 18). Based on these findings, we hypothesize that perimenopause/menopause leads to irregular or reduced levels of estrogen in the brain. This irregularity or reduction of estrogen availability decreases the stimulation of ERα, ERβ and GPER1 mediated estrogen signaling. The attenuated ERβ signaling leads to decreased levels of BDNF protein in the hippocampal region of the brain. The decreased levels of BDNF and thus reduced BDNF-TrkB signaling weakens the synaptic and neurogenic strength thus rendering the brain to a weakened adaptation to environmental stressor resulting in increased risk to depression. Attenuated BDNF/TrkB signaling also increases the protein levels of 5HT2A receptors, which could also play a role in the increased susceptibility for depression associated with perimenopause as 5HT2A antagonism has been shown to relieve the symptoms of depression. In addition, we propose that timely intervention with an ERβ-targeted modulator could potentially attenuate this susceptibility and reduce the risk or ameliorate the clinical manifestation of this brain disorder.

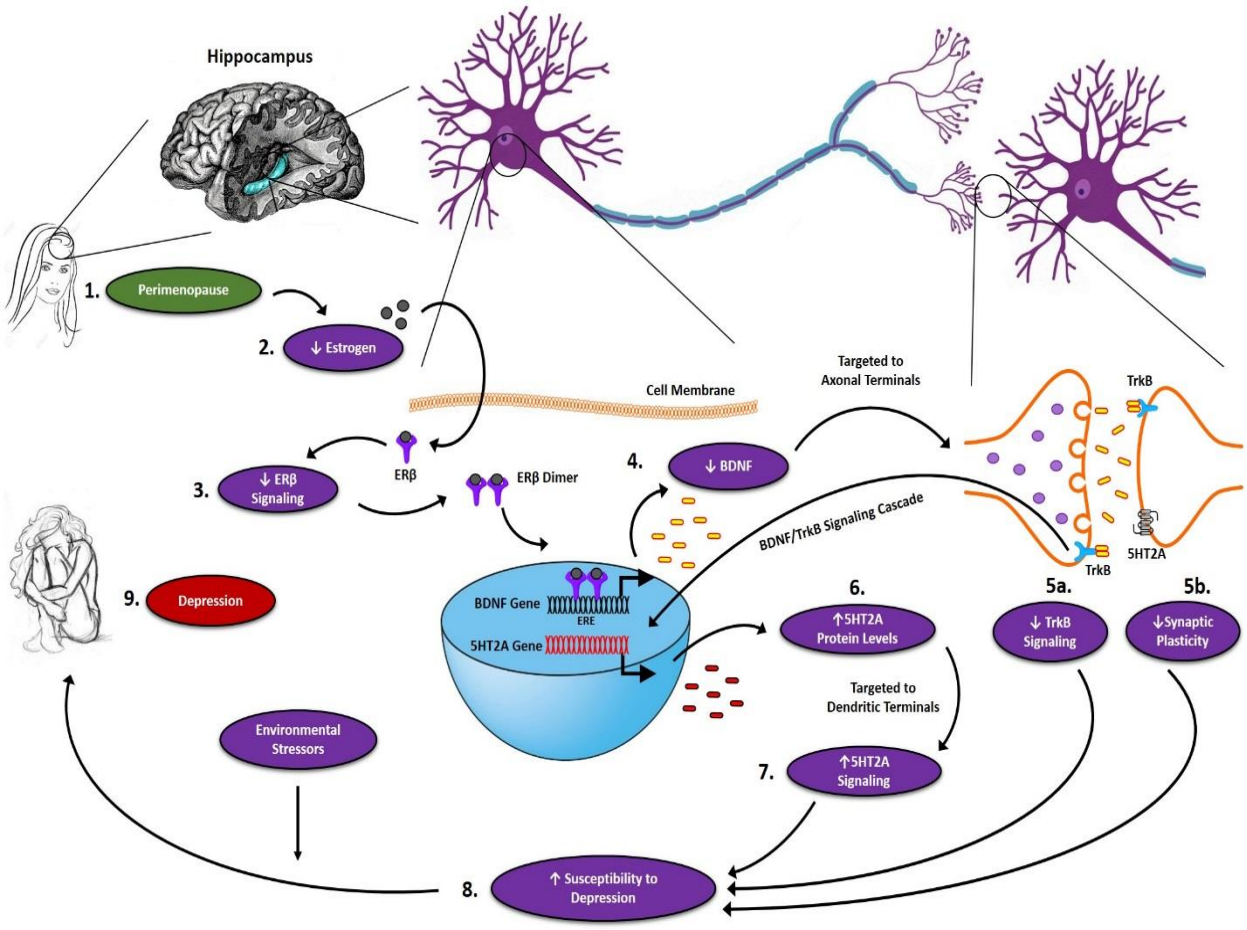

Figure 18 Proposed Hypothesis: ERβ regulation of BDNF-5HT2A signaling in female brain. 1) Perimenopausal onset results in 2) irregular profile of endogenous estrogen in the brain. Reduced estrogen homeostasis 3) decreases the stimulation of ERβ-mediated signaling in the brain. Reduced ERβ signaling results in reduced transcription of the BDNF gene, thus 4) decreasing BDNF protein levels in the hippocampus. Decreased BDNF protein levels lead to 5a) decreased pre- and post-synaptic TrkB signaling and a 5b) weakening of synaptic strength. The decrease in BDNF/TrkB signaling through unknown mechanisms upregulates the transcription of the 5HT2A gene thus 6) increasing 5HT2A protein expression. The increased 5HT2A receptor expression leads to 7) an increase in postsynaptic 5HT2A signaling. Decreased BDNF/TrkB and increased 5HT2A signaling along with decreased synaptic plasticity predisposes the brain to 8) an increased risk of developing depression, which could be exacerbated by environmental stressors leading to 9) the onset of depression.

Chapter 2

ERβ Interacts with APOE in Regulation of BDNF-5HT2A Signaling in Female Brain: Potential Link between Depression and AD

2.1. Abstract

Depression has been reported to be commonly manifested in Alzheimer's disease (AD) patients and is considered to be a risk factor for AD. Human apolipoprotein E (APOE) gene exists in three major isoforms (coded by ε2, ε3, and ε4) and the ε4 allele has been associated with a greater incidence for both depression and AD. Although mounting evidence has pointed to the potentially complex interaction between these two brain disorders in which APOE may play a role, the underlying mechanisms are largely unknown. In this study, using human APOE gene-targeted replacement mouse models, we investigated the role of APOE isoforms and how they might interact with estrogen receptor (ER) β to modulate the brain signaling involved in depression. Our initial analyses in 6-month-old female hAPOE mice demonstrated that APOE regulated the expression of BDNF and 5HT2A receptor in a genotype dependent manner, with APOE4 brain exhibiting the lowest level of BDNF and the highest level of 5HT2A. In addition, both pre- and postsynaptic proteins were downregulated indicating a synaptic deficit in APOE4 brain. Our subsequent analyses revealed that a 3-month chronic treatment with an ERβ-targeted phytoestrogenic diet induced a number of changes in APOE2 and APOE3 brains, including a significant decrease in the expression of 5HT2A receptor and an increase in BDNF/TrkB and synaptic proteins. In contrast, APOE4 brain was largely unresponsive to the treatment, with only select synaptic proteins increased in the treated group. Taken together, these results indicate that APOE4 negatively impacts BDNF-5HT2A signaling in female brain, which could in part underlie the APOE4-mediated increased risk for depression. In a larger context, this mechanism could serve as a molecular link between depression and AD particularly associated with APOE4. Enhancing ERβ activity could provide a greater therapeutic benefit in non-APOE4 carriers than in APOE4 carriers in the intervention of these brain disorders.

2.2. Introduction

2.2.1. Prevalence and economic burden of Alzheimer's disease

Alzheimer's disease (AD) is currently recognized as one of the most common and feared neurodegenerative diseases, effecting nearly 44 million people across the globe, including 5.4 million Americans [373]. According to newly accepted diagnostic criteria, AD is defined as a slow and gradual neurocognitive disease with major symptoms including amnestic behavior, visuospatial deficits, delusionary behavior and social withdrawal [374-377]. There are two types of AD: early-onset familial AD (fAD) and late-onset sporadic AD (LOAD). fAD is rare (5% of AD cases) and mostly caused by inherited genetic mutations that result in abnormal overproduction of neurotoxic β-amyloid (Aβ) peptides and senile plaques, a major neuropathological hallmark of AD. Alternatively, LOAD is the most common form of AD representing more than 95% of all human cases. It generally develops after age 60 and involves a heterogeneous and multifactorial etiology that likely includes a combination of genetic and environmental factors that influence a person's risk for developing the disease. Of the 5.4 million Americans currently living with AD, 5.2 million are over the age of 65. This number is expected to triple by the year 2050 reaching a prevalence of approximately 13.8 million Americans. The direct and indirect costs associated with AD result in a huge economic burden which amounted to $236 billion in 2015; a number estimated to reach 1 trillion by 2050 [373]. In addition, it was estimated that 15.9 million family members and unpaid caregivers provided an estimate of 18.1 billion hours of care to patients with AD and other kinds of dementia, resulting in a cost of $221.3 billion in 2015 [373]. As the number of AD cases is expected to triple by 2050 and no cure or successful treatment is currently available, it is imperative that research focuses on the identification of a treatment option to better combat AD.

In fact, it has been estimated that a treatment that delays the onset of AD by just 5 years could reduce the number of people with the disease by nearly 50% in 50 years [378].

2.2.2. Association between AD and Depression

Depression has been reported to occur in up to 50% of AD patients depending on the diagnostic criteria used. Moreover depression is considered to be present in all stages of AD [113, 114]. A study by Starkstein et al. reported that both minor (26%) and major (26%) depression is present in AD subjects. This study also reported that the severity of neurological impairment was significantly increased with the severity of depression in AD subjects [113]. A study by Suh et al. reported that depression is an independent predictor of mortality in AD patients in both community-based and nursing home-derived populations [379]. Comorbid depression in AD patients has also been significantly associated with increased need of institutionalization [380], greater health care utilization [381] and decreasing care giver's wellbeing [382, 383].

AD and depression have been found to share some determinant factors at some extent at the anatomical and molecular levels. Studies have reported neurodegeneration in frontal limbic and frontal subcortical circuits in the pathogenesis of idiopathic depression whereas a similar degeneration in cerebral frontal subcortical pathways has been proposed to contribute to depression in AD [384-387]. AD patients with history of depression have been shown to have more neuritic plaques and neurofibrillary tangles (NFTs) when compared to AD patients with no history of depression [388]. Imaging of Aβ deposits using Pittsburgh compound B radiotracer (PiB) revealed that retention of the compound in the depressed patients was comparable to that of AD patients [389]. The studies have revealed that a higher plasma ratio of $A\beta_{40}$ to $A\beta_{42}$ is

associated with a higher risk of developing AD [390, 391]. A study by Sun et al. reported that depressed subjects exhibited a higher plasma ratio of $A\beta_{40}$ to $A\beta_{42}$ when compared with non-depressed patients in the absence of cardiovascular diseases and antidepressant treatment. [392]. Another study by Namekawa et al. conducted in 100 Japanese depressed subjects reported that serum ratio of $A\beta_{40}$ to $A\beta_{42}$ was significantly higher in patients with both early-onset and late-onset depression when compared to control subjects [393]. Moreover, the dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis has also been reported in AD patients [394, 395]. An analysis of more than 150 studies reported hyper-activity of the HPA axis in 43% of depressed patients and 67% of depressed psychotic patients [396]. Alternatively, a study by Gil-Bea et al. reported higher CSF cortisol and lower glucocorticoid receptor mRNA level in frontal cortex, indicating HPA dysfunction [397]. Vascular risk factors such as hypertension, diabetes dyslipidemia, cardiovascular disease and stroke have been associated significantly both with late life depression [398] and AD [399]. Thus there seems to be an interaction between these two diseases at some molecular levels and the mechanism of depression in AD seems to be a complex one.

