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Mechanisms of Estrogen Receptor Alternative Splicing and the Consequences for Aging in the Female Brain

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MECHANISMS OF ESTROGEN RECEPTOR ALTERNATIVE SPLICING AND THE CONSEQUENCES FOR AGING IN THE FEMALE BRAIN

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LIST OF ABBREVIATIONS

3βAdiol	5α-Androstane-3β, 17β-diol
AP-1	activator protein-1
AVP	arginine vasopressin
ERαKO	ERα knockout mice
ERβKO	ERβ knockout mice
CEE	conjugated equine estrogens
CELF	CUG-Binding Protein Elav-like family member
CORT	corticosterone
CRH	corticotrophin releasing hormone
DBD	DNA-binding domain
DHT	dihydrotestosterone
DPN	diaryl propriolnitrile
ERE	estrogen response element
E2	17β-estradiol
ERβ	estrogen receptor beta
ERα	estrogen receptor alpha
ESE	exon splicing enhancer
ESS	exon splicing silencer
ET	estrogen therapy

GSK3β	glycogen synthase kinase-3
GPER	G protein-coupled estrogen receptor
HNRNP	heterogeneous nuclear riboprotein
HT	hormone replacement therapy
ISE	intron splicing enhancer
ISS	intron splicing silencer
KEEPS	Kronos Early Estrogen Prevention Study
LBD	ligand binding domain
MPA	medroxyprogesterone acetate
NMD	nonsense-mediated decay
NOVA	neuro-oncological ventral antigen
PI3K	phosphoinositol-3-kinase
PPT	propyl pyrazole triol
PVN	paraventricular nucleus
RBP	RNA-binding protein
SF1	splicing factor 1
SNRPS	small nuclear ribonucleoproteins
SR	serine-rich
SUMO	small ubiquitin like modifier
TOP1	topoisomerase I
WHI	Women's Health Initiative

CHAPTER I

STATEMENT OF THE PROBLEM

The advances in healthcare and scientific knowledge have resulted in longer life expectancies in both men and women; the average life expectancy of a woman in United States is now 81.2 years old [1]. These advanced ages in women now means that they are experiencing the effects of age-related changes in the body for much longer periods of time, mainly reproductive senescence, resulting in the loss of circulating ovarian hormones. The age at which menopause occurs has not changed, resulting in women now living over a third of their lives in a postmenopausal state.

The major circulating estrogen produced by the ovaries, 17β-estradiol (E2), has many homeostatic effects in the body like neuroprotection, cognition, stress responses, cardioprotection, and bone density. During aging and reproductive senescence, all these physiological processes are affected and generally result in negative outcomes for women: osteoporosis, cardiovascular disease, stroke, and cognitive impairment. Hormone replacement therapy (HT) was to become the standard in treating women undergoing reproductive senescence in order to abrogate the negative effects associated with the decline in circulating E2. These effects, as well as HT efficacy, were investigated in the Women's Health Initiative (WHI) and Women's Health Initiative Memory Studies (WHIMS). Both studies were aimed at investigating how HT would impact health issues in aging women through a large-scale study based on previous experiments conducted in

animals that had demonstrated the positive effects of HT. While the WHI studies were ended abruptly due to negative consequences associated with the study, later analysis from these studies brought forth some interesting, yet important, observations. The adverse effects of HT occurred mainly women who were at least 10 years removed from menopause [2-5]. These adverse effects included cardiovascular and coronary disease, breast cancer, stroke, and cognitive impairments. However, younger women up to 5 years postmenopause benefited from the treatments [6, 7]. These findings were later supported by the Kronos Early Estrogen Prevention Study (KEEPS) and KEEPS Cognitive and Affective ancillary study that observed positive effects of early estrogen therapy (ET) in peri-menopausal and early postmenopausal women on cognition, mood, and cardiovascular disease [8, 9].

These studies, along with others [10, 11], led to the idea of a therapeutic window in which ET is beneficial, known as the "timing hypothesis" [12], pointing to age-related adjustments that occur during and after this critical period of declining E2 levels [13]. However, the mechanisms underlying the changing molecular environment of the aging brain in response to E2 deprivation still remains elusive. *Therefore, this dissertation aims at understanding the molecular changes that occur in the brain in response to age and diminishing E2 levels and how these changes contribute to the physiology and adaptive mechanisms the brain undergoes in this critical period of adjustment.*

E2 is known to regulate transcription through an important class of nuclear steroid receptors called estrogen receptors (ERs). ERα and ERβ mediate the actions of E2 upon binding through interactions within the promoter region of ER-regulated genes. Both ER

subtypes are subject to alternative splicing, and it is through this process that ERβ splice variants arise altering the receptor function and responsiveness to E2 in the brain. Our laboratory has previously demonstrated that the effects of ERβ splice variants on mediating gene transcription. We have also demonstrated the effects of E2 deprivation on microRNA (miRNA) expression and ERβ protein:protein interactions that may impact ER gene regulation. Therefore, I hypothesized that aging and diminished E2 levels affect the alternative splicing of ERβ in the aged female brain through altered expression of ER-regulated splicing factors.

In order to test this hypothesis, three specific aims were developed (summarized in Figure 1):

1. Quantify the mRNA expression of ERβ splice variants in various brain regions in young and aged animals following E2 deprivation and acute E2 treatment. There are several splice variants of ERβ expressed in the rat, mouse, and human brain. Previous studies describing the mRNA distribution of ERβ splice variants have been conducted in the rat, yet the effects of aging and reproductive senescence on splice variant expression have not been thoroughly investigated. I hypothesize that aging and E2 deprivation increase alternative splicing events, resulting in an increase of ERβ splice variants, while treatment with E2 will decrease splice variant expression. Chapter III will explore this aim.

2. Quantify the mRNA expression of the Nova1 splicing factor within various regions of aging brain and determine the effects of E_2 treatment on the expression of this splicing factor during E_2 withdrawal. Increases in Nova1-regulated alternative

splicing events were observed in aged male cortex due to an age-related decrease in Nova1 expression. I hypothesize that age-related decline in Nova1 expression along with decreased levels of E2 result in the downregulation of Nova1 and treatment with E2 results in upregulation of Nova1 needed for effective splicing regulation, resulting in decreased ERβ splice variant expression. Chapter IV will explore this aim.

3. Elucidate a) whether Nova1 regulates ERβ splicing, and how b) E2 affects RNA-binding proteins responsible for mediating transcription. ERβ pre-mRNA transcripts contain several Nova1 recognition motifs, meaning Nova1 may regulate alternative splicing of ERβ. ERβ alternative splicing may be enhanced as a result of altered splicing kinetics. I hypothesize that Nova1 binds to ERβ pre-mRNA during spliceosome assembly to mediate exon exclusion that results in decreased ERβ variant expression. Also, E2 may directly or indirectly affect global alternative splicing events by affecting mRNA expression and activity of RNA polymerase II (RNAPII), as well as other splicing factors and spliceosome components. Chapters III and IV will explore this aim.

The molecular mechanisms by which HT efficacy changes with age and increasingly longer periods of E2 deprivation are unknown. I predicted that E2 efficacy in the brain is decreased with advanced age in females, due to increased levels of ERβ splice variants. The results from this dissertation show that ERβ alternative splicing is altered in a brain region-specific manner, along with RNAPII mRNA expression and activity. These data also demonstrate a role for Nova1 in the regulation of ERβ alternative splicing and provide putative molecular mechanisms by which the therapeutic window of HT efficacy is affected.