Depression has also been reported to be an independent risk factor for the development of AD. A systematic review and meta-analysis by Ownby et al. reported that depression increased the risk of developing AD by nearly two fold in all studies examined [400]. Another meta-analysis which included 23 community-based prospective cohort studies concluded that late life depression increased the incidence of AD by 1.65-fold [401]. A longitudinal epidemiologic study by Garcia et al. conducted in 4803 subjects aged 55 years and older reported severe depression to be a risk factor of AD [402]. Honolulu Asia Aging Study conducted in 1932 cognitively healthy men aged from 71 to 90 years reported that the depressed subjects with APOE4 allele had a 7.1 fold greater

risk of developing AD when compared to depressed subjects without APOE4 allele [403]. Taken together, these studies indicate that a history of depressive behavioral episodes is linked with the subsequent risk of developing LOAD. A prospective study by Steenland et al. performed in 5607 subjects between 2005 and 2011 concluded that depression was a strong risk factor for progression from normal to moderate cognitive impairment and a borderline significant risk factor for progression from mild cognitive impairment (MCI) to AD [404]. A strong association between AD and depression came from a study conducted by Wilson et al. in 650 elderly catholic clergy members without the initial clinical signs of AD. Most of the subjects participating in the study had on an average one depressive symptom whereas 1% of the subjects were severely depressed at the starting of the study. Interestingly after 7 years of follow up, the subjects who developed AD were the subjects who reported most depressive symptoms in the cohort. The study reported that with an increase of each depressive symptom the risk of AD was increased by approximately 20% [405]. In nut shell these studies point towards a possible and complex interaction of depression and AD, with depression being a risk factor for the later development of AD.

2.2.3. Apolipoprotein E: Role in AD and Depression

2.2.3.1. Apolipoprotein E: From Structure to Function

Human apolipoprotein E (APOE) is a 299 amino acid lipoprotein containing an N-terminal receptor binding region (amino acids 136-150) and a C-terminal lipid binding region (amino acids 244-272) linked by a flexible hinge region [406]. The APOE gene, located on chromosome 19, is comprised of four exons which are transcribed into an 1180 nucleotide long APOE mRNA transcript. The APOE gene contains several SNP's distributed across the gene [407, 408]. Two

SNPs in exon 4 of APOE gene lead to formation of three common APOE protein isoforms differing by one or two amino acids at residues 112 and 158; APOE2 (cys112, cys158), APOE3 (cys112, arg158) and APOE4 (arg112, arg158) with population frequencies of 8% 75% and 14% respectively [409].

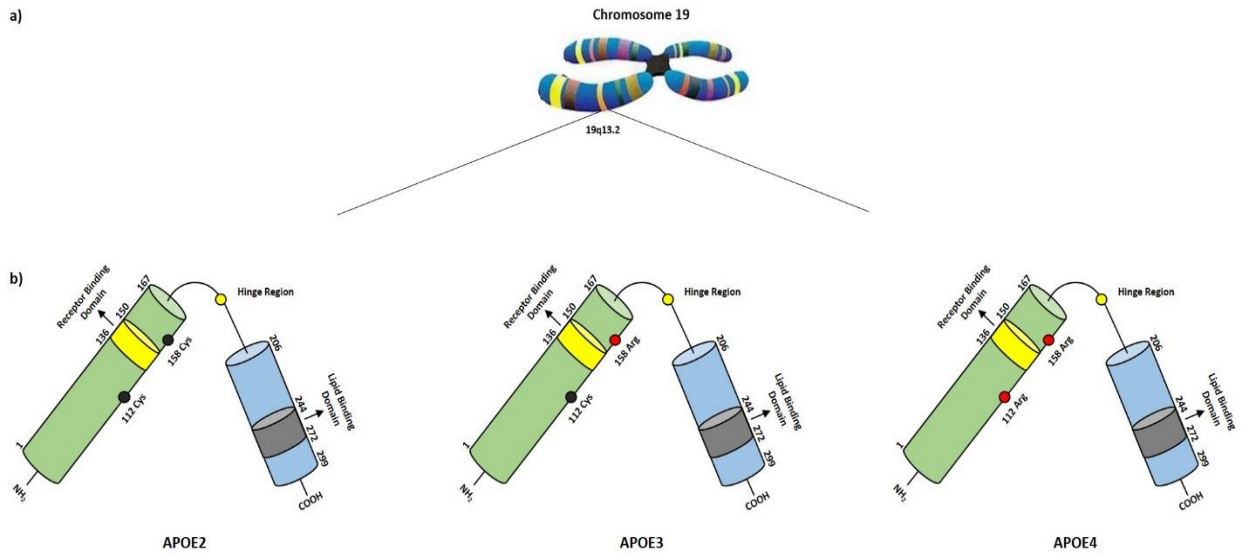

Figure 19. Structural Difference between Different APOE isoforms. a) Location of APOE on chromosome 19, position 13.2 has been illustrated. **b)** APOE is a 299 amino acid long protein having a receptor binding domain (residues 136-150) in N terminal region (residues 1-167) and a lipid binding domain (residues 244-272) in C terminal region (residues 206-299). Three major APOE isoforms, APOE2, APOE3 and APOE4 structurally differ by one or two amino acids. APOE2 has Cys residues located at both 112 and 158 positions, APOE3 has Cys residue at position 112 but Arg residue at position 158 whereas APOE4 has Arg residues at both 112 and 158 positons. The structural differences among different isoforms alters the expression and function of APOE protein significantly. Modified from [2, 4].

Although there is a mere difference of one or two amino acids, these differences alter APOE structure and function. In addition to the two structural domains (receptor and lipid binding domain) present in APOE2 and APOE3, APOE4 contains an extra domain interaction between Arg-61 and Glu-225 that renders APOE4 more susceptible to proteolysis [410, 411]. In a similar

manner this minor difference also effects the function of APOE. APOE concentrations in plasma and CSF have been reported to be dependent on the APOE isoform present (APOE2/APOE2 > APOE3/APOE3 > APOE2/APOE4 > APOE3/APOE4 > APOE4/APOE4) [412]. Hepatic parenchymal cells are major APOE producing cells, however, APOE mRNA has been found in multiple tissues including spleen, kidneys, lungs, ovaries, testes and adrenal glands [413]. In the central nervous system (CNS), astrocytes have been recognized as the major source of APOE. However, microglia, pericytes and neurons have also been reported to synthesize APOE under certain pathological conditions [413-415]. APOE mediates lipid transport in both the periphery and CNS. APOE is critical in CNS as it is the major apolipoprotein found in the brain. APOE serves an endocrine like function as it participates in the transport of cholesterol and other lipids from the site of lipid synthesis to the cell types where it is used, stored or excreted [414]. In addition to these functions, APOE can also serve paracrine like functions as APOE takes up the lipids generated after neuronal degeneration and redistributes them to cells requiring lipids for proliferation, membrane repair or myelination of new neurons [416, 417].

2.2.3.2. APOE in Late-onset Alzheimer's disease

In 1993 it was demonstrated that APOE binds to Aβ with high affinity and ApoE4 is at higher frequency in LOAD compared to unrelated age-matched controls [418]. Presence of one ε4 allele increased the risk of AD by 2-3 fold whereas presence of two ε4 alleles increased the risk for AD by 12 fold when compared to individuals with no ε4 alleles [419]. Alternatively, a study by Corder et al. reported that the APOE2 isoform reduced the risk of LOAD by 50% [420]. Another study by Farrer et al. reported that AD risk was significantly increased for subjects with the ε2/ε4 , ε3/ε4, or ε4/ε4 genotypes whereas the risk was decreased for subjects possessing the ε2/ε2 or ε2/ε3

genotypes when compared with subjects possessing the ε3/ε3 genotype [409]. ApoE4 also significantly impacts the age of onset of AD. In general, one copy of the ε4 allele shifts the risk curve for AD 5 years earlier, two copies of the ε4 allele shifts the curve 10 years earlier whereas one copy of APOE2 allele shifts the curve 5 years later [421]. A population-based study by Meyers et al. reported that 55% of APOE4/APOE4 group developed AD by age of 80 compared to 27% of APOE3/APOE4 and 9% of ApoE3/ApoE3 group [422]. While several genome-wide association studies have identified many other loci associated with the development of LOAD, all studies have confirmed that APOE is the strongest genetic risk factor associated with the development of LOAD [423-427].

Based on the association between APOE and Aβ in brains of AD patients, it was hypothesized that APOE may function as Aβ binding protein that induces a pathological β sheet conformational change in Aβ [428]. A lot of research studies have supported the hypothesis and suggested that indeed APOE4 allele dosage in association with increased Aβ burden in the brain [429-431]. Corroborating the results, in vitro aggregation studies have reported that all three APOE isoforms promoted Aβ42 fibrillation, with the effect most severe in APOE4 and least in APOE2 isoform [432]. Animal studies have added also pointed in the same direction and concluded that Aβ aggregation is dependent on specific APOE isoform. Research study by Bales et al in PDAPP mice bred to APOE2 (PDAPP/TRE2), APOE3 (PDAPP/TRE3) and APOE4 (PDAPP/TRE2) knock in mice an increase in Aβ levels in an isoform specific manner, PDAPP/TRE4 having highest Aβ burden and PDAPP/TRE2 having the lowest Aβ burden in cortex and hippocampus [433]. In addition to this study, intracerebral administration of lentivirus expressing APOE2 into the brains of PDAPP mice has been shown to significantly reduce the hippocampal Aβ burden. PET imaging studies have corroborated the results and reported that APOE4 allele increases Aβ burden in a dose

dependent manner both in cognitively normal elderly patients [434] and in prodromal AD patients [435]. Moreover, APOE is best characterized Aβ chaperone and has been heavily implicated in the clearance of Aβ [436-441]. The cellular and molecular mechanism of this modulation is not clearly understood but certain mechanisms of action have been proposed. Interestingly these proposed mechanistic propositions have been reported to work in an isoform dependent manner, with APOE2 having positive and APOE4 having negative functional effects. It has been proposed that APOE induces Aβ degradation by lowering the microglial cholesterol levels which facilitates the intracellular trafficking of Aβ to lysosomes for degradation with APOE2 having strongest while APOE4 having weakest cholesterol efflux activity[442]. It also has been reported that APOE facilitates Aβ binding and lysosomal trafficking in neurons in an isoform dependent manner with APOE3 being more efficient at it than APOE4 [443]. Unbound APOE can help in Aβ clearance by eliminating Aβ across blood brain barrier in an isoform dependent manner [444]. A study by Jain et al postulated that APOE can act both within microglia and in extracellular space to promote the proteolysis of Aβ through proteinases [445]. The study showed that APOE4 exhibited least facilitation of Aβ clearance when compared to APOE3. APOE has also been reported to differentially regulate dendritic spine density and morphology in an isoform dependent manner [446]. APOE4 TR mice have been shown to have fewer and smaller dendritic spines when compare to APOE2 or APOE3 TR mice [446]. Thus any alteration in the function of APOE as seen in the different isoforms of APOE can lead to the onset of pathological conditions which can probably precipitate to lead to diseases such as AD.

APOE genotype has been reported to be sexually dimorphic when being tested for the incidence of AD risk between males and females, showing increased interaction with female subjects compared to male subjects. The earliest evidence of this interaction was a report by Payami et al.

which used logistic regression analysis conditioned on sex and APOE genotype in 52 families with LOAD and reported that the risk of developing AD was significantly lower in ε4 males when compared with ε4 females [447]. The significance of the interaction was replicated in an independent bigger study, a meta-analysis done by Farrer et al. conducted in 5930 AD patients and 8607 control patients reported that at most ages and across all genotypes, women are more likely to develop AD than men. The author revealed that females having ε3/ε4 genotype had double the risk of developing AD when compared to the men with same genetic background [409]. Two additional studies corroborated the findings and concluded that APOE4 females have significantly greater risk of developing AD when compared to their male counterparts with same allele. The first study performed by Bretsky et al. in 195 patients used a logistic regression model to show that the presence of one or more ε4 alleles confers a substantially greater risk to women when compared to men [448]. The second study by Breitner et al. reported that the main effect of sex on AD was vanished when the study data was controlled for sex-ε4 interaction, with the association between female sex and AD applying exclusively to female ε4 carriers [449]. A recent prospective cohort study by Altman et al. conducted in 8,084 subjects aimed at examining the APOE4- sex interaction in conversion from healthy to MCI/AD or from MCI to AD reported that females having ε3/ε4 genotypes are at a significant increased risk of developing AD when compared with males having the same genotype In addition the study reported a significant APOE4-sex interaction on CSF tau levels, p-Tau levels and Tau Aβ Ratio indicating a more AD like pathology in female APOE4 carriers when compared to male APOE4 carriers [450]. Moreover APOE2 allele, anti-AD allele has been found to be more protective in men when compared to women. Altman and colleagues reported a significant interaction between APOE genotype and sex in which APOE2 allele trended to being more protective in males when compared to females [450].

Neuroimaging biomarker studies, which focused on the brain volume as the endpoint for analyzing the APOE-sex interaction are telling the same story. The above mentioned interaction between APOE4 and female sex was evident in the research study by Juottonen et al. which reported a 33% decrease in the volume of entorhinal cortex in ε4 females when compared with the males carrying the same APOE allele, even after controlling for age, duration of the disease and MMSE scores for AD detection [451]. A study by Fleisher et al. reported that women subjects having one ε4 allele have significantly reduced hippocampal volume when compared to MCI subjects with no ε4 allele, whereas men only showed a significant reduction in the hippocampal volume when carrying two ε4 alleles [452].

Following the trail of clinical studies the post mortem studies, although less in number revealed similar findings. An autopsy research study by Corder et al. which analyzed the extent of AD brain lesions for men and women at each decade between 25 to 95 years in more than 5000 subjects reported that women had more extensive senile plaques deposition throughout the brain when compared with men. Specifically, the gender gap was reported to be larger and limited to the women carrying APOE4 allele [453]. The results were corroborated by a study conducted by Damoniseaux et al. in 2012 which reported that female ε4 carriers showed a reduction in the functional connectivity in cuneal cortex, precuneus, and posterior cingulate gyrus when compared with male ε4 carriers [454].

The neurobiological significance of this interaction is quite evident as APOE-sex interaction seen in the clinical studies has been observed and replicated in animal studies too. Most of the animal studies studied the sex-APOE interaction and observed memory and cognitive performance as the end point to derive the correlation. First animal study to report this interaction was done by Raber et al. in 1998, which reported that female (NSE)-apoE4 animals had decreased performance day

to day learning capacity as evident in the water maze test when compared with male (NSE)-apoE4 animals indicating a deficit in spatial learning when comparing females and males [427]. A number of studies consolidated the results and reported that apoE4-TR female mice have significantly decreased cognition and memory function as evident from water maze test when compared to the males of same genotype background [455, 456]. A study by Rijpma et al. provided a probable reason for the observed deficit in memory function and reported that female but not male mice have decreased presynaptic density in the hippocampus specifically in the inner molecular layer of dentate gyrus [457]. In addition to the observed sexual dimorphic APOE behavior in clinical and animal models, the efficacy of AD treatment varies by gender and APOE status too. A placebo controlled double blinded study by Farlow et al. reported that tacrine was less effective in APOE4 carrying women but equally effective in the men irrespective of the APOE genotype [458].

The fact that AD prevalence, first of all being much higher in females when compared to males and in addition the observed skewed impact of APOE4 allele, the biggest risk factor of AD towards females, puts females at an immensely increased risk for developing AD when compared to the their male counterparts.

2.2.3.3. APOE in Depression

APOE, apart from being a proven risk factor of AD, has also been implicated in the etiology of depressive disorders. The first report of APOE involvement in depression came in the year 1996 when Ramachandran et al. reported that AD patients with APOE3/APOE4 genotype had more than 3 fold increase in the signs of depression when compared with APOE3/APOE3 genotype subjects [459]. Another research study that came out a year later also reported a positive correlation

between APOE4 genotype and depression in AD patients [460]. A relatively recent research study conducted in 323 AD patients also reported a significantly higher prevalence of APOE4 genotype in depressed AD patients compared to non-depressed patients. The observed effect was primarily significant for women, wherein women subjects possessing APOE4 allele were 4 times more likely to be depressed when compared to women carrying an APOE allele other than APOE4. Moreover the presence of APOE4 in men subjects did not correlate with the onset of depression implicating a sex-based association of this effect [461]. Although these studies were quite informative, they were not able to segregate and determine whether APOE4 is an independent risk factor for depression or interacting with the AD to produce the observed correlation.

Addressing this important shortcoming two independent studies reported high prevalence of ApoE4 in depressed patients having no sign of clinical AD. First study performed by Rigaud et al. in 140 subjects divided into the categories of AD subjects, early onset depressive subjects and late onset depressive subjects reported that APOE4 frequency was significantly higher in late onset depressed patients when compared to normal healthy non depressed controls implicating a possible interaction of APOE4 and depression [462]. Second study which was a community based study carried out in a UK African-Caribbean population aged 55-75 years old reported the presence of APOE4 in 69% of individuals with depression and the interaction of these two factors was shown to be significantly associated further with subjective memory impairment in those subjects [463]. A research study by Butters et al. comparing the distribution of APOE2, APOE3 and APOE4 alleles in late life depressive patients reported that the mean age of onset of first lifetime depression episode in APOE4 carriers was significantly lower than the mean age of onset of non-carrier patients [464]. Despite these findings in the support of the notion of interaction between APOE genotype and depression, there are quite a few studies that tell otherwise [465-467].

These earlier findings are further supported by two recent clinical studies. A clinical research study by Sureshkumar et al. conducted in 31 cases of late-onset depression aged above 50 years and 31 age matched controls revealed a significant association between the APOE4 allele and depression when compared to control patients. The study concluded that the elderly in India who possess the ε4 allele have 4.7 times more risk of developing depression when compared to the age-matched control subjects [468]. The findings were corroborated in a larger study performed by Skoog et al. which recruited 839 men and women aging between 70-92 years old and having no signs of clinical depression or dementia at the start of the study reported a significant association between APOE4 status and onset of depression. The study concluded that APOE4 allele was significantly associated with incident minor depression, severe depression or depressive symptom of any kind, implicating that APOE4 allele can potentially be used to identify people at high risk of clinical depression [469].

In addition to the proven increased risk of depression in the presence of APOE4 allele, APOE2 genotype has been associated with protective effect against the onset of depressive disorders. For instance a study by Fan et al in 273 Taiwanese patients with depression and 429 healthy controls reported lower frequency of APOE2 allele in depressed patients when compared to other two APOE isoforms implicating a possible protective role of APOE2 against depression [470]. The results were further corroborated in a study performed by Julain and colleagues which revealed that presence of APOE2 allele significantly predicted the increased positive effects and was associated with decreased depressive symptoms when compared with subjects without APOE2 allele [471].

The neuroimaging studies although very few in number tell the same phenomenon. Neuroimaging biomarker studies mainly focused on the brain volume as the endpoint for analyzing the APOE-

depression interaction. For instance, a study by Kim et al. compared 45 depressed patients for genotype related differences in hippocampal volume and reported that subjects with APOE4 allele showed significant reduction in hippocampal volume, even after controlling for age, gender, MMSE scores and baseline cerebral volume [472]. The results were further strengthened by an MRI study which reported that the depressed subjects with one copy of APOE4 allele showed more pronounced hippocampal atrophy when compared to the depressed subjects lacking the APOE4 allele copy [473]. Yuan et al reported similar set of results but significant in more brain regions, the study revealed significantly smaller volumes of right medial frontal gyrus, left middle frontal gyrus and left inferior occipital gyrus in APOE4 carrying depressed patients when compared with non-carriers [474]. In nut shell these studies point towards a possible complex interaction of APOE, AD and depression but the underlying mechanistic molecular etiology of this interaction is still unknown and thus provides a compelling frontier for research.

2.2.4 Phytoestrogens

Phytoestrogens are nonsteroidal plant-derived compounds that structurally or functionally mimic endogenous mammalian estrogens especially E2 [475-477]. Phytoestrogens, which are divided into three classes (isoflavones, lignans and coumestans), were first reported to exhibit estrogenic activities in 1926 [478]. Specifically, phytoestrogens have been shown to elicit a biochemical response by binding to estrogen receptors (ERs), through a phenolic ring present in the chemical structure [479, 480]. However, while phytoestrogens function via ER-mediated signaling, these compounds exhibit 102 to 105 times less estrogenic activity when compared with human estrogens [476]. Interestingly, phytoestrogens can function both as agonists and antagonists depending on the tissue, ER and concentration of circulating endogenous estrogens.

There are several classes of phytoestrogens: steroidal estrogens, found in few plants and the more ubiquitous phenolic estrogens, isoflavones, coumestans and lignans. Among the three classes of phytoestrogens, isoflavones comprise the largest class containing more than 1000 members and are the most well-known of the phytoestrogens. Naturally occurring isoflavones that have shown estrogenic activity are: the aglycones (daidzein (4′,7-dihydroxyisoflavone) and genistein (4′,5,7-trihydroxyisoflavone)), the glycosides (daidzin and genistin) [476, 481]. Isoflavones are usually found in legumes such as soy, chickpeas, clover, lentils and beans [476]. After mammals consume isoflavones, daidzein and genistein are metabolized in the gastrointestinal tract, daidzein gets metabolized to O-desmethylangolensin and eqoul to exert its biological effects [481]. Coumestans are another group of plant phenols that show estrogenic activity. Coumestrol was first reported in 1957 by Bickoff and coworkers as a new phytoestrogen that was isolated from ladino clover, strawberry clover and alfalfa or Lucerne [482]. The main coumestans with phytoestrogenic effects are coumestrol and 4′-methoxycoumestrol. Coumestans are less common in the human diet than isoflavones [483], yet similar to isoflavones, in that they are also found in legumes, particularly food plants such as sprouts of alfalfa and mung bean [484, 485]. The third class of phytoestrogens, lignans were first identified in plants and later in biological fluids of mammals [486]. The most well-known phytoestrogenic lignans are secoisolariciresinol and matairesinol and are found in cereal, rye bread, oilseeds, black tea or coffee and food legumes [486].

Phytoestrogens have been reported to contribute towards a lower incidence of menopausal symptoms [487], cardiovascular diseases [488], breast cancer [489], prostate cancer [490] and osteoporosis [491] when compared with normal age-matched control subjects. In addition, use of phytoestrogens has been shown to be neuroprotective against the Aβ induced damages in AD. A study by Choi et al. demonstrated that green tea extract, a source of flavonoids attenuates Aβ

induced neurotoxicity in cultured hippocampal neurons. The study showed that co treatment of primary hippocampal cells with neurotoxic Aβ and green tea extract increased the cell survival and decreased caspase activity when compared with vehicle treated group [492]. Another study replicated these results using black tea extracts and demonstrated that the black tea extract inhibited the Aβ aggregation and Aβ derived neurotoxic ligands, thus inhibiting the apoptotic effects induced by Aβ [493].

Table 2 Binding Affinity of Estrogenic Compounds

Compounds	ERα		ERβ		Selectivity	GPER1	
	IC_{50}	RBA	IC_{50}	RBA	(β/α)	IC_{50}	RBA
	μM	%	μM	%		μM	%
17β-Estradiol	0.0253	100.00	0.0325	100.00	0.78	0.0178	100.00
Genistein	4.7355	0.5343	0.0789	41.12	60.00	0.133	13.41
Daidzein	26.65	0.0949	1.738	1.867	14.27	-	-
Equol	5.876	0.4306	0.5825	5.571	10.09	-	-
G+D	9.896	0.2557	0.1574	20.62	62.87	-	-
G+D+E	15.71	0.1610	0.1902	17.06	82.60	-	-

Table 2 Binding Affinity of Different Estrogenic Compounds. Table showing binding affinity and relative binding affinity of individual class of phytoestrogens and different combinations. RBA refers to relative binding affinity of the compound that is expressed as a percentage of the binding affinity of 17β-Estradiol. Data taken and modified from [3]

Moreover Levites et al. revealed that the same green tea extract enhances the release of non-amyloidogenic soluble form of amyloid precursor protein (sAPPalpha) into the culture medium of human SH-SY5Y neuroblastoma and rat pheochromocytoma PC12 cells implicating its possible role in decreasing disease pathogenesis [494]. Moreover isoflavones such as gycitein, genistein and daidzein have been shown to bind the monomeric form of transthyretin protein, a protein that binds to Aβ and prevents plaque formation [495]. Phytoestrogens have been shown to have more

binding affinity towards ERβ than ERα [496, 497] (Table 2). In addition ERβ is expressed more in hippocampus when compared to ERα [498, 499] and thus has been implicated in mediating the beneficial effects of phytoestrogens.