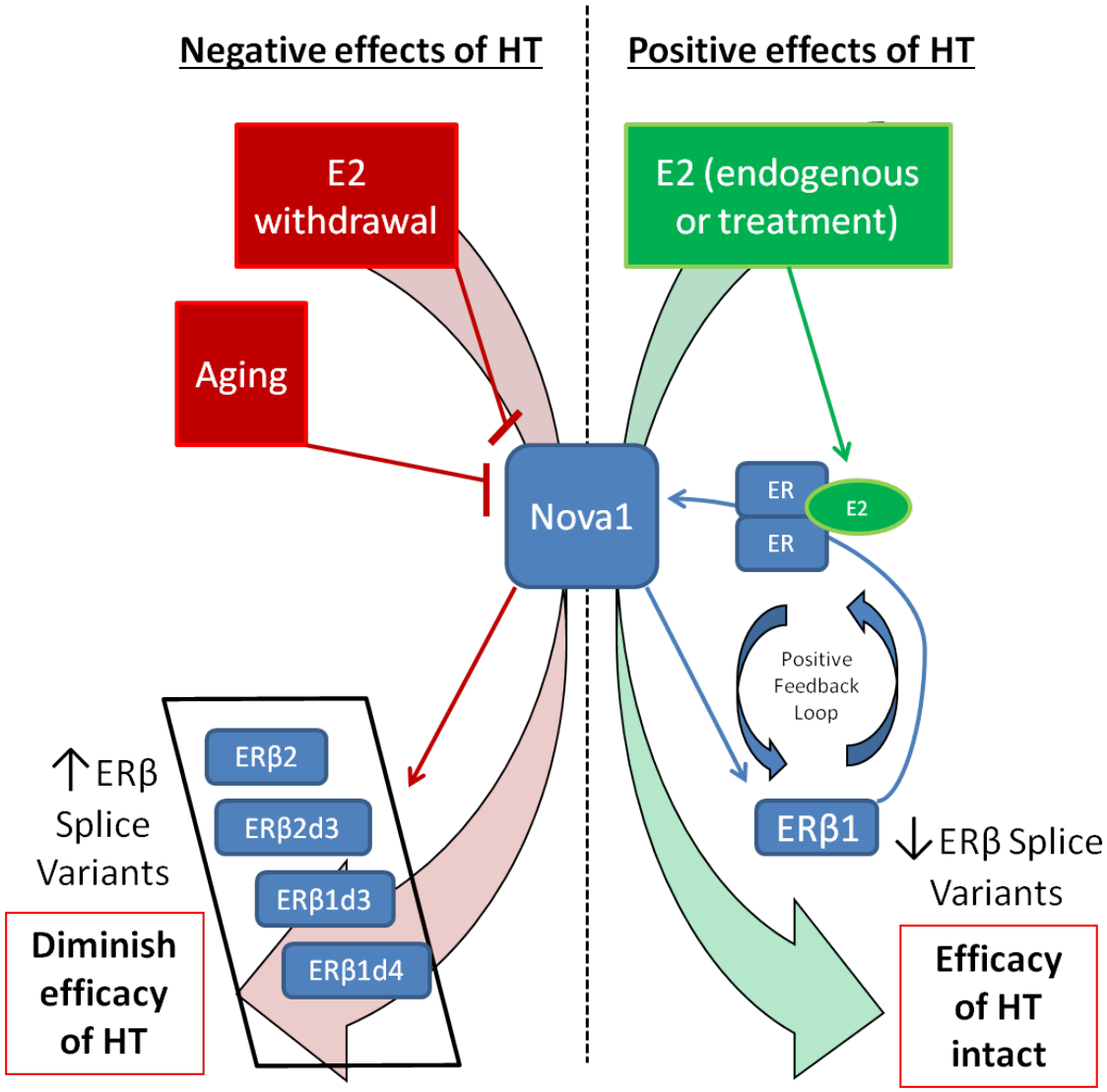

Figure 1. Hypothesis of dissertation: a model. Aging and decreased circulating E2 levels affect the alternative splicing of ERβ in the aged female brain through altered expression of ER-regulated splicing factors (i.e. Nova1), thereby contributing to the decreased efficacy and negative effects of late HT in women. HT = hormone replacement therapy; ERβ = estrogen receptor beta; E2 = 17β-estradiol; Nova1 = Neuro-Oncological Ventral Antigen 1.

CHAPTER II

REVIEW OF THE LITERATURE

Menopause and Aging

Menopause, also known as reproductive senescence, is the end of a woman's reproductive years due to termination of the menstrual cycle. The average age of menopause is approximately 51 years of age, although the National Institutes of Health (NIH) and National Institute of Child Health and Development (NICHD) report that this transition can occur between the ages of 45-55 [14]. Prior to the event of menopause, the production of ovarian hormones begins to deviate from normal reproductive cyclicity due to loss of hormone-producing ovarian follicles. Levels of the major circulating estrogen produced by the ovaries, 17β-estradiol (E2), can fluctuate rapidly during this period of decline, which manifest in a variety of negative physiological symptoms including hot flashes, memory impairment, and anxiety [15, 16]. Decreased circulating levels of progesterone also occurs during the menopausal transition due to the lack of corpora lutea formation. While this transition occurs at a relatively consistent age in women, life expectancy has steadily increased. The CDC reports that the average life expectancy for a woman in the United States is about 81.2 years [1]. These statistics indicate that women are now living over a third of their lives post-menopause, which increases their risk for developing cognitive disorders, cardiovascular diseases, and breast cancer [17-19]. The loss of E2 due to menopause is considered a key factor associated with these increased

health risks, further compounding the effects of aging in women. Previous studies using animal models have provided evidence that E2 has positive effects on cognition, memory, anxiety, depression, cardiovascular health, and bone density [8, 20-24].

The Women's Health Initiative Studies and Hormone Replacement Therapy

The Women's Health Initiative (WHI), the Women's Health Initiative Study on Cognitive Aging (WHISCA), and the Women's Health Initiative Memory Studies (WHIMS) were conducted to measure the effects of hormone replacement therapy (HT) in postmenopausal women. At the time, HT was not a novel therapy, as nearly 40% of women in the U.S. had undergone treatment. The trials enrolled over 27,000 post-menopausal women between the ages of 50-79, including those with prior hysterectomy. Contrary to earlier work in animal models, the clinical data revealed that HT had little to no effect on cognitive function in women, and that combined treatments of conjugated equine estrogen and medroxyprogesterone acetate (CEE/MPA) decreased global cognitive functioning and increased the risk for mild cognitive impairment [2, 3, 25-27]. The first WHI clinical trials had to be terminated prematurely due to increased incidence of cardiovascular disease, stroke, and breast cancer, to which the underlying cause was studied later through multiple meta-analyses and additional clinical trials. Post-hoc analysis of the WHI trials revealed that the age of the participants was an integral factor that predicted whether the effects of HT were beneficial or detrimental, however other factors contributed to the negative outcomes associated with these studies including choice of reference group, such as women who had previously undergone HT, and

hormone preparation [6, 28-31]. Additional basic science and observational clinical studies since the initial WHI have supported the beneficial effects of HT [9, 32-35]. These studies include the recent Kronos Early Estrogen Prevention Study (KEEPS) which saw improvement in mood and anxiety [8]. Further meta-analysis by several groups observed cognitive and additional health benefits in women who received HT for short term treatments and most notably during perimenopause or early postmenopause [7, 36, 37].

The combined efforts of both scientists and clinicians have yielded the observation that the age at which a woman undergoes HT is vital to its efficacy. These observations led us to what we now know as the "timing hypothesis": women who were more than 10 years removed from the event of menopause were associated with the negative outcomes of HT, while women who were perimenopausal, or up to 5 years postmenopause, benefited from HT [12]. Therefore, the window of opportunity for HT is a very short period of time in relation to a woman's life expectancy, and missing this critical period may have detrimental health effects [11, 38-41].

Estrogen Receptors

Estrogens actions are mediated primarily through two steroid receptors, Estrogen Recepter (ER) α and ERβ. These receptors are class I members of the ligand-activated nuclear receptor superfamily and act as transcription factors for a wide variety of genes [42]. Early studies on ERs in the 1960s by Jensen and colleagues provided the classical model of the two-step hypothesis: cytoplasmic-localized ERs bind ligand, undergo

conformational changes, dimerize, and then translocate to the nucleus to regulate gene transcription through DNA-binding and/or association with other transcription factors [43]. Since 1968, our understanding of ERs and their signaling pathways have been greatly expanded.

For instance, the discovery that there are two distinct ERs, encoded by separate genes, provided additional mechanisms through which E2 can mediate its actions [44, 45]. Dimerization of the receptors can occur in a homo- or hetero-typic manner where ERβ can dimerize with itself or with ERα [46]. Following dimerization, these receptors then bind to estrogen response elements (EREs) located within the promoter regions of ER-responsive genes to regulate transcription. High affinity studies of ERα with EREs described a canonical inverted repeat sequence *AGGTCAnnnTGACCT*, where 'n' represents any nucleotide [47]. This exact sequence rarely occurs naturally, and many times EREs are variations of this high affinity sequence, including ERE half-sites [48-50]. Transcriptional modulation can also occur in an ERE sequence-independent manner, where increased nucleotide variation results in less activity at these non-canonical EREs [51]. ERβ is not only able to act in a *cis* manner at an ERE, but can also act in *trans* at an AP-1 site when associated with other transcription factors like c-Jun and c-Fos [52].

The structure of ERβ in the rat (rERβ) contains 6 domains. Each of these domains, designated A-F, have different functions that work together to transcriptionally regulate gene expression (Figure 2). The A/B domains comprise the activator function-1 (AF-1) domain that binds to co-regulatory proteins required for transcriptional regulation. Homology of the A/B domain between ERα and ERβ is only 17%, making this the least

conserved domain [44, 45] and likely contributes to the distinct functions of each receptor subtype. The C domain is the DNA-binding domain (DBD), which recognizes the ERE sequences within a gene promoter through two zinc fingers motifs. The spacing of these two zinc fingers allows the helix-loop-helix structure to bind and recognize canonical ERE sites. The D domain is the nuclear localization domain, which acts as a hinge region important for conformational changes that occur during ERβ dimerization and subsequent binding to DNA. This region is highly conserved between ERα and ERβ. The E domain is the ligand-binding domain (LBD) and the AF-2 region, which is important for interactions between the dimerized receptors and other transcription factors. The LBD is comprised of 12 ordered alpha helices, which are integral for ligand specificity [53]. The true function of the F domain in ERβ remains relatively unknown. The F domain of ERα is larger than ERβ, and they share about 18% homology, and while it isn't required for transcriptional activation, it can modulate the activity of ERα [44, 54, 55]. Our laboratory has observed similar effects in human ERβ (hERβ) splice variants that lack an F domain, but they are still able to activate transcription as measured using reporter gene assays [56]. Comparatively, rERβ is highly homologous to hERβ, sharing between 80% homology in the A/B domains and up to 98.5% homology in the C domain [57].

ERα is derived from a different gene than ERβ, but acts in a similar manner as a transcriptional regulator of ER-sensitive genes. ERα was the first described ER. Unlike ERβ, ERα shows little ligand-independent activity, but the AF-1 domain is very active under ligand stimulation in ERE reporter-gene expression studies when compared to ERβ

[58]. In the brain, ERα has been shown to play a critical role in regulating reproductive neuroendocrine function and sexual behavior [59]. Studies done by Dupont and colleagues engineered ERα (ERαKO) and ERβ (ERβKO) null mice to elucidate the relative contribution of each receptor in the reproductive system. These mouse models revealed that ERαKO female mice are infertile, while ERβKO mice are only subfertile [60]. However, ERβKO mice suffered from neurological deficits that weren't exhibited in ERαKO mice. These results are consistent with a lesser distribution of ERα in the brain that is not as extensive as ERβ, yet prominent in female reproductive organs. An interesting finding from these ER-null mouse models is they delineated an important relationship between ERα and ERβ function: when one of these receptors is non-functional, the other can compensate for some of the lost function. In many tissues, ERα and ERβ are co-expressed, suggesting that they can influence each other's action in a given cell. Indeed, ERβ has been shown to oppose the effects of ERα in some breast cancer models, yet it can act synergistically on EREs [61, 62].

There also exists a G protein-coupled estrogen receptor GPER (formerly called GPR30), which mediates non-genomic effects of E2 [63]. Early studies showed that intrauterine E2 treatment resulted in a rapid increase in intracellular cAMP [64]. Nearly three decades later there was evidence that E2 stimulated adenylyl cyclase, leading to second message signaling by calcium (Ca2+) and inositol triphosphate (IP3) [65-68]. The discovery of GPER led to the novel understanding that E2 not only regulates gene transcription, but can also regulate important second messenger signaling pathways that mediate cell growth and survival [69].

ERβ Distribution in the Brain

The distribution of ERβ expression in the brain varies according to specific region and sub-nuclei within those regions [70-72]. For instance, ERβ can be found throughout the hypothalamus, an important brain region which regulates stress, mood and other autonomic functions [59]. The hypothalamus also contains several sexually dimorphic nuclei, meaning that there are clear differences between both structure and function between males and females [59, 73]. This part of the brain also responds differently to sex steroid hormones in males and females, which is in part due to differential expression of ERs in this region. A high density of ERβ expressing cells has been shown in two particular subnuclei of the hypothalamus, the preoptic area (POA) and paraventricular nucleus (PVN) [70]. These two regions are important for mediating the release of neuroendocrine hormones, such as gonadotrophin-releasing hormone (GnRH) and corticotrophin-releasing hormone (CRH), respectively [74, 75].

Estrogen receptors also play important regulatory roles in learning and memory, which are processes mediated in large part by the hippocampus; a brain region that expresses high levels of ERβ [70]. The two major divisions of the hippocampus, the dorsal and ventral hippocampus, have differing functions. The dorsal hippocampus is associated with spatial memory, verbal memory, and learning of conceptual information, while the ventral hippocampus functions in fear conditioning and affective processes [76-78]. The importance of E2 in mediating cognitive functions within these regions has been described through synaptic plasticity and cell proliferation in the hippocampus, and loss

of E2 signaling can negatively affect these functions as observed in multiple behavioral and cellular studies [79-85].

Another important sexually dimorphic region in which ERβ expression is observed in high density is the amygdala, which performs a primary role in processing of emotional memories [86]. Divided into four subdivisions (medial, cortical, central and basolateral), all four of these divisions express ERβ [70]. Anxiety disorders have been linked to dysfunction of the amygdala, a disorder which is more prevalent in females than in males [87, 88]. Amygdala dysfunction has been attributed to disrupted connections with the hypothalamus and cerebellum. Furthermore, analysis of the distribution of ERβ in the cerebral cortex revealed a high density of ERβ expressing cells within layers IV-V [70]. Moderate ERβ expression can be found also within layers II-IV. Expression of ERβ in the cortex has been linked to homeostasis of the cortex, including increased spine density and modulation of synaptic signaling [89, 90].

ERβ Alternative Splicing

Like many multiexonic proteins, ERβ is subject to alternative splicing. These alternative splicing events can result in the loss or addition of exons that can alter the function of the receptor. Shortly after the discovery of ERβ, a second isoform was discovered from cDNA libraries of rat ovary that encoded a longer form of ERβ [91]. This was quickly followed by the discovery of hERβ splice variants in testis cDNA libraries [92]. Rat (r)ERβ2 has an 18 amino acid insert within the ligand binding domain that decreases its affinity for estrogen up to 30-fold [93]. Shortly thereafter brought the

discovery of additional splice variants in the rat that followed different alternative splicing patterns than those observed in humans [94, 95]. The delta variants, delta3 (d3) and delta4 (d4), result from an exclusion of exon 3 or 4, respectively. A deletion of exon 3 results in the loss of the receptor's ability to bind DNA, with 39 amino acids deleted from the DBD. Deletion of exon 4 not only eliminates its ability to bind ligand, but also alters the cellular localization of the receptor. As observed by Price and colleagues this variant appears to be abundantly present in the cytoplasm and lacks a nuclear localization signal [94]. The d3 variant appears to occur independently of ERβ2 alternative splicing due to the coexistence of both an ERβ1d3 variant and ERβ2d3 variant. The d4 variant only appears to occur as ERβ1d4, although it's been postulated that ERβ2d4 may also exist.

By comparison, alternative splicing of human (h)ERβ yields splice variants that are all truncated by varying lengths at the C-terminus, which eliminates the F domain and portions of the LBD [92]. In the brain, there are at least 4 splice variants described in humans and 5 in rat. An additional human splice variant, hERβ3, has also been described and its expression is restricted to the testis [92].

The expression of the ERβ splice variants occur throughout the brain, and they tend to be expressed in a region-specific manner [96]. For instance, the PVN and the supraoptic nucleus (SON) both highly express ERβ splice variants [94, 97]. In addition, the hippocampus expresses the ERβ2 splice variant along with less observed ERβ1d4 variant [94]. Interestingly, increased expression of the ERβ splice variant ERβ2 has also been linked to anxiety disorders in female ovariectomized (OVX) rats [79] and studies