In the light of these findings, a specific combination of clinically relevant phytoestrogens which include genistein, daidzein and eqoul has been developed by Zhao et al. in 2009, and has been reported to exhibit an 83 fold binding selectivity for ERβ over ERα [3]. The formulation known as phyto-β-SERM when chronically administered has been reported to improve physical health, prolonged survival, improve spatial recognition memory and attenuate Aβ deposition and plaque formation in the brains of triple transgenic female mice [500]. Collectively, as phytoestrogens elicit the same beneficial effects as ET while limiting the adverse side effects phytoestrogen formulations could possibly be used as a suitable alternative for the treatment of AD in the place of conventional ET [151, 501, 502].

Thus the goals of the second part of my thesis were to elucidate the probable mechanism that underlies the association between ApoE4 genotype and depression by examining the differential modulation of serotonergic signaling, BDNF signaling and synaptic function by different ApoE isoforms. The major hypothesis of my study was that the presence of ApoE4 isoform dysregulates serotonergic and BDNF signaling pathways when compared to ApoE3 isoform. On the other hand we predicted that the presence of ApoE2 isoform will protect the subjects from dysfunctional molecular manifestations of depression. The study also analyses the possibility of ERβ activation to be used as a therapeutic target to reduce the deficits observed in the animals. The data overall provides a probable mechanistic rationale for the increased risk of depression associated with ApoE4 genotype which further increases the susceptibility of developing AD. In addition the data

points towards the use of the ERβ-targeted phyto-β-SERM as possible therapeutic agent for reducing the risk of depression, thereby reducing the susceptibility of an individual to develop AD.

2.3. Materials and Methods

2.3.1. Animal Model

Human ApoE Gene Targeted Replacement Mouse Model. The mouse lines were created by gene targeting and carry one of the three human APOE alleles in place of the endogenous murine ApoE gene while retaining the endogenous regulatory sequences required for modulating hApoE expression. These mice share a C57BL/6J genetic background and express the human APOE protein at physiological levels; thus, they provide a complete in vivo system that allows direct measurement and comparison of hApoE isoform-specific effects [503, 504]. The following experiments were conducted on cortical brain tissue collected from 6-month-old hApoE2, hApoE3, and hApoE4 female mice (n=5 for each genotype group).

2.3.2. Phyto-β-SERM Treatment. Both rodent diets were custom manufactured by Harlan Laboratories (Madison, WI). The base/control diet was prepared from Teklad Global 16% Protein Rodent Diet (Harlan Laboratories), which was ground and repelleted. This diet has a fixed formula and is nutritionally balanced, containing 16% protein and 3.6% fat that support the growth and maintenance of rodents and not containing alfalfa or soybean meal, thus minimizing the levels of natural phytoestrogens. The phyto-β-SERM diet was prepared by adding equal parts of genistein, daidzein, and equol (LC Laboratories, Woburn, MA) to the base diet. A total of 100 mg (genistein, daidzein, and equol) was added per 1,000-g diet. This diet would deliver to mouse a daily intake of 0.25 mg of added phyto-β-SERM formulation (genistein, daidzein and equol), or 10 mg/kg (body weight [BW]) mouse per day, assuming a 25-g mouse eating a 2.5-g diet per day. The diet was designed to deliver to the mice a total amount of added phytoestrogens that is biologically equivalent to a daily intake of 50 mg in humans. The conversion of human dose to mouse equivalent dose was based on the conversion factor of equivalent surface area dose from human to

mouse18: 50 mg/60 kg (BW, human) * 12 (human to mouse conversion factor) = 10 mg/kg (BW, mouse). The treatment was started when mice were 3 months old for a span of 3 months and at the end of the treatment, mice were sacrificed immediately and brain tissues were collected.

2.3.3. Tissue Protein Extraction. Tissue samples were homogenized using the Bullet Blender 24 Homogenizer (Next Advance, NY, USA) in T-PER reagent (Pierce Biotechnology, IL, USA) supplemented with protease and phosphatase inhibitors (Roche Applied Science, IN, USA) and 100 µL 0.5 mm glass beads (Next Advance, NY, USA) at speed 8 for 3 min at 4°C followed by centrifugation at 12,000 rpm for 8 min at 4°C. Supernatant was transferred to a new micro centrifuge tube and protein concentration was determined via BCA Assay (Pierce Biotechnology, IL, USA).

2.3.4. Western blotting. Equal amounts of total protein (20 µg/lane) were loaded and separated by 10% SDS-PAGE. Resolved proteins were transferred to 0.2 µm pore-sized PVDF membranes (Bio-Rad, CA, USA) and blocked with 5% Blotting Grade Blocker (BioRad, Hercules, CA) in TBST (100 mL 10X 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, USA); rabbit polyclonal anti-β Actin (1:3000; Thermo Scientific, MA, USA); rabbit polyclonal anti-TrkB (1:1000; Abcam, MA, USA); mouse monoclonal anti-β Tubulin (1:3000; Thermo Scientific, MA, USA); rabbit polyclonal pTrkB (1:1000; Bioworld Technology Inc., MN, USA); mouse monoclonal anti-MAP2 (1:750; Thermo Scientific, MA, USA); rabbit monoclonal anti-NeuN (1:3000; Abcam, MA, USA); rabbit polyclonal anti- PSD95 (1:500; Abcam, MA, USA); rabbit monoclonal anti-Synaptophysin (1:1000; Abcam, MA, USA); mouse monoclonal anti-SHANK3 (1:1000; NeuroMab, CA, USA) and rabbit polyclonal anti-Synaptobrevin2 (1:1000; Enzo Lifesciences, NY, USA).

2.3.5. Statistical Analysis. The statistical analyses were conducted using GraphPad Prism version 5.0 (Graph Pad software Inc., CA, USA) and is presented as mean±standard deviation. For data comparisons between two groups, Student's *t*-test and for analysis involving multiple groups, one-way ANOVA/two way ANOVA followed by *post* hoc Bonferroni's test was used. For all purposes, $p<0.05$ was considered as statistically significant.

2.4. Results

2.4.1. 5HT2A but not 5HT1A receptor expression is regulated by ApoE in a genotype-dependent manner.

In order to elucidate the differential role of ApoE genotype in regulating serotonergic signaling we used human ApoE2, ApoE3 and ApoE4 gene targeted replacement mouse model (ApoE2, ApoE3, and ApoE4). Cortical tissues were harvested from female 6-month-old ApoE2, ApoE3, and ApoE4 mice and probed for 5HT1A and 5HT2A immunoreactivity. Our rationale for choosing to examine only 5HT1A and 5HT2A receptors lies in the fact that these two receptors are most well characterized serotonergic receptors in the field of depression.

The data indicate no significant differential regulation of 5HT1A receptor expression by ApoE genotype (Figure 20a, $F(2,6) = 0.4805$, $p=0.6404$, NS). In contrast, we observed a differential regulation of 5HT2A receptor expression among three ApoE genotypes. Our data revealed an up-regulation of 15%-20% in 5HT2A expression level in ApoE3 brain when compared to ApoE2 brain whereas an up regulation of 30% in 5HT2A expression level was evident in ApoE4 animals when compared to ApoE2 animals (Figure 20a, $F(2,6) = 12.76$, $p=0.0046$; ApoE2 vs ApoE3, $p=0.0483$; ApoE2 vs ApoE4, $p=0.0021$; ApoE3 vs ApoE4, $p=0.1701$, NS).

2.4.2. BDNF but not TrkB receptor expression is regulated by ApoE in a genotype-dependent manner. In addition to serotonergic signaling, we examined the differential regulation of BDNF signaling by ApoE genotypes. The data indicate a significant decrease in BDNF expression levels in ApoE4 animals when compared to ApoE2 animals ($p=0.0021$), whereas no significant differences were found when comparing ApoE2 with ApoE3 animals ($p=0.0483$). The data also

indicates a decrease of approximately 10% in BDNF levels in ApoE3 animals when compared to ApoE4 animals (Figure 20b, p=0.1701). The data in addition also reveals no differential regulation of TrkB expression level among different ApoE genotypes (Figure 20b, F(2,6) = 0.2108, p=0.8157, NS).

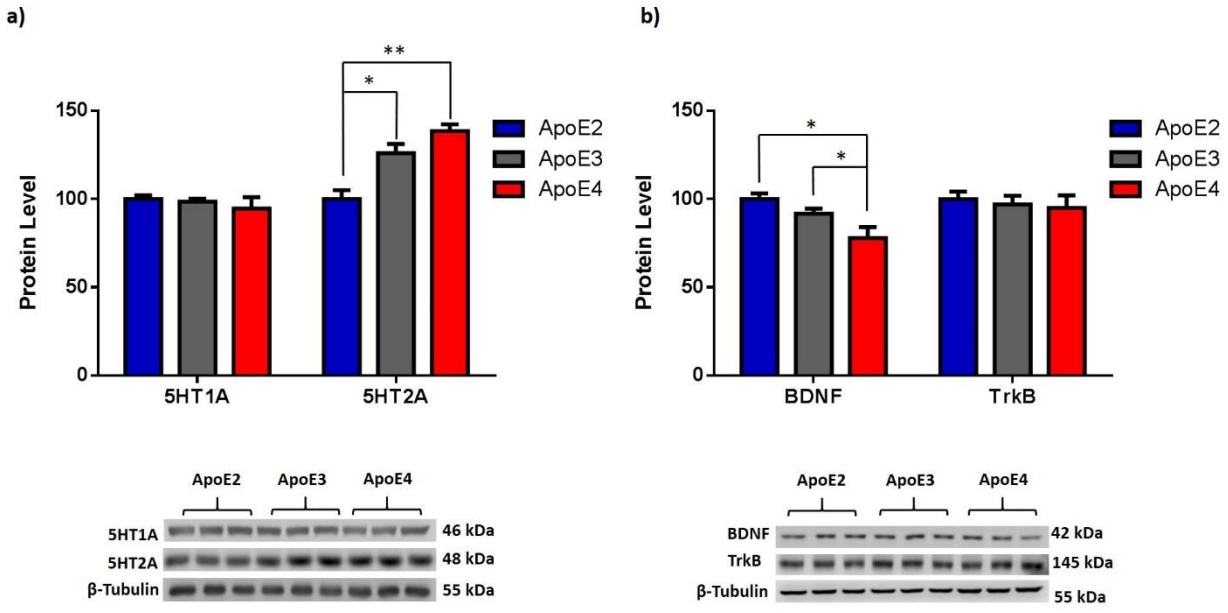

Figure 20. 5HT2A and BDNF/TrkB Expression Levels are regulated in an ApoE Genotype – dependent Manner. Expression levels of (a) 5HT1A and 5HT2A and (b) BDNF and TrkB protein were examined in the cortex of human ApoE2, ApoE3 and ApoE4 gene targeted replacement mouse model. The integrated density value of the bands in western blots was determined using densitometry and data was normalized to an internal loading control (β Tubulin) and to wild type group. Data is shown as mean ± standard deviation, n=3. One-way ANOVA with Bonferroni post hoc test *p<0.05, **p<0.01.

2.4.3. Functional but not structural presynaptic proteins are regulated by ApoE in a genotype-dependent manner. Depression has been shown to down regulate the expression levels of presynaptic proteins; a phenomena which is reversed in patients treated with antidepressants [505]. In order to elucidate the possible differential regulation of presynaptic proteins among ApoE genotypes, cortical tissues of hApoE-TR animals were probed for synaptophysin, a synaptic vesicle structural protein, and synaptobrevin 2, a SNARE involved in the docking of vesicles to the membrane.