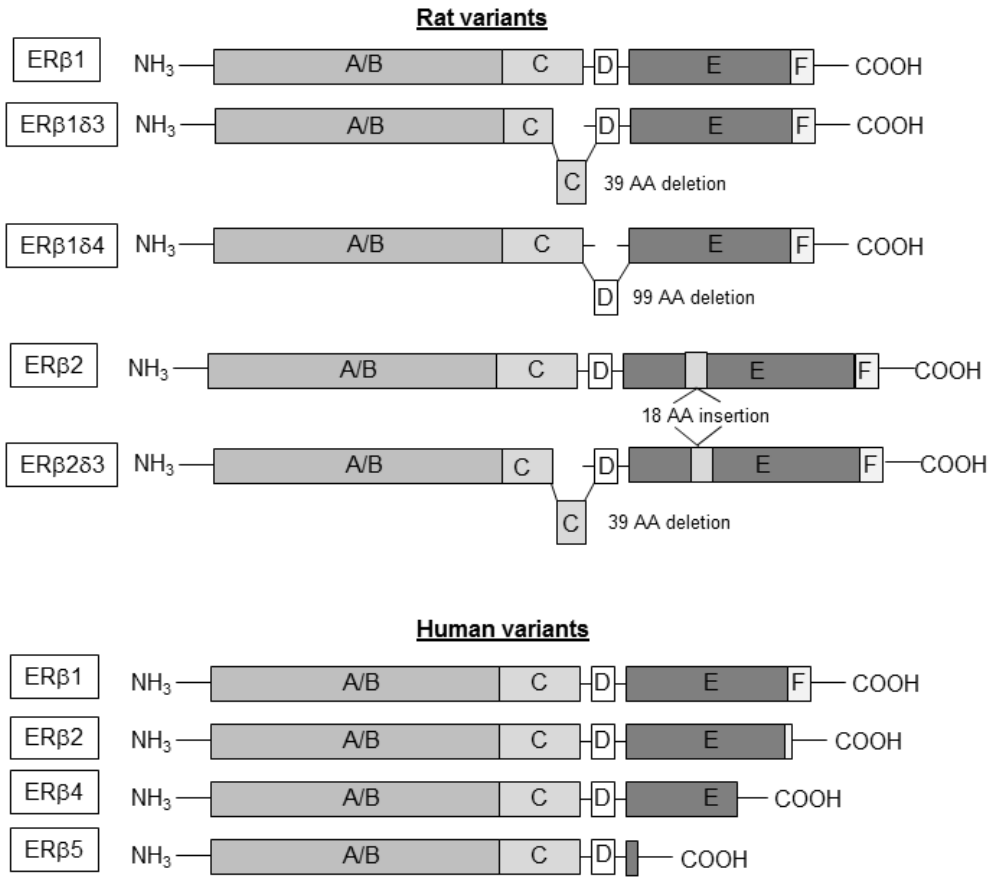

Figure 2. Diagram of rat and human ERβ splice variants. Structural depiction comparing the differences between rat and human splice variants of ERβ. A/B = AF-1 domain, C = DNA binding domain, D = hinge domain, E = AF-2/ligand binding domain, F= undefined.

have confirmed that the amygdala expresses the ERβ2 splice variant, along with ERβ1d3 and ERβ2d3 [94]. The cortex also expresses the ERβ splice variants ERβ2 and ERβ1d3 [94, 95]. Taken together, these brain-region specific patterns of ERβ splice variant expression suggests that each splice variant has distinct mechanisms regulating their alternative splicing.

The ERβ splice variants are both structurally and functionally different from the wild-type ERβ1. Our laboratory revealed that ERβ2 had weaker effects on ERE- and activator protein (AP)-1-mediated luciferase reporter gene activity in the presence of E2, and the ERβ-selective agonist diaryl propriolnitrile (DPN), when compared to ERβ1 [98]. Unlike ERβ1, ERβ2 was also unresponsive to the androgen metabolite 5α-Androstane-3β, 17β-diol (3βAdiol), further revealing the effects of alternative splicing on the LBD. These differential functions were also demonstrated using complex gene promoters, such as GnRH and arginine vasopressin (AVP). Specifically, ERβ1d3 increased GnRH promoter activity both in the presence and absence of E2, which differed from the effects of ERβ1 or any of the other splice variants [99]. Both ERβ1 and ERβ2 increased reporter activity through ligand-independent actions, but E2 treatment blocked this ligand-independent effect. However, the use of selective estrogen receptor modulator (SERM) raloxifene inhibited GnRH the promoter E2-dependent increase observed with ERβ1d3. These opposite effects of treatment on ERβ1d3-mediated transcription suggested that the structural differences resulting from alternative splicing alters the protein conformation upon ligand binding. Ligand-induced changes in protein conformation are critical for exposing docking sites to recruit the necessary coregulatory proteins required for

mediating transcriptional activity. Thus, the ERβ splice variants might recruit a different suite of coregulatory proteins, or might have decreased ability to form stable protein:protein interactions, resulting in differential signaling through these alternative forms of the receptor.

Further studies of the rERβ splice variants, ERβ2 and ERβ1d3, showed that they were less effective at stimulating AVP promoter activity in the absence of ligand when compared to ERβ1 [100]. However, ERβ2, but not ERβ1d3, stimulated AVP promoter activity equally well when treated with either E2 or the ERβ-selective agonist 3βAdiol. The ERβ1d3 splice variant was shown to have no effect on AVP promoter activation, either in the presence or absence of ligand binding. The production of a novel ERβ2 antibody by Chung and colleagues increased our understanding of ERβ2 coexpression with GnRH and oxytocin neurons in the brain, and also expanded the field of ERβ splice variant studies [101]. A recent study using this ERβ2 antibody showed that increased ERβ2 protein expression resulted in negative effects on hippocampal neurogenesis and depressive-like behaviors in aged female rats [79]. These studies also detected ERβ2 protein in white blood cells, uncovering a possible marker for the study of E2 efficacy of HT in the context of the timing hypothesis. Together, these functional differences demonstrate that increased expression of ERβ splice variants with age and/or decreased circulating levels of E2 could have profound impacts on ERβ-mediated gene expression.

Our laboratory has also studied the function of hERβ splice variants using neuronal cell lines, and have showed that these human variants are able to activate ERE-regulated promoter activity in a ligand-independent manner similar to rERβ, but are

largely unresponsive to ligand [56]. Functional studies of hERβ splice variants in the brain are prohibitive and we must rely on rat models to provide a basis for their speculative roles in human. However, studies have used human breast, ovarian, and prostate cell and tissue models to understand the role of the hERβ splice variant in those contexts [102-109]. Moreover, 5 additional hERβ disease-related splice variants have been described in a recent mini-review by Taylor *et al.* [110].

ERα is also subject to alternative splicing, yet it is relatively unknown if these splice variants are expressed as proteins [110-112]. To date, 18 splice variants have been described in the brains of patients who suffered from schizophrenia [113], and more than 20 variants have been found in breast cancer cell lines and tumors [114]. Interestingly, a recent study found that nuclear protein E3-3 (NPE3-3) regulates alternative splicing of several of these ERα variants [115]. It is unclear if this nuclear factor regulates ERβ alternative splicing.

Regulation of Alternative Splicing

Protein diversity is created through the mechanism known as alternative splicing. Alternative splicing is the process by which exons located within a pre-mRNA transcript may be excluded from, or included within, the final gene product depending on a number of factors that influence this post-transcriptional process. These alternatively spliced transcripts may result in translated proteins that have similar activity to wild-type proteins, functionally immature proteins, or proteins that have lost critical domains that are required for proper physiological function. An estimated 90-95% of multiexonic

proteins undergo alternative splicing from the approximately 20,000 genes encoded within the human genome [116]. Alternative splicing should not be confused with general RNA splicing events that result in wild-type mature mRNA products for translation.

The process by which alternative splicing occurs is via *trans*-acting factors binding to *cis*-regulatory sites on the pre-mRNA [117-119]. Exon and intron splicing enhancers (ESEs and ISEs, respectively) are RNA sequences that promote the use of a weak or gene-regulated splice site, mediated by a complex containing serine-rich (SR) proteins, which can recruit other splicing factors. Exon and intron splicing silencers (ESSs and ISSs, respectively) are sequences which repress the use of splice site via recruitment of factors such as heterogeneous nuclear ribonucleoproteins (hnRNPs) that block spliceosomal assembly at the site [120].

Trans-acting proteins, called small nuclear ribonucleoproteins (snRNPs), form a complex with small nuclear RNAs (snRNAs) called the spliceosome. The spliceosome complex is generally responsible for removing the introns from pre-mRNA transcripts in order to produce mature mRNA for further processing. The snRNPs, designated U1, U2, U4, U5, and U6, mediate splicing through a succession of enzymatic reactions, along with assistance from U2 auxiliary factors (U2AF), splicing factor 1 (SF1) and SR proteins [118, 121].

U1 binds to the 5' intronic GU sequence along with accessory proteins and enzymes, which is called the spliceosome E complex (Figure 3) [122]. U2 binds to the branch site, resulting in the hydrolysis of ATP. This step forms the A complex, a critical step in determining the site at which splicing will occur by defining the intron to be

removed and the exon to be included. When the U4/U5/U6 trimer complex assembles, U5 binds to the 5' exon and U6 binds to U2, forming the B complex [123]. U1 is released from the complex, initiating a shift whereby U5 moves from exon to intron and U6 binds to the 5' splice site, which subsequently forms the C complex. U4 is then released and U6/U2 catalyzes transesterification at the 5' splice site to form the lariat structure at the branch point [124]. With the U5/U6/U2 complex still bound, the 3' site is cleaved and ATP hydrolysis drive ligation of the opposing exons. The spliced RNA is then released, and the remaining spliceosome complex disassembles.

Spliceosome assembly and alternative splicing events are also facilitated by RNA-binding proteins (RBPs). RBPs recognize specific sites located within the pre-mRNA transcript that can regulate splicing decisions made by the spliceosome, resulting in alternative splicing events. As mentioned previously, hnRNPs can enhance splice site silencing through binding of ISS and ESS sequences that can counteract the effects of other RBPs by blocking their ability to enhance splicing [125, 126]. RBPs are responsible for a host of interactions that coordinate general RNA splicing. RNA helicases, like DEAD-Box RNA helicase Ddx17 and Ddx5 are important for altering RNA secondary sequences, alternative splicing choices, and transcription initiation [127].