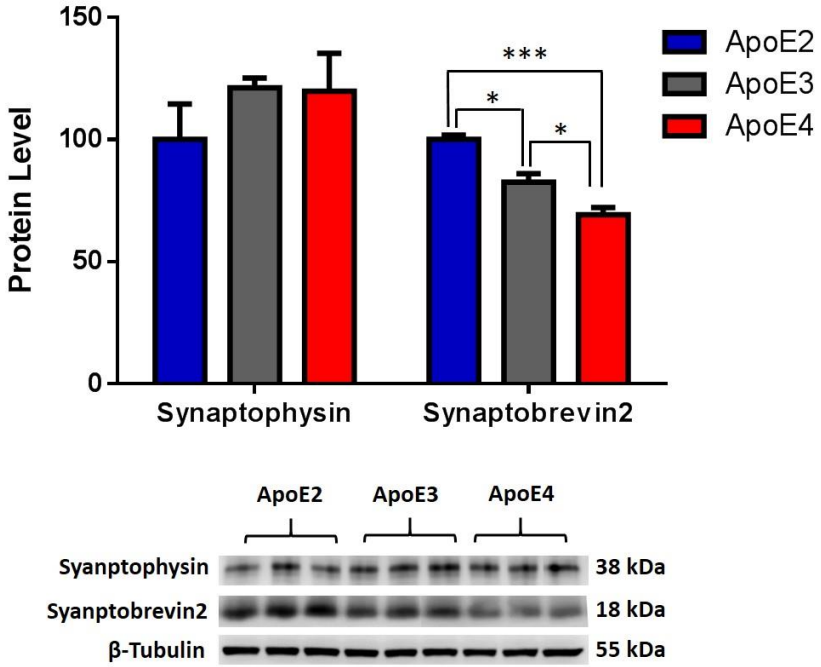

Figure 21. Functional, but not Structural Presynaptic Proteins are regulated in an ApoE Genotype Dependent Manner. Expression levels of presynaptic proteins, synaptophysin and synaptobrevin2 were examined in the cortex of human ApoE2, ApoE3 and ApoE4 gene targeted replacement mice. The integrated density value of the bands in western blots was determined using densitometry and data was normalized to an internal loading control (β Tubulin) and to the ApoE2 group. Data is shown as mean ± standard deviation, n=5. 10% SDS-PAGE gels. One-way ANOVA with Bonferroni post hoc test *p<0.05, **p<0.01, ***p<0.001.

The results indicate that synaptophysin was not regulated by ApoE genotype as no significant difference in expression levels of synaptophysin were seen when comparing the three animal groups (Figure 21, $F(2,6) = 0.8986$, $p=0.4557$, NS). In contrast, our data indicate that synaptobrevin 2 levels were differentially modulated by ApoE genotype. Specifically, the data demonstrated a 15% decrease in synaptobrevin 2 expression in ApoE3 animals when compared to ApoE2 animals ($p=0.0111$), a 30% decrease in ApoE4 animals when compared to ApoE2 animals ($p=0.0010$), and a 15% decrease in ApoE4 animals vs ApoE3 animals (Figure 21, $F(2,6) = 29.10$, $p=0.0436$).

2.4.4. Postsynaptic proteins are downregulated in ApoE4 brain. In addition to presynaptic proteins, function and expression levels of post synaptic proteins have also been shown to decrease in depression [506]. Therefore, to elucidate the possible differential regulation of postsynaptic proteins by ApoE genotypes, we examined the protein expression of PSD95 and SHANK3 in cortical tissues from ApoE animals. The data indicates that PSD95 was differentially regulated by ApoE isoforms. Specifically, we observe a 15% decrease in the expression levels of PSD95 in ApoE4 animals when compared to both ApoE2 and ApoE3 animals with no significant difference occurring between ApoE2 and ApoE3 animals (Figure 22, $F(2,6) = 7.857$, $p=0.0211$; ApoE2 vs ApoE3, $p=0.8833$, NS; ApoE2 vs ApoE4, $p=0.0003$; ApoE3 vs ApoE4, $p=0.0445$).

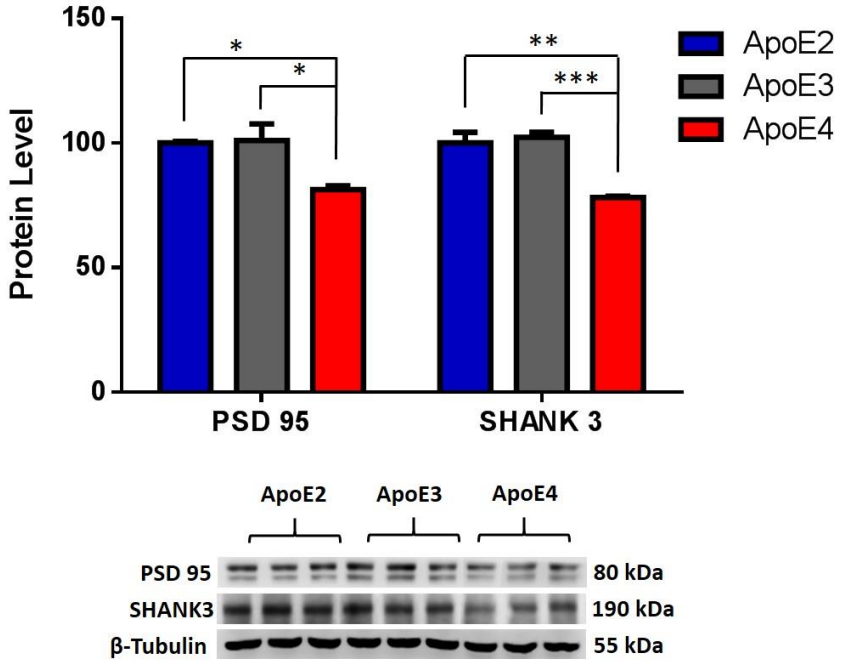

Figure 22. ApoE isoforms differentially regulate expression level of posy synaptic proteins. Expression levels of postsynaptic proteins, PSD95 and SHANK3 was examined in the cortex of human ApoE2, ApoE3 and ApoE4 gene targeted replacement mouse model. The integrated density value of the bands in western blots was determined using densitometry and data was normalized to an internal loading control (β Tubulin) and to the ApoE2 group. Data is shown as mean ± standard deviation, n=5. 10% SDS-PAGE gels. One-way ANOVA with Bonferroni post hoc test *p<0.05, **p<0.01, ***p<0.001.

Similar to these findings, SHANK3 expression levels were also decreased by 20% in ApoE4 animals when compared to both ApoE3 and ApoE2 animals with no difference occurring between ApoE2 and ApoE3 animals (Figure 22, $F(2,6) = 24.88$, $p=0.0007$; ApoE2 vs ApoE3, $p=0.6153$, NS; ApoE2 vs ApoE4, $p=0.0073$; ApoE3 vs ApoE4, $p=0.0002$).

2.4.5. Phyto-β-SERM treatment significantly decreases 5HT2A expression in ApoE2 and ApoE3 brains but not in ApoE4 brain. Estrogen use has been implicated in alleviating the mood-related symptoms in depression as well in improving cognition and memory-related deficits in AD. To examine the beneficial effects of ERβ-mediated signaling, our transgenic animals were administered a control diet or a phyto-β-SERM-supplemented diet for 3 months and sacrificed at 6 months of age. Our data indicate that phyto-β-SERM treatment significantly decreased the expression levels of 5HT2A receptor in both ApoE2 and ApoE3 animals, but not in ApoE4 animals (Figure 23, ApoE2, p=0.0414; ApoE3, p=0.0075; ApoE4, p=0.3206, NS).

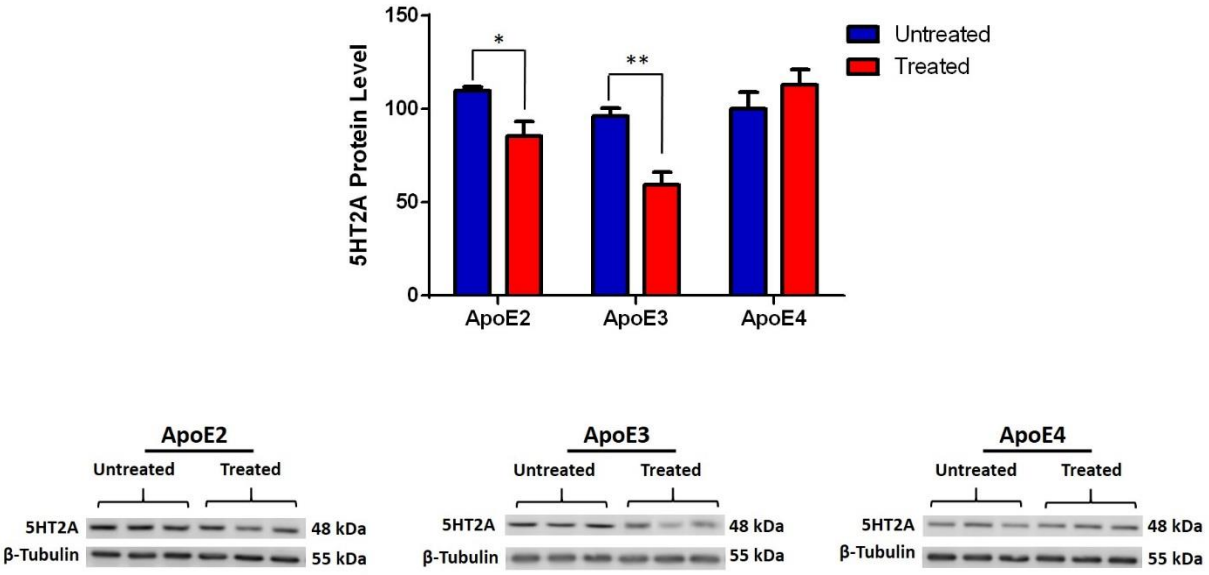

Figure 23. ERβ Activation Leads to Decreased Levels of 5HT2A in hApoE2-TR and hApoE3-TR animals, but not in hApoeE4-TR Animals. Female hApoE2-TR, hApoE3-TR, and hApoE4-TR were administered a daily Phyto-β-SERM supplemented diet or control diet for 3 months and sacrificed at the age of 6 months. Cortical tissues were probed for 5HT2A immunoreactivity. The integrated density value of the bands in western blots was determined using densitometry and data was normalized to an internal loading control (β Tubulin) and to the untreated group of each genotype. Data is shown as mean ± standard deviation, n=5. 10% SDS-PAGE gels. t test *p<0.05, **p<0.01.

2.4.6. Phyto-β-SERM treatment significantly increases BDNF/TrkB signaling in ApoE2 and ApoE3 brains but not in ApoE4 brain. . In addition to 5HT2A receptor expression levels, we also examined the effect of phyto-β-SERM treatment on the expression of BDNF and TrkB. Our data indicate that phyto-β-SERM treatment resulted in a significant increase in the expression levels of BDNF in ApoE3, but not in ApoE2 animals and ApoE4 animals (Figure 24; ApoE2, p=0.2586, NS; ApoE3, p=0.0422; ApoE4, p=0.7721, NS). The data also revealed that 3 month treatment with Phyto-β-SERM increased the expression levels of TrkB receptor in ApoE2 and ApoE3 animals, but not in ApoE4 animals (ApoE2, p=0.0283; ApoE3, p=0.0033; ApoE4, p=0.6400, NS).