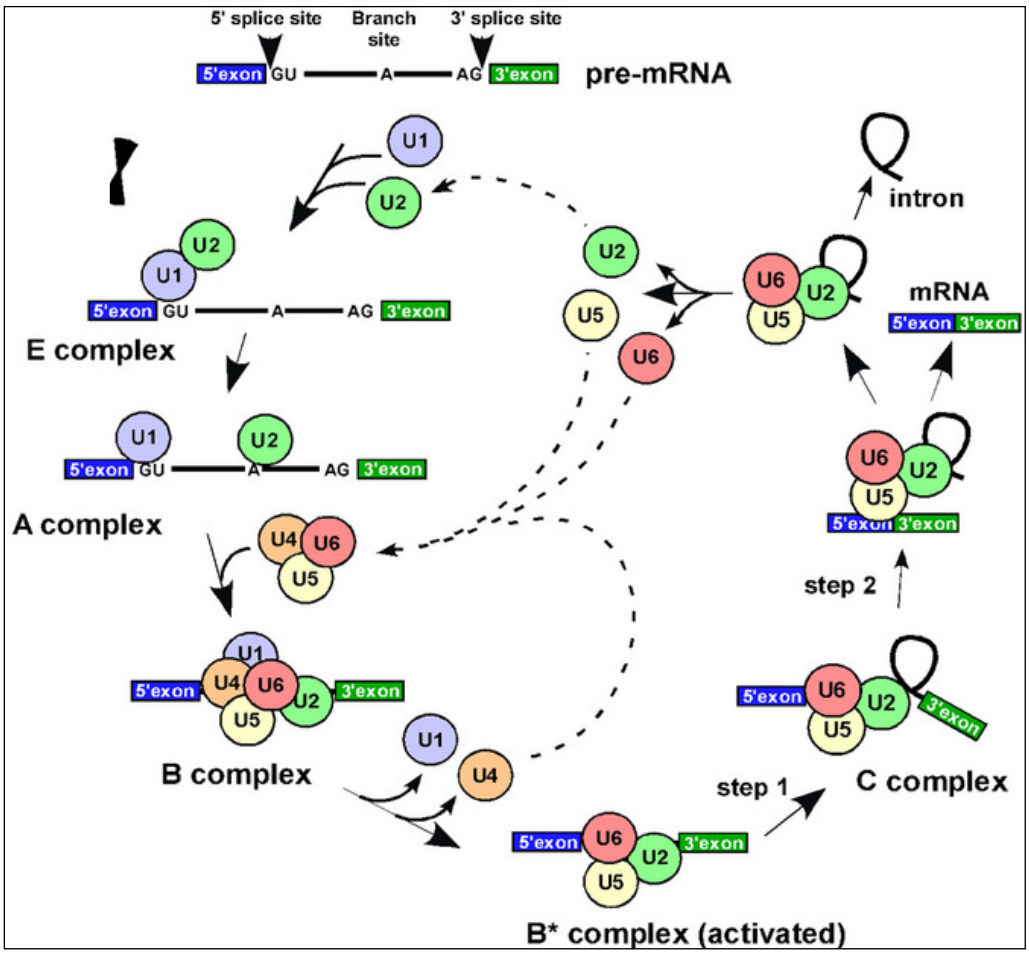

Figure 3. Schematic of spliceosome assembly and RNA splicing (adapted from H. Urlaub, Max Planck Institute). A generic pre-mRNA transcript is processed by the spliceosome complex in a highly ordered set of sub-complexes and chemical reactions.

Other RBPs like RNA binding protein fox-1 (Rbfox1), CUG-Binding Protein Elav-like family member (Celf)4, Celf5, and neuro-oncological ventral antigen 1 (Nova1) are important for development and maintaining splicing patterns in the brain [13, 128-131]. Loss or altered expression of these RBPs affect splicing patterns which can affect synaptic plasticity, neuron excitability, behavior, and development [129, 130]. Alternatively spliced exons can also affect its translation into a protein, and the loss of these RBPs can result in nonsense-mediated decay (NMD) of the mRNA. NMD allows the cell to control the translation of mRNAs that would result in a gain of function or dominant negative form of the protein [132]. However, certain splicing events can result in mRNA that bypasses the NMD pathway, which results in increased expression of dominant negative variants.

The alternative splicing of ERβ results in a set of splice variants that can alter the efficacy of E2-regulated physiological processes. However, the majority of studies have focused on ERβ splice variants function, and there is little known about the factors regulating their expression in various tissues [56, 94, 95, 98-100]. The regulatory mechanisms by which 54bp are inserted into ERβ2, or how exons 3 and 4 are removed from the pre-mRNA transcripts, along with the possible splicing factors associated with these alterations have yet to be described. Interestingly, the most commonly studied splice variant, ERβ2, has sequence characteristics suggesting that it is not a splice variant, but perhaps is the wild-type form of ERβ. Analysis done in our laboratory of the structure of ERβ2 shows that the 54bp insert is located within an intron between exons 5 and 6,

which together comprise the LBD. In order to qualify as an exon, the sequence must be present in the mature mRNA and also contains part of the open reading frame (ORF) of the coded protein [133]. Analysis shows no stop codons that would be read if translation were out of frame. Further analysis shows weak 5' and 3' splice site upstream and downstream of the insert (Figure 4) [134, 135]. The regions both contain respective branch points at which the U2 snRNP may recognize to assist in recruitment of the U4/U5/U6 trimer. Therefore, these observations suggest that the ERβ2 insert is not an intronic element, but actually an alternative exon encoded within the pre-mRNA transcript of ERβ.

Figure 4. 5’ and 3’ weak splice sites located downstream of Exon 5. Weak splice sites (underlined text) were detected using SplicePort splice site prediction software (http://spliceport.cbcb.umd.edu/). The computational donor (5') score was 1.46 and the acceptor (3') score was 0.851, where a score of 0.8 or greater is considered probable.

Nova1 Splicing Factor

Neuro-oncological ventral antigen 1 (Nova1) is a protein that was observed in patients with paraneoplastic neurological degenerations (PND) to induce an autoimmune response due to its abnormal expression in tumor cells [136]. It wasn't until after its discovery as a tumor antigen that continued work done by Darnell and colleagues elucidated that it was a neuron-specific RNA-binding protein that mediated the alternative splicing of several receptors in the brain [13, 137]. Nova1 regulates the expression of many important receptors in the brain, including glycine, GABA, and dopamine receptors [13, 128, 138, 139]. Studies on RBPs were revolutionized by the HITS-CLIP method, an UV-crosslink immunoprecipitation experiment for high throughput studies on RBPs in the brain [140, 141]. These studies were developed by the same group that discovered Nova1, so naturally the neuron-specific RBP was used for the development of this method. These studies along with computational analysis led to the discovery of over 700 splicing events that are regulated by Nova1 in the brain, signifying the importance of this RBP in neurons [142, 143].

Structurally, Nova1 contains three KH-domains responsible for binding single-stranded RNA [144]. Pre-mRNA transcripts regulated by Nova1 contain a consensus sequence (YCAY, where Y indicates a pyrimadine) that, when recognized by Nova1, results in the binding of this protein to the transcript at this location [128]. For efficient Nova1 binding, these YCAY consensus sequences need to be clustered together. Therefore, YCAY clusters located in an intron or exon dictates the outcome of splicing of

the Nova1-regulated mRNA. Intronic YCAY clusters located either upstream or downstream of the Nova1 alternatively spliced exon mediate enhanced spliceosome assembly and inclusion of the exon (Figure 5) [145]. However, intronic YCAY clusters located immediately upstream of the exon blocked U1 snRNP binding, resulting in exon exclusion. This was also true for YCAY cluster found within the alternatively spliced exon itself. However, recent evidence from Park and colleagues found that Nova1 binding to exonic YCAY clusters can block binding of hnRNP M, a known splicing inhibitor [139]. Degrees by which Nova1 binds these YCAY cluster may lead to a modulatory or fine-tuning mechanism that coincides with its ability to enhance or repress alternative splicing. Analysis of ERβ pre-mRNA transcripts show that several YCAY clusters are located upstream of the ERβ2 insert (Figure 6). Therefore, Nova1 may be an important regulator of ERβ alternative splicing.