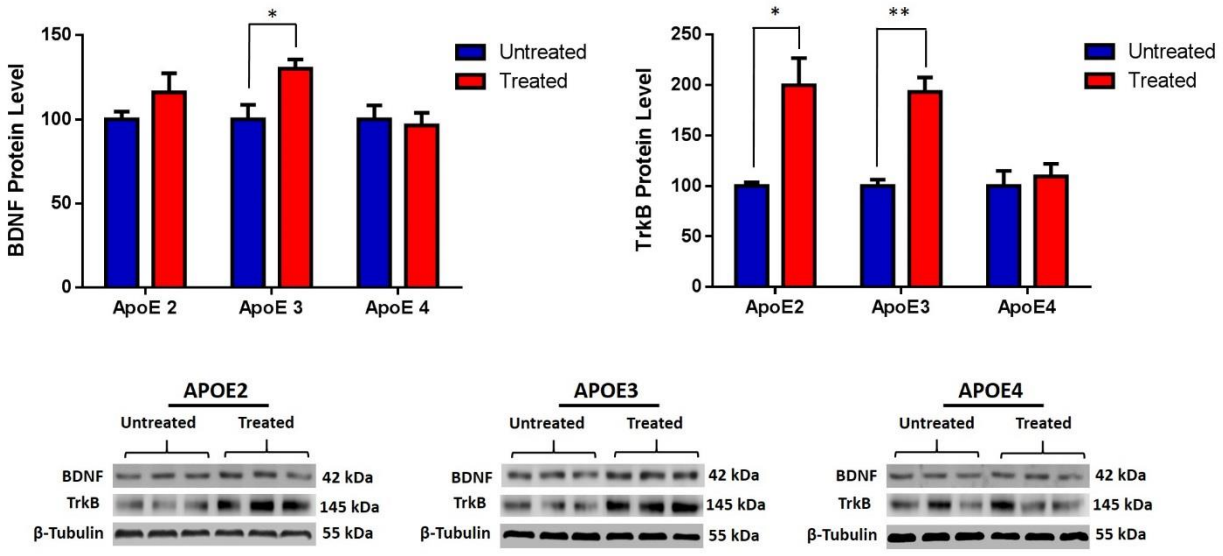

Figure 24. ERβ Activation Upregulates BDNF and TrkB expression in hApoE2-TR and hApoE3-TR animals, but not in hApoeE4-TR Animals Female hApoE2-TR, hApoE3-TR, and hApoE4-TR were administered a daily Phyto-β-SERM supplemented diet or control diet for 3 months and sacrificed at the age of 6 months. Cortical tissues were probed for BDNF and TrkB immunoreactivity. The integrated density value of the bands in western blots was determined using densitometry and data was normalized to an internal loading control (β Tubulin) and to the untreated group of each genotype. Data is shown as mean ± standard deviation, n=5. 10% SDS-PAGE gels. t test *p<0.05, **p<0.01.

2.4.7. Phyto-β-SERM treatment regulates the expression of presynaptic proteins in an ApoE genotype-dependent manner. In context of the deleterious down regulation of functional presynaptic proteins in ApoE4 animals, we next examined whether the diet can modulate the aforementioned pre-synaptic proteins in 6-month-old female ApoE mice. Our data revealed that phyto-β-SERM treatment resulted in a significant increase in the expression levels of synaptophysin in ApoE2 and ApoE3 animals, but not in ApoE4 animals (Figure 25, ApoE2, p=0.0085; ApoE3, p=0.0010; ApoE4, p=0.3499, NS). In contrast, the expression levels of synaptobrevin 2 were increased in all animals administered phyto-β-SERM diet regardless of ApoE genotype (Figure 25; ApoE2, p=0.0001; ApoE3, p=0.0001; ApoE4, p=0.0002).

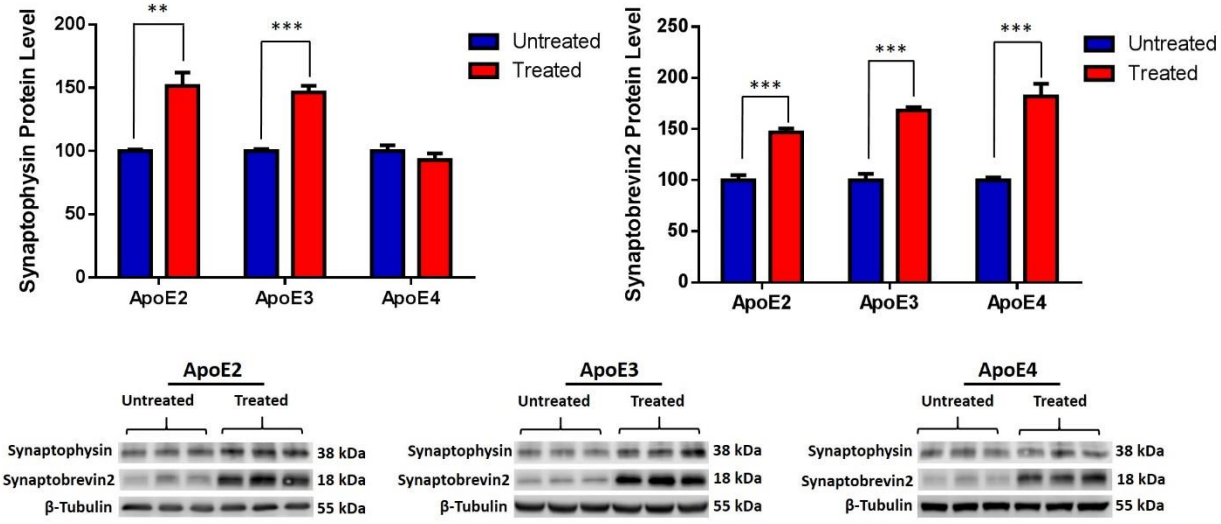

Figure 25. ERβ Activation Leads to an Upregulation of Presynaptic Proteins in an ApoE Genotype Manner. Female hApoE2-TR, hApoE3-TR, and hApoE4-TR were administered a daily Phyto-β-SERM supplemented diet or control diet for 3 months and sacrificed at the age of 6 months. Cortical tissues were probed for Synaptophysin and synaptobrevin 2 immunoreactivity. The integrated density value of the bands in western blots was determined using densitometry and data was normalized to an internal loading control (β Tubulin) and to the untreated group of each genotype. Data is shown as mean ± standard deviation, n=5. 10% SDS-PAGE gels. t test *p<0.05, **p<0.01, ***p<0.001.

2.4.8. Phyto-β-SERM treatment regulates the expression of postsynaptic proteins in an ApoE genotype-dependent manner. In addition to presynaptic proteins, our data revealed that treatment with phyto-β-SERM can lead to a significant increase in the expression levels of PSD95 in ApoE2, ApoE3, and ApoE4 animals (Figure 26; ApoE2, p=0.0101; ApoE3, p=0.3293, NS; ApoE4, p=0.0013). In contrast, the expression levels of SHANK3 were not altered following chronic treatment with phyto-β-SERM (Figure 26; ApoE2, p=0.3485; ApoE3, p=0.33, NS; ApoE4, p=0.5121).

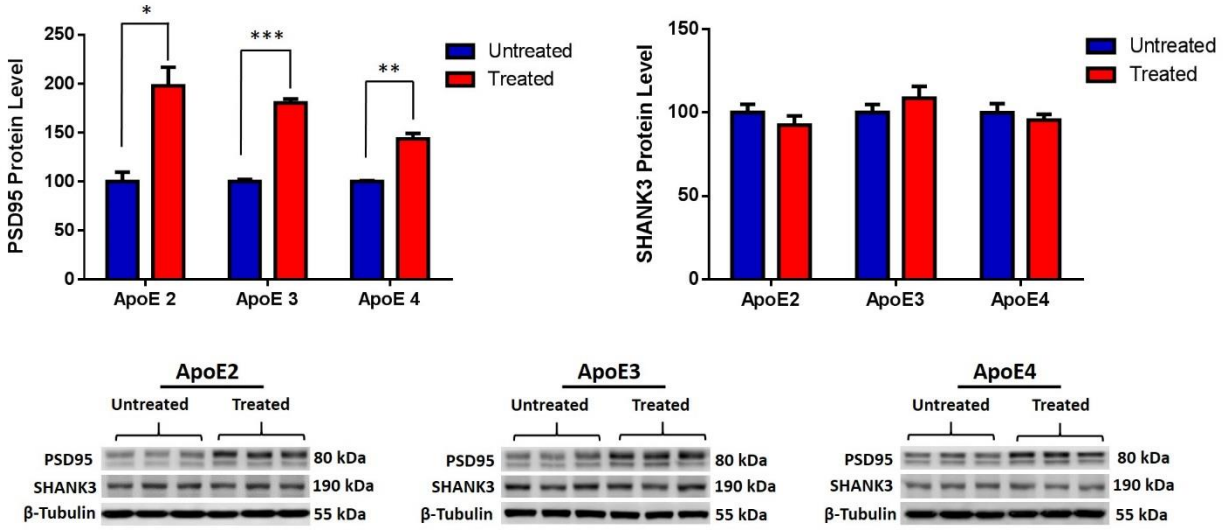

Figure 26. ERβ Activation Leads to an Upregulation of Postsynaptic Proteins in an ApoE Genotype Manner. Female hApoE2-TR, hApoE3-TR, and hApoE4-TR were administered a daily Phyto-β-SERM supplemented diet or control diet for 3 months and sacrificed at the age of 6 months. Cortical tissues were probed for PSD95 and SHANK3 immunoreactivity. The integrated density value of the bands in western blots was determined using densitometry and data was normalized to an internal loading control (β Tubulin) and to the untreated group of each genotype. Data is shown as mean ± standard deviation, n=5. 10% SDS-PAGE gels. t test *p<0.05, **p<0.01, ***p<0.001.

2.5. Discussion

Recent clinical studies have found an association between depression and ApoE4, a major risk factor for AD. Our study aimed at determining how ApoE isoforms may differentially regulate the molecular signaling pathways implicated in depression. The major hypothesis of our study was that the ε4 allele dysregulates serotonergic and neurotrophic pathways when compared to the ε3 allele. In contrast, we predicted that possession of the ε2 allele will protect the subjects from dysfunctional molecular manifestations of depression. In our analysis, we used 6-month-old human ApoE2, ApoE3 and ApoE4 gene targeted replacement mice in an effort to elucidate whether or not our hypothesis holds true in this animal model.

Our analysis in the cortical tissue harvested from female 6-month-old ApoE2, ApoE3, and ApoE4 mice revealed that 5HT2A, but not 5HT1A receptor expression was modulated in an ApoE genotype dependent manner (Figure 20 a). Our data indicated a 20% and 25% increase in the expression level of 5HT2A receptors when comparing ApoE2 with ApoE3 animals and ApoE2 with ApoE4 animals respectively. These findings correspond with clinical findings that have reported an increase in 5HT2A receptor expression in the depressed patients. A radiolabeled binding study from Mann et al., which used [3H]spiperone as a 5HT2A ligand, reported that the binding potential of 5HT2A receptors is higher in post-mortem brains of suicidal depressed patients compared to healthy controls [229]. These results have been replicated in several studies that used different 5HT2A ligands but reported a similar increase in the binding potential of 5HT2A receptors primarily in the prefrontal cortex of depressed patients compared to healthy control subjects [230]. Additionally, subjects with high scores on the dysfunctional attitude scale exhibit higher 5HT2A binding in frontal cortex; a finding that was independently confirmed in a study in euthymic, medication free, depressed patients [231]. Protein and mRNA expression

studies have further corroborated these results. Research studies by Pandey et al., Escriba et al., and Shelton et al. showed higher 5HT2A protein/mRNA expression in subjects suffering from major depressive disorder compared with healthy controls [230, 232, 233]. Thus, our finding of higher 5HT2A expression level in ApoE4 animals possibly implicates that ApoE4-mediated increased risk of developing depression could be partially attributed to its upregulation of 5HT2A signaling when compared to other two genotypes.

In addition to determining the probable dysfunctional serotonergic signaling in ApoE4 animals, we also examined the probable modulation of BDNF signaling by different ApoE isoforms. The data indicated a significant decrease in BDNF expression levels when comparing ApoE4 animals with ApoE2 and ApoE3 animals (Figure20 b). A post mortem study by Diwedi et al. reported a significant decrease in mRNA and protein levels of BDNF and TrkB in depressed subjects compared to normal subjects [507]. A recent study by Ray et al. corroborated these results and reported a 30% decrease in BDNF mRNA in depressed patients compared to normal subjects [318]. Thus our finding of decreased BDNF expression levels in ApoE4 animals strengthens the finding of recent clinical studies linking depression and ApoE4 genotype at a molecular level, implicating that downregulation of BDNF signaling could also contribute to the increased risk for depression associated with ApoE4.