Since the discovery of Nova1 nearly two decades ago, there have been several studies on the regulation of this splicing factor. One of the first studies on Nova1 expression revealed that Nova1 can autoregulate itself through removing the E4 exon that contains a phosphorylation domain and KH domain that recognizes the YCAY repeats [146]. Nova1 expression has been shown to be regulated by hormones like glucocorticoids (GC) and also by miRNA 181b-5p in the context of astrocytomas [147, 148]. A recent study on aging in the brain revealed Nova1 expression declining with advanced age, both in healthy and neurodegenerative male patients [149]. These decreases in Nova1 expression resulted in significant increases in alternative splicing

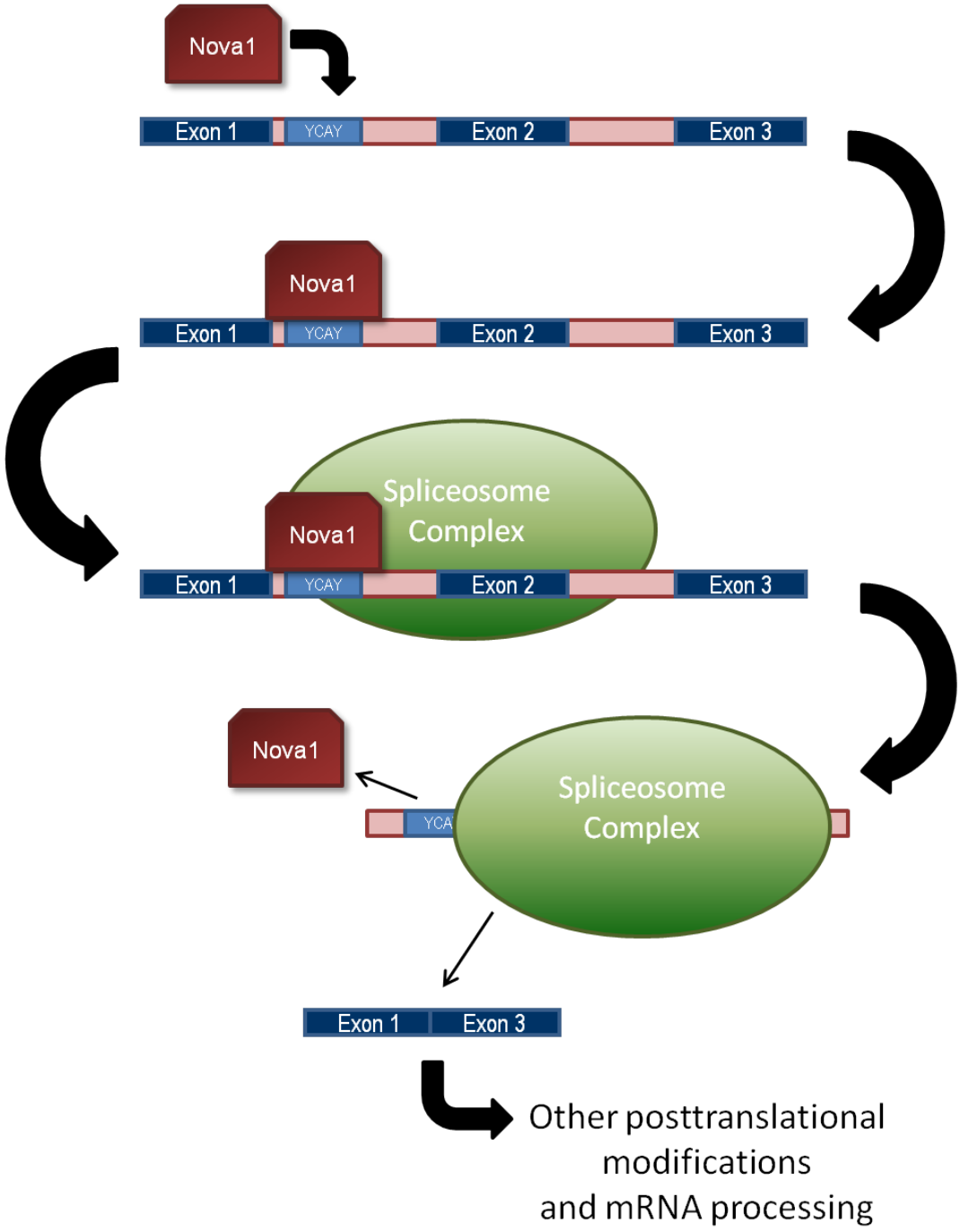

Figure 5. A simplified model of Nova1 exon exclusion mechanism. Nova1 recognizes YCAY consensus sequence located within the intron upstream of an exon. Upon binding, Nova1 enhances spliceosomal removal of the exon, along with the flanking intronic element. The excluded elements are removed from the mRNA transcript through RNA splicing outlined in Figure 3.

events in proteins associated with neurodegeneration. To date, there have been no studies determining the effects E2 on Nova1 expression.

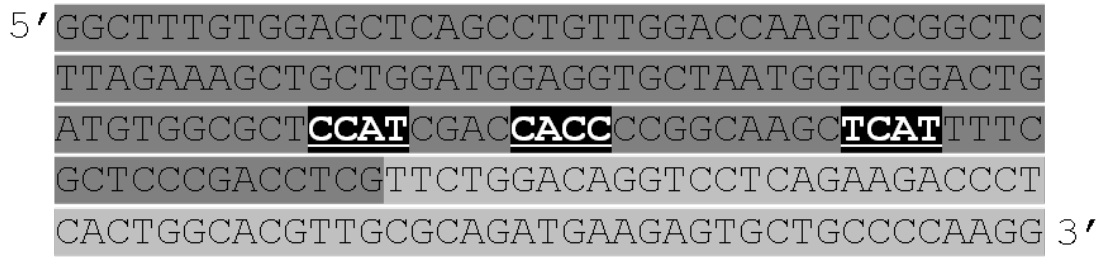

Figure 6. Nova1 consensus sequences in ERβ exon 5. Full-length ERβ mRNA was analyzed for the presence of Nova1 YCAY consensus sequences using RBPmap (http://rbpmap.technion.ac.il/index.html). Highlighted (black boxes, white text) regions indicate consensus YCAY sequences located in the depicted exon 5 (highlighted in dark gray). The ERβ2 alternatively spliced insert is highlighted in light gray box. Scores assigned to Nova1-binding probabilities must be greater than 0.895 (out of 1.00) based on weighted rank in order to be detected (Paz et al., Nucleic Acids Res. 2010).

CHAPTER III

AGING AND LOSS OF CIRCULATING 17B-ESTRADIOL ALTERS THE ALTERNATIVE SPLICING OF ERB IN THE FEMALE RAT BRAIN

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Hormone replacement therapy (HT) has become routine in abrogating the negative effects associated with the decline in circulating 17β-estradiol (E2) in women postmenopause. The efficacy of HT in mediating these negative effects is temporally dependent, as inferred from data obtained in the Women's Health Initiative (WHI) and Women's Health Initiative Memory Study (WHIMS) studies. Specifically, HT was beneficial or neutral for most parameters measured (cognition, memory, cardiovascular) in younger postmenopausal women, whereas older women, 10 or more years postmenopause, experienced adverse effects including cardiovascular and coronary disease, stroke, cognitive impairment, and dementia [2-5, 150, 151]. Other studies have suggested the idea of a therapeutic window in which HT is beneficial, known as the "timing hypothesis", pointing to age-related adjustments that occur during and after this critical period of declining E2 levels [6, 7, 10-12, 38-41]. Although the benefits of HT on mood in postmenopausal women are controversial, recent studies have yielded optimism about the beneficial effects of HT, especially on mood and anxiety [8, 32, 152, 153]. However, the mechanisms by which the molecular environment of the aging brain changes during this period in response to E2 withdrawal remain unknown. We

hypothesized that differential effects of E2 could be due to changes in alternative splicing of the estrogen receptor.

The physiological effects of E2 are mediated primarily through estrogen receptor α (ERα) and β (ERβ). These receptors act as transcriptional regulators for genes that are functionally important for a variety of processes in the brain including memory, reproduction, and stress [20, 24, 33, 35, 59, 154, 155]. Similar to many other proteins, ERβ is subject to posttranscriptional alternative splicing. There are several identified ERβ splice variants that naturally occur in both humans and in rodents [91, 92, 94, 95, 156]. ERβ1 is the wild-type isoform that is primarily expressed throughout the human (hERβ1) and rat (rERβ1) brain. This isoform also has the highest affinity for E2, the major circulating estrogen during the reproductively competent period of the lifespan [157]. At least four hERβ splice variants are expressed in the human brain, and five splice variants of rERβ have been described in the rat brain [95, 158]. The physiological relevance of these ERβ splice variants has been highlighted in a recent study where increased expression of the rat dominant negative ERβ2 isoform diminished the effectiveness of HT in OVX female rats [79]. This study also revealed that withdrawal of E2 over time might increase the expression of ERβ2, further implicating the actions of these receptors when circulating E2 levels reach the nadir that occurs with menopause. Thus, the increase in alternative splicing of ERβ may negatively impact E2-regulation of important processes due to the structural and functional differences that occur in these variants.