Synaptic proteins have been found to be decreased in the depressed patients. A study by Kang et al. reported a significant decrease in synapse related genes in the pre-frontal cortex of depressed patients compared to control subjects [505]. In order to elucidate the probable dysfunctional synaptic plasticity as seen in the depressed patients, we examined the modulation of expression levels of pre- and post-synaptic proteins in ApoE4 animals when compared with ApoE2 and ApoE3 animals. The data indicates a genotype-based decrease in the expression levels of

synaptobrevin2 when comparing ApoE4 animals with ApoE2 and ApoE3 animals (Figure 20). Interestingly the expression levels of another presynaptic protein synaptophysin were not different when comparing the three animal groups (Figure 21). It is likely that the ApoE genotype only regulates functional but not structural pre-synaptic proteins. Thus synatophysin which is a structural protein present on the synaptic vesicles and involved in vesicle kinetics and recycling is not regulated on the basis of ApoE isoform but synaptobrevin2 which is a functional protein and is involved in the vesicle docking and fusion is modulated in an ApoE genotype dependent manner. Thus in ApoE4 animals the docking and fusion of synaptic vesicles is significantly decreased leading to decreased neurotransmitter exocytosis and thus decreased synaptic function. In addition to pre-synaptic proteins, our data indicate a down regulation of nearly 20% in the expression levels of postsynaptic proteins PSD95 and SHANK3 when comparing ApoE2 and ApoE3 with ApoE4 animals (Figure 22). These data are quite similar to what has been observed with respect to presynaptic proteins and therefore implicates a probable dysfunctional synaptic function at both pre- and post-synaptic levels. Taken together, these data suggest that ApoE4 animals are more susceptible to the risk of developing depression and a probable loss of cognition and memory function as evident by decreased levels of both pre- and post-synaptic proteins.

ET has been reported to exert promising effects in the treatment of depression and mood-related symptomatology [141-143]. Despite its beneficial effects in improving behavioral deficits associated with depression, use of ET as main stream therapeutic option has not been a possibility because of its dire side effects. ET has been reported to increase the reoccurrence of endometrial cancer [508] and venous thromboembolism in women with previous episodes of the disease. Additionally, a study by the Women's Health Initiative (WHI) demonstrated that ET increased the risk of non-fatal cardiovascular diseases and breast cancer in older women [501]. Thus, due to the

substantial risks associated with ET [151, 502] it is imperative to find alternative approaches that possess the beneficial effects of ET without eliciting the potentially fatal side effects. A specific ERβ-targeted combination of plant derived phytoestrogens, Genistein, Daidzein and Equol has been shown to be such an alternative. The combination has been shown to prolong survival and improving spatial memory in an Alzheimer's disease model [500]. Thus we decided to elucidate whether this combination of phytoestrogens (Phyto-β-SERM) would be able to reverse the serotonergic, neurotrophic and synaptic dysfunction seen in ApoE4 animals. The data indicate that a 3-month treatment with phyto-β-SERM diet induced a significant reduction in the expression levels of 5HT2A receptor in ApoE2 and ApoE3, but not in ApoE4 animals (Figure 23). The data is quite intriguing as Phyto-β-SERM treatment worked in the same manner as chronic treatment with antidepressants, suggesting a possible beneficial role for Phyto-β-SERM in improving depression-related behavior in these animals. Interestingly, the treatment did not lead to any significant change in the expression levels of 5HT2A receptor levels in ApoE4 animals,. The chronic Phyto-β-SERM treatment also resulted in an increase in the levels of BDNF and its receptor TrkB when comparing treated transgenic animals with non-treated control animals (Figure 24). These results also strengthen the probable beneficial effects of Phyto-β-SERM treatment in treating depressive phenotype as antidepressants have been shown to induce a similar increase in the expression level of BDNF and TrkB in several clinical and molecular studies. Interestingly again the treatment did not induce any significant changes in the levels of BDNF/TrkB expression levels in ApoE4 animals, suggesting that ApoE genotype modulates ERβ activity in the regulation of these brain signaling involved in depression.

Antidepressants have been reported to increase the expression and function of proteins related to synaptic plasticity. A study by Li et al. reported a startling increase in the expression levels of

Synapsin1, PSD95 and GluR1 in synaptoneurosomes from prefrontal cortex (PFC) after ketamine administration. Two independent studies by Leary et al. and Sairanen et al. reported that chronic imipramine and fluoxetine treatment induced an increase of synaptic proteins in the hippocampus and PFC of rats respectively. Moreover, we witnessed a significant decrease in the levels of both pre- and post-synaptic proteins in ApoE4 animals, thus we decided to elucidate whether or not chronic Phyto-β-SERM will work in a similar manner as antidepressants to reverse the observed deficits. Our analysis revealed that the Phyto-β-SERM treatment increased the expression levels of the pre-synaptic proteins, Synaptophysin and Synaptobrevin2 by 50% in all three transgenic animal models when compared with untreated control animals (Figure 25). In addition to the upregulation of pre-synaptic proteins, the expression levels of post-synaptic protein PSD95 was also increased by nearly two folds in treated transgenic animals irrespective of the ApoE isoform when compared with control untreated animals (Figure 26). These results strongly suggest that Phyto-β-SERM treatment may improve the cognitive and memory-related deficits occurring in LOAD.

2.6. Limitation of the Study

Overall, the findings of our study are very informative and enlightening but there remains a number of key issues that need to be kept in mind and addressed.

- **Pro BDNF Levels**

In similarity with the first chapter, we did not measure the levels of pro BDNF at any point of time in this study. However, as already mentioned although the measurement of proBDNF in the study model is important, the absence of these data does not diminish the significance of the findings as we saw significant change in the expression of mature form of BDNF. The decline or rise in mature form of BDNF in our animal or cell culture models directly demonstrates a dyshomeostasis in the equilibrium and thus would elicit deleterious or beneficial effects with respect to depression.

- **APOE Regulation of 5HT Signaling**

Again in similarity with the first study, this study too focused only on 5HT2A receptors thus leaving aside a number of other serotonin receptors that could have also been studied, therefore limiting the translational ability of the study. Although the results of first study serve as a starting point for this study, the absence of investigation of rest of the serotonin receptors in first study itself limits the findings of this study to an extent too.

- **Binding Specificity of phyto-β-SERM Diet**

One of the other weaknesses of this study is that the binding specificity of the phyto-β-SERM to GPER1 is not known. The phytoestrogenic diet, having equal amounts of eqoul, daidzein and genistein was formulated in a manner that it has more binding affinity towards ERβ receptor in comparison to ERα receptor. Although the diet is 83 folds more selective for ERβ when compared

to ERα, the relative binding affinity of the diet towards GPER1 is unknown. One of three components of the formulation, genistein has been reported to have lower binding specificity for GPER1 in comparison to ERβ receptor but the binding affinities of the other two components towards GPER1 is still unknown (Table 2). Hence there is a probability that the effects reported in the study can be mediated via GPER1 instead of ERβ receptor. However this probability is very low as the end points reported in the first study after specific agonism of ERβ by DPN are similar to the results observed in this study, thus highly implicating that the beneficial effects of phyto-β-SERM diet are very well mediated through ERβ receptors.

2.7. Future Direction

Overall, findings of the study are very informative and enlightening but a significant amount of future research is needed to corroborate and further substantiate the results. For instance broader investigation of the regulation of 5HT signaling system by APOE isoforms is needed to be delved into. In addition future research should be well aimed at understanding the mechanism underlying the ERβ mediated differential effects in three different APOE brains. Last and most importantly, translational investigation of the role of APOE genotypes and ERβ modulation in the animal models of depression associated with APOE isoforms should be carried out. More specifically the future studies would be aimed at determining whether or not ApoE4 brain is more susceptible to depression in comparison to ApoE2 and ApoE3 brains. In addition it would be very interesting to determine whether activation of ERβ signaling would be able to reverse the probable deficits.

2.8. Conclusion

Our findings illustrate a possible mechanism involving BDNF-5HT2A pathways by which ApoE4 increases the risk for depression (Figure 27). In a larger context, this mechanism could underlie the link between depression and AD associated with APOE4. Enhancing ERβ activity could provide a greater therapeutic benefit in non-APOE4 carriers than in APOE4 carriers in the intervention of these brain disorders. Further studies in animal models of depression and AD will be needed to examine the pharmacological significance of these mechanistic findings at translational level.

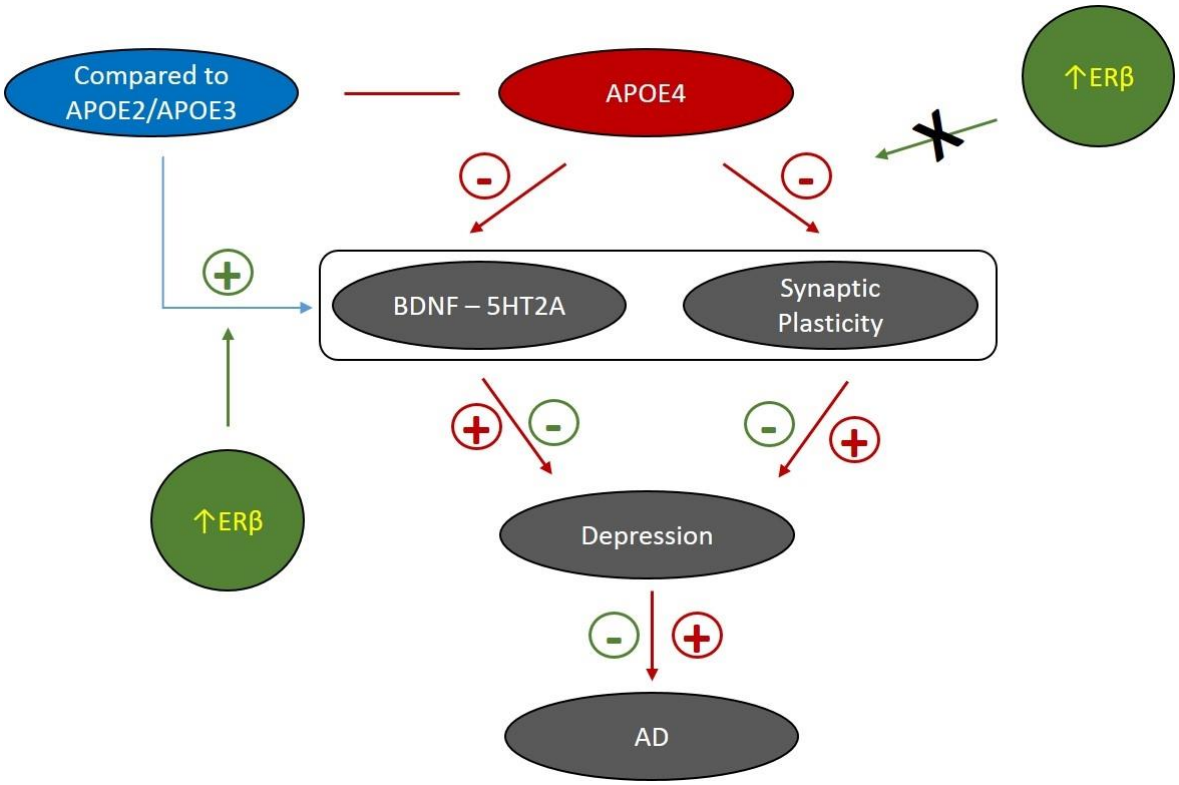

Figure 27. ERβ Interacts with APOE in Regulation of BDNF-5HT2A signaling in female brain. A schematic illustration showing differential effects of APOE genotype on BDNF-5HT2A signaling and synaptic plasticity. ApoE4 carriers showed significant alterations in 5HT2A and BDNF signaling along with synaptic function, which renders this genotype to the greatest risk of developing depression and AD when compared to ApoE2 and ApoE3 carriers. In addition ERβ activation reversed these effects only in ApoE2 and ApoE3 brains, but not in ApoE4 brains thus implicating that ApoE4 brains are least treatment responsive among three genotypes.