Alternative splicing of ERβ in humans differs from that in rats: hERβ variants are the result of truncations from the C-terminal end of the mRNA transcript, while rERβ

splice variants arise from insertions and deletions within the mRNA transcript (Figure 2) [92, 94, 95]. Nevertheless, both human and rodent ER variants are functionally different from the wild-type, can form heterodimers with the wild-type, and ultimately alter downstream E2-mediated signaling pathways [46, 56, 100, 158]. More importantly, these splice variants have also been shown by our laboratory to be constitutively active in the absence of ligand binding, suggesting they might be particularly important postmenopause. Ligand-independent effects of ERβ impact a variety of genes that are regulated by ERs, including arginine vasopressin (AVP) and corticotrophin releasing hormone (CRH) [56, 98, 100, 159], two hypothalamic hormones important for regulating stress and anxiety [160]. As it pertains to memory, the dorsal and ventral hippocampus are important regions that also express ERβ splice variants. The dorsal hippocampus is important for spatial and verbal memory, while the ventral hippocampus is important for fear conditioning and affective processes [161]. Memory impairment, overall declines in cognitive function, and increased anxiety are all possible negative effects that coincide with loss of circulating E2 in postmenopausal women [2, 3, 8, 19, 20, 162]. Therefore expression of these alternatively spliced receptors during menopause, a time when E2 is no longer being produced endogenously by the ovaries, may have a variety of implications within many different brain regions, including the hypothalamus and hippocampus.

Alternative gene splicing contributes to increased biodiversity of proteins that can be expressed from a limited set of genes within our genome, as over 95% of multi-exonic genes are alternatively spliced [116]. Recent evidence has suggested that alternative

splicing events increase with age in the brain [149]. Gene transcripts that contain multiple coding exons can undergo splicing that may exclude these exonic sequences from the final mRNA transcript to be translated [119]. These alternative splicing events occur through mechanisms that involve either *cis*-sequences encoded within the pre-mRNA transcript, or by *trans*-acting splicing factors, such as RNA binding proteins [163, 164]. The rate at which alternative splicing occurs can also influence exon inclusion events [165, 166]. For instance, interference of RNA Polymerase II (RNAPII) elongation rates resulted in inhibition-dependent changes in alternative splicing, whereby exons that were located 3' downstream of short intronic sequence were included within the mature mRNA transcript [167]. It is becoming widely accepted that alternative splicing events likely occur at the site of transcription, and in cooperation with transcription, and that these coordinated actions between *trans*-acting factors and RNAPII are heavily involved in determining the fate of pre-mRNA transcripts [168, 169].

The purpose of this study was to determine how E2 deprivation, as occurs during menopause, affects the alternative splicing of ERβ in the aging brain. We hypothesized that ERβ2 expression would increase in a tissue-specific manner due to age-related and E2 deprivation-related changes in alternative splicing, and treatment with E2 would abrogate these effects. To test this hypothesis, we OVX Fisher344 rats at 3 and 18 months of age to study the effects of aging (1 week OVX) and E2 deprivation (1-12 weeks OVX) followed by acute E2 treatment in these animals (Figure 7). Moreover, changes in the expression or activity of RNAPII have not been previously demonstrated in aged animals, or in relation to E2 treatment. Therefore, we also assessed expression of

RNAPII and its activity in these animals to determine how this ubiquitous enzyme might influence alternative splicing. Lastly, we assessed how RNAPII interference may specifically affect ERβ2 alternative splicing in a hypothalamic neuronal cell line. Our data revealed that alternative splicing events are influenced by aging and E2 deprivation in a brain region-specific manner. We also observed that interference of RNAPII increased expression of ERβ2, and that expression and activity of RNAPII changes with aging and E2 deprivation in a brain region-specific manner.

Briefly, 3 and 18 month old animals underwent OVX and subjected to our E2 deprivation paradigm and then treated with vehicle or E2 (Figure 7). Circulating E2 concentration was measured in blood to determine levels of hormone prior to studies. A separate group of 18 mo. animals were treated with ERα and ERβ-selective agonists. To determine the effects of aging and E2 deprivation on ERβ alternative splicing, total ERβ and ERβ2 mRNA expression were measured in the hypothalamus, dorsal hippocampus, and ventral hippocampus of these animals. A set of intact animals were used to compare expression levels of ERβ in the hypothalamus. An *in vitro* set of studies were conducted in hypothalamic neurons treated with the TOPI inhibitor camptothecin, E2, or a combination of both to determine the effects of splicing kinetics on ERβ alternative splicing. To determine if aging and E2 deprivation had an effect on RNAPII, expression and activity of RNAPII were measured in the 18+ mo. animals subjected to the deprivation paradigm only via qRT-PCR and western blot.

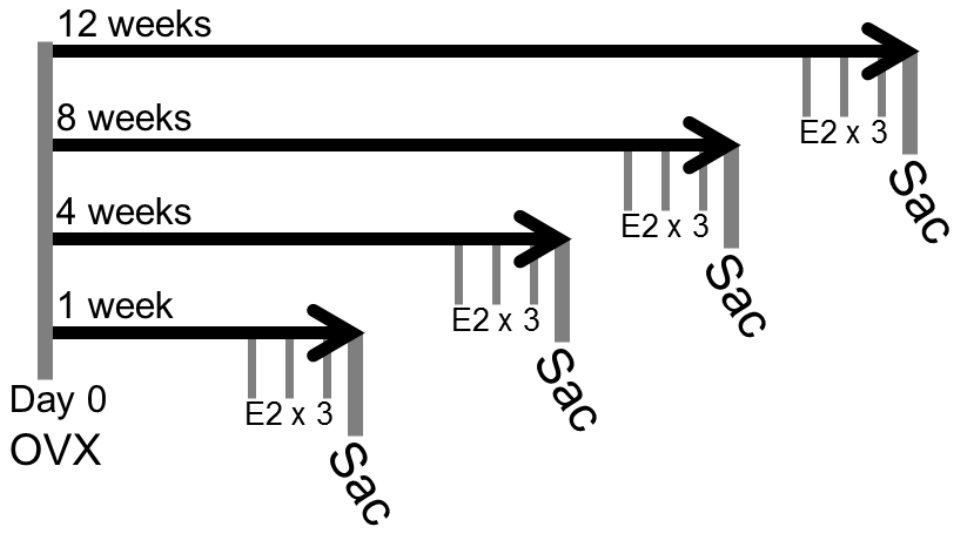

Figure 7. Diagram of the E2 deprivation paradigm. Animals were ovariectomized at day 0 and then separated into 4 deprivation groups (N=16-20/deprivation group) that were subjected to increasingly longer periods of hormone deprivation (1, 4, 8, and 12 weeks). Following deprivation, animals were treated with either vehicle (safflower oil) or E2 (2.5 µg/kg) by subcutaneous injection once daily for 3 consecutive days (n=6-10/treatment group). Animals were sacrificed 24 hours following the last treatment.

Results

ERβ alternative splicing in the aging brain

No changes in total ERβ or ERβ2 expression were detected in ovarian intact animals whose ages corresponded with either 3-mo. old OVX animals, or 18-mo. old OVX subjected to the E2 deprivation paradigm (Figure 8D). There was a significant decrease in total ERβ expression between the 3-mo. and 18-mo. intact animals. Comparison of 3-mo. vs. 18-mo. OVX animals treated with vehicle or E2 revealed a statistically significant main effect of age ($F(1,18)=7.838$, $p<0.015$) on ERβ2 expression in the hypothalamus (Fig. 9A, hatched region), but not the dorsal or ventral hippocampus (Fig. 9B,C). In the hypothalamus, ERβ2 expression was not affected by E2 treatment in 3-mo old animals (Fig. 9A). By contrast, ERβ2 expression was significantly decreased in 18-mo. old vehicle-treated animals compared to 3-mo old animals, yet it was significantly increased following E2 treatment. Further, E2 treatment significantly decreased total ERβ expression only in the dorsal hippocampus and there were no differences in 18-mo. old animals between treatment groups (Fig. 9B). The other previously identified rat ERβ splice variants, ERβ1d3, ERβ1d4, and ERβ2d3, were undetectable in either age group for all brain regions tested (data not shown).