Final Conclusion

Depression is among the most burdensome disorders worldwide, leading to 52 million YLDs per year and amounting to $210 billion around the globe. Women are 2.5 times more likely to experience major depression than men, with most cases occurring during the endocrine transition from pre- to perimenopause. Cohen et al reported that perimenopausal women are twice as likely to develop depression compared with women who remained in the premenopausal phase [509]. The hormonal changes that occur during perimenopausal onset are now well associated with an increased vulnerability for psychiatric disorders, such as depression, however, the mechanistic understanding of the phenomenon is poorly understood. ERs have been implicated to play a role in the pathophysiology of depression but lack of consistent results and considerable number of contradictory reports have limited the path to a definite conclusion. The aim of first part of the thesis was to elucidate the role of ERα and ERβ in the regulation of BDNF and 5HT signaling in the female brain. Our analyses in ERα and ERβ knockout ($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 response was brain region-specific. The brain region-specific regulation of BDNF is intriguing as it is parallel to the clinical observations in depressed subjects. Post mortem studies have reported a significant decrease in mRNA and protein levels of BDNF and TrkB in the hippocampus of depressed subjects compared to normal subjects [269], which was reversed with antidepressant treatment [270]. Thus our results in mice animal model demonstrate brain region specific regulation of BDNF by ERβ, pointing towards a probable pathway for the phenomenon seen in clinical and basic science research studies. These data were further validated in two different $ER\beta^{-/-}$ rat models; one with targeted deletion of exon 3 and the other with a targeted deletion of exon 4 in the ERβ gene. As expected, ERβ deficiency in both Exon $3^{-/-}$ and Exon $4^{-/-}$

models resulted in a significant reduction in the levels of both monomeric and trimeric forms of BDNF. Our *in vivo* findings were further validated in primary hippocampal neurons. Analyses in primary hippocampal neurons indicated that ERβ activation by DPN significantly enhanced BDNF-TrkB signaling. In addition, ERβ agonism lead to a significant increase in the pre-synaptic vesicle protein Synaptophysin and post density protein, PSD95 when compared to vehicle treated neurons. These findings are in line with the research outcomes of a study from Liu et al. which reported a similar increase in levels of synaptic proteins in the hippocampus of mice following treatment with ERβ, but not ERα specific agonists [341], implicating that specific activation of ERβ can modulate the expression levels of synaptic proteins in a similar manner to those of antidepressants. Soon after the discovery of adult hippocampal neurogenesis in humans [343], it was theorized that basal deficits in hippocampal neurogenesis may underlie symptoms of psychiatric disorders, particularly depression [344]. This theory was largely based on the findings indicating decreased hippocampal volume in depressed patients [345, 346] and increased neurogenesis with the administration of antidepressant medication and therapies [344, 347]. So in the light of these findings, we decided to determine whether specific activation of ERβ can similarly increase and/or modulate the expression levels of neurogenic proteins. Our analysis revealed nearly 50% increase in molecular markers pertaining to neurogenesis in DPN-treated neurons compared to vehicle treated neurons. These findings point towards a therapeutic possibility that agonism of ERβ can be used to alleviate the depressive symptomatology, as adult hippocampal neurogenesis has been shown to be sufficient enough to relive depressive phenotype in mice [355].

Our finding of ERβ regulation of BDNF in hippocampus prompted us to further examine signaling pathways related to mood disorders. The monoamine hypothesis is the oldest hypothesis postulated

for depression [211] and 5-HT is an important player in this hypothesis. Moreover, there are not many studies that have analyzed the probable molecular mechanism underlying the interaction of estrogen signaling, BDNF signaling and 5-HT signaling; three signaling pathways thought to converge in the hippocampus (for review see [332, 356]). Our analyses in 6-month-old ERβ knockout rats revealed a 20% increase in expression levels of 5HT2A, but there was no significant change in the expression level of 5HT1A receptor. These findings were further validated in 10-month-old ERβ knockout rats. These results correspond with clinical findings that have reported an increase in 5HT2A receptor expression in depressed patients [228-230] and antidepressants ranging from SSRIs to MAOIs have been reported to induce a downregulation of binding sites of these receptors in post synaptic regions in brain [234]. Thus there appears to be a possibility that perturbed ERβ signaling in perimenopause/menopause leads to a dysfunction in both BDNF and 5HT2A signaling which in turn increases the vulnerability for the onset of depression. Our study demonstrated that BDNF is not being regulated by 5HT2A at least at the levels of protein regulation but 5HT2A is being regulated by BDNF in hippocampus probably via signaling pathways that regulate the gene expression of the receptor as there was no evident regulation of 5HT2A receptor expression level with acute agonism of TrkB signaling. Thus it is possible that during perimenopause/menopause declined BDNF signaling leads to an increased 5HT2A expression level which increases the susceptibility for mood disorders. Moreover the 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 5HT2A receptor signaling.

Our findings illustrate a possible mechanism underlying the increased susceptibility for depression associated with perimenopause and menopause in women. Based on these findings, we

hypothesize that perimenopause/menopause leads to irregular or reduced levels of estrogen in the brain. This irregularity or reduction of estrogen availability decreases the stimulation of ERα, ERβ and GPER1 mediated estrogen signaling. The attenuated ERβ signaling leads to reduced transcription of BDNF gene thus decreasing the levels of BDNF protein in the hippocampal region of the brain. The decreased levels of BDNF and thus reduced BDNF-TrkB signaling weakens the synaptic and neurogenic strength thus rendering the brain to a weakened adaptation to environmental stressor resulting in increased risk to depression. Attenuated BDNF/TrkB signaling also increases the activity of 5HT2A, which could also play a role in the increased susceptibility for depression associated with perimenopause as 5HT2A antagonism has been shown to relieve the symptoms of depression. These molecular alterations are escalated in the presence of environmental stressors and lead to the development of depression in perimenopausal females.

Studies have shown comorbidity between depression and other major diseases such as coronary heart disease (CHD), myocardial infarction (MI), type 2 diabetes (T2DM), stroke, and Alzheimer's disease (AD), which convolutes the treatment strategies. Depression has been reported to be commonly manifested in Alzheimer's disease (AD) patients and is considered to be an independent risk factor for AD. Human apolipoprotein E (APOE) gene exists in three major isoforms (coded by ε2, ε3, and ε4) and the ε4 allele has been associated with a greater incidence for both depression and AD. Although mounting evidence has pointed to the potentially complex interaction between these two brain disorders in which APOE may play a role, the underlying mechanisms are largely unknown. The aim of the second chapter of the thesis was to elucidate the possible interaction between APOE genotype and BDNF-5HT2A signaling, so as to gain a better understanding of underlying molecular mechanism of depression being a risk factor for AD.

Our analysis in the cortical tissue harvested from female 6-month-old ApoE2, ApoE3, and ApoE4 mice revealed that 5HT2A, but not 5HT1A receptor expression was modulated in an ApoE genotype dependent manner (Figure 1a). Our data indicated a 20% and 25% increase in the expression level of 5HT2A receptors when comparing ApoE2 with ApoE3 animals and ApoE2 with ApoE4 animals respectively. These findings correspond with clinical findings that have reported an increase in 5HT2A receptor expression in the depressed patients. A radiolabeled binding study from Mann et al., which used [3H]spiperone as a 5HT2A ligand, reported that the binding potential of 5HT2A receptors is higher in post-mortem brains of suicidal depressed patients compared to healthy controls [229]. These results have been replicated in several studies that used different 5HT2A ligands but reported a similar increase in the binding potential of 5HT2A receptors primarily in the prefrontal cortex of depressed patients compared to healthy control subjects [230]. Additionally, subjects with high scores on the dysfunctional attitude scale exhibit higher 5HT2A binding in frontal cortex; a finding that was independently confirmed in a study in euthymic, medication free, depressed patients [231]. Protein and mRNA expression studies have further corroborated these results. Research studies by Pandey et al., Escriba et al., and Shelton et al. showed higher 5HT2A protein/mRNA expression in subjects suffering from major depressive disorder compared with healthy controls [230, 232, 233]. Thus our finding of higher 5HT2A expression level in ApoE4 animals possibly implicates that ApoE4-mediated increased risk of developing depression could be partially attributed to its upregulation of 5HT2A signaling when compared to other two genotypes. In addition the data indicated a significant decrease in BDNF expression levels when comparing ApoE4 animals with ApoE2 and ApoE3 animals, thus strengthening the finding of recent clinical studies linking depression and ApoE4

genotype at a molecular level and implicating that downregulation of BDNF signaling could also contribute to the increased risk for depression associated with ApoE4.

Synaptic proteins have been found to be decreased in the depressed patients [505]. In order to elucidate the probable dysfunctional synaptic plasticity as seen in the depressed patients, we examined the modulation of expression levels of pre- and post-synaptic proteins in ApoE4 animals when compared with ApoE2 and ApoE3 animals. The data indicates a genotype-based decrease in the expression levels of synaptobrevin2, but not synaptophysin when comparing ApoE4 animals with ApoE2 and ApoE3. This observation made us conclude that ApoE genotype only regulates functional but not structural pre-synaptic proteins. In addition to pre-synaptic proteins, our data indicate a down regulation of in the expression levels of postsynaptic proteins PSD95 and SHANK3 when comparing ApoE2 and ApoE3 with ApoE4 animals. These data are quite similar to what has been observed with respect to presynaptic proteins and therefore implicates a probable dysfunctional synaptic function at both pre- and post-synaptic levels. Taken together, these data suggest that ApoE4 animals are more susceptible to the risk of developing depression and a probable loss of cognition and memory function as evident by decreased levels of both pre- and post-synaptic proteins.

ET has been reported to exert promising effects in the treatment of depression and mood-related symptomatology [141-143] but due to the substantial risks associated with ET [151, 502] it is imperative to find alternative approaches that possess the beneficial effects of ET without eliciting the potentially fatal side effects [501, 508]. Thus we decided to use a specific ERβ-targeted combination of plant derived phytoestrogens, Genistein, Daidzein and Equol to elucidate whether this combination of phytoestrogens (Phyto-β-SERM) would be able to reverse the serotonergic, neurotrophic and synaptic dysfunction seen in ApoE4 animals. Our data indicated that a 3-month

treatment with phyto-β-SERM diet induced a significant reduction in the expression levels of 5HT2A receptor in ApoE2 and ApoE3, but not in ApoE4 animals (Figure 4). The data is quite intriguing as Phyto-β-SERM treatment worked in the same manner as chronic treatment with antidepressants, suggesting a possible beneficial role for Phyto-β-SERM in improving depression-related behavior in these animals. In addition, Phyto-β-SERM treatment also resulted in an increase in the levels of BDNF and its receptor TrkB when comparing treated transgenic animals with non-treated control animals. These results also strengthen the probable beneficial effects of Phyto-β-SERM treatment in treating depressive phenotype as antidepressants have been shown to induce a similar increase in the expression level of BDNF and TrkB in several clinical and molecular studies. Interestingly again the treatment didn't induce any significant changes in the levels of BDNF/TrkB expression levels in ApoE4 animals, suggesting that ApoE genotype modulates ERβ activity in the regulation of these brain signaling involved in depression.

In the light of our finding of a significant decrease in the levels of both pre- and post-synaptic proteins in ApoE4 animals, we decided to elucidate whether or not chronic Phyto-β-SERM will work in a similar manner as antidepressants to reverse the observed deficits. Our analysis revealed that the Phyto-β-SERM treatment increased the expression levels of the pre-synaptic proteins, Synaptophysin and Synaptobrevin2 by 50% in all three transgenic animal models when compared with untreated control animals. In addition to the upregulation of pre-synaptic proteins, the expression levels of post-synaptic protein PSD95 was also increased by nearly two folds in treated transgenic animals irrespective of the ApoE isoform when compared with control untreated animals. These results strongly suggest that Phyto-β-SERM treatment may improve the cognitive and memory-related deficits occurring in LOAD.

Collectively, these findings implicate that the reduction in ERβ signaling homeostasis during perimenopause leads to dysregulation of BDNF-5HT2A receptor network and weakened synaptic plasticity, which together predispose the female brain to a susceptible state for depression. Timely intervention with an ERβ-targeted modulator could potentially attenuate this susceptibility and reduce the risk or ameliorate the clinical manifestation of this brain disorder. In addition we demonstrated a significant regulation of BDNF 5HT2A signaling by APOE genotype status, thus pointing towards a possible mechanistic pathway resulting in the reported association between depression and AD. In addition, our results suggest that ERβ agonism can possibly reduce depressive phenotype in ApoE2 and ApoE3 carriers but not in ApoE4 carriers. These findings can be very useful in treating perimenopausal depression and thus reducing the risk of developing AD later.

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