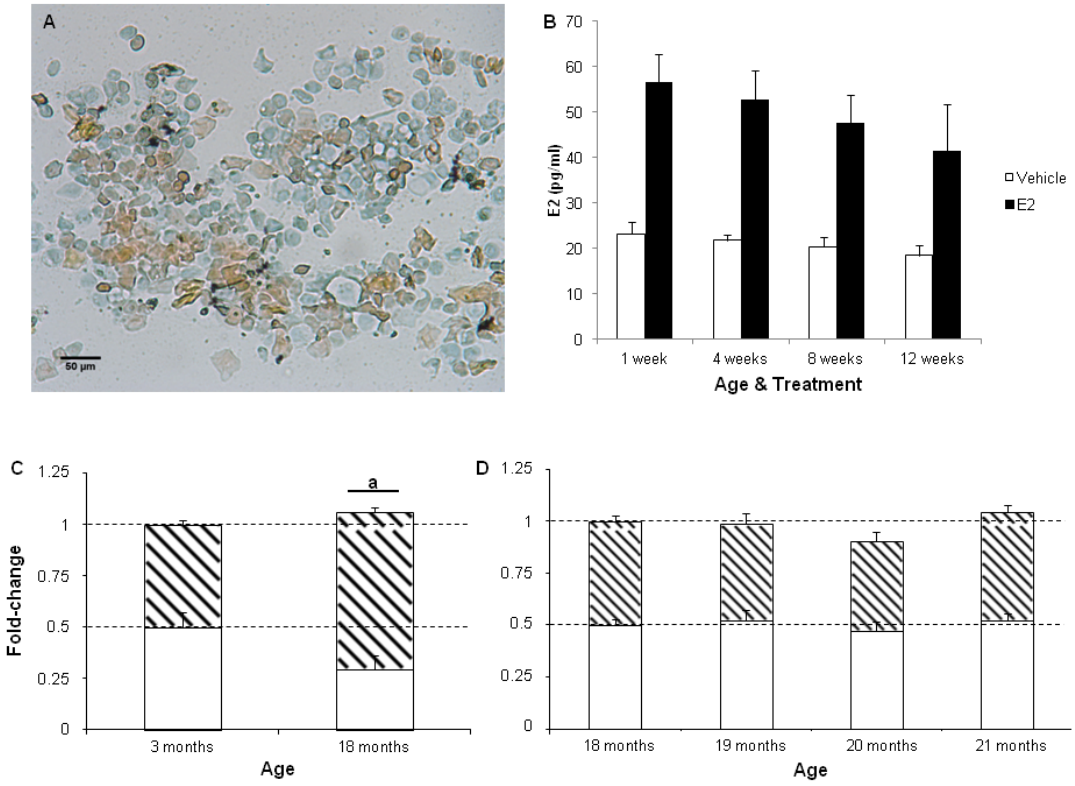

Figure 8. **Vaginal cytology, E2 plasma concentrations, and alternative splicing of ERβ in intact animals.** (A) Representative image (20x) from vaginal smears that were obtained from 18-mo. old female rats daily for seven days before OVX (N = 6). Cells were stained with Papanicolaou stain before imaging. Orange G (orange) stains keratinized squamous epithelial cells. Eosin azure (blue) staining cells represent non-keratinized squamous epithelial cells, neutrophils, and red blood cells (if present). (B) Concentration (pg/ml) of plasma E2 in vehicle- and E2-treated animals during hormone deprivation paradigm. E2 concentration was analyzed by 17β-Estradiol high sensitivity ELISA Kit. 18-mo. animals prior to OVX had low circulating E2 levels (35.0 ± 7.1 pg/ml, N = 6). (C) Total ERβ (white region) and ERβ2 (hatched region) mRNA expression was measured in the hypothalamus of intact (non-OVX) 3 and (D) 18-21 month old female rats. a, indicates statistically significant difference ($p < 0.05$) between groups as determined by paired t-test. Data are expressed as mean ± SEM.

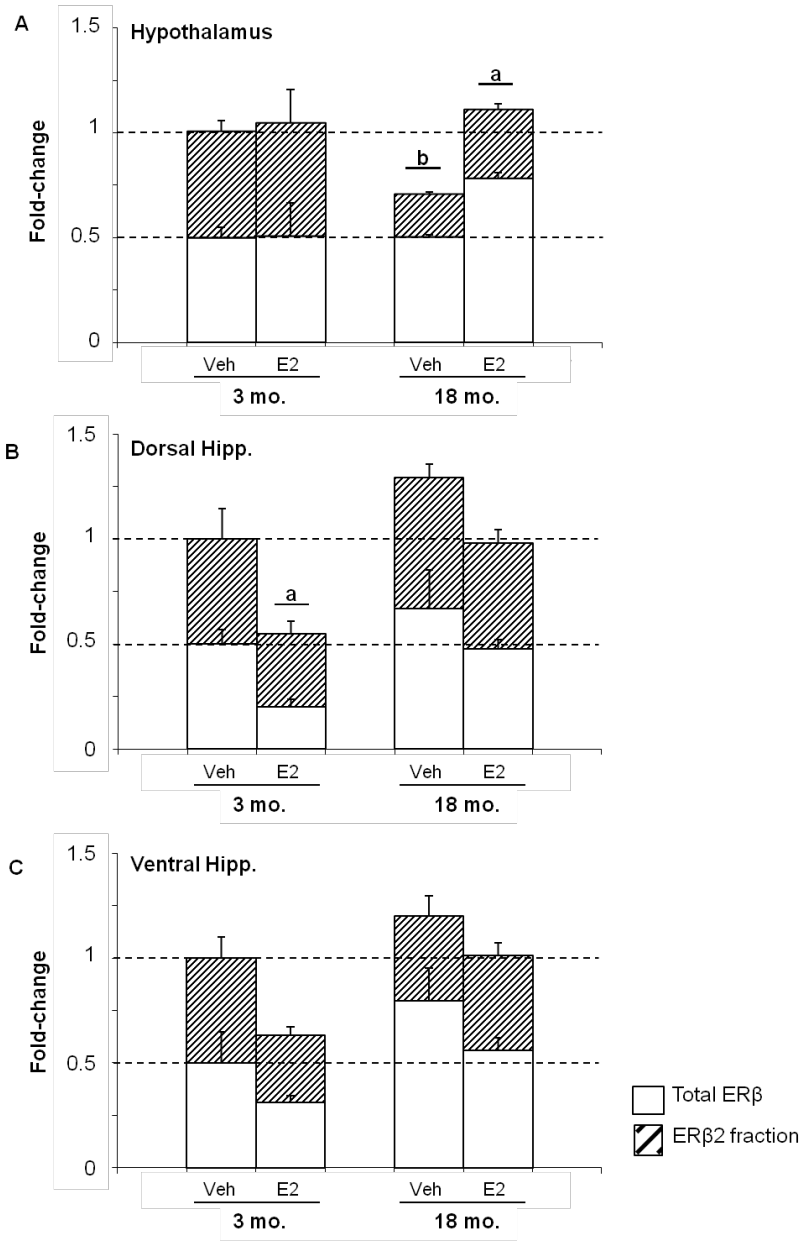

Figure 9. Comparison of total ERβ and the fraction that represents ERβ2 (hatched region) mRNA expression in the young and aged female rat brain. ERβ2 expression was measured in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1 week post-OVX in 3 and 18-mo. old female rats (N = 6/age/treatment group). Animals were OVX and 1 week later treated with either vehicle (safflower oil) or 2.5 µg E2 (dissolved in safflower oil). The letter "a" indicates a statistically significant difference ($p < 0.05$) in total ERβ and the letter "b" indicates a statistically significant difference ($p < 0.05$) in ERβ2 as determined by Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

Brain region-specific changes in ERβ splice variant expression following varying lengths of E2 deprivation in aged female rats

To assess the effects of varying lengths of E2 deprivation in the aged brain, 18-mo. old animals were OVX followed by administration of vehicle or 2.5 ug/kg E2 once/day x 3 consecutive days at varying time points (Fig. 7). Two-way ANOVA revealed a significant interaction between the length of E2 deprivation and subsequent E2 treatment in the hypothalamus ($F(3,40)=3.943$, $p<0.015$) (Fig. 10A). There was also a significant main effect of length of E2 deprivation alone (i.e. vehicle treatment) on ERβ2 expression in both the dorsal and ventral hippocampus ($F(3,42)=7.714$, $p<0.001$ & $F(3,41)=8.409$, $p<0.001$, respectively) (Fig. 10, B and C), as well as a significant main effect of treatment in the dorsal hippocampus ($F(1,42)=5.115$, $p<0.05$) (Fig. 10B), although no statistically significant interaction was revealed in these brain regions. Treatment with E2 significantly increased ERβ2 at the one-week deprivation time point in the hypothalamus (Fig. 10A), consistent with our earlier-described experiment comparing 3-mo and 18-mo. animals (Fig. 9A). E2 treatment also significantly increased ERβ2 expression in the hypothalamus at 12, but not 4 or 8 weeks deprivation (Fig. 10A).

Analysis of total ERβ expression revealed a significant interaction between E2 deprivation and subsequent E2 treatment in both the dorsal and ventral hippocampus ($F(3,42)=12.932$, $p<0.001$ & $F(3,41)=33.585$, $p<0.001$, respectively) (Fig. 10, B and C). In addition, a significant main effect of E2 treatment on total ERβ expression was observed in the hypothalamus ($F(1,40)=6.378$, $p<0.016$). Significant increases in total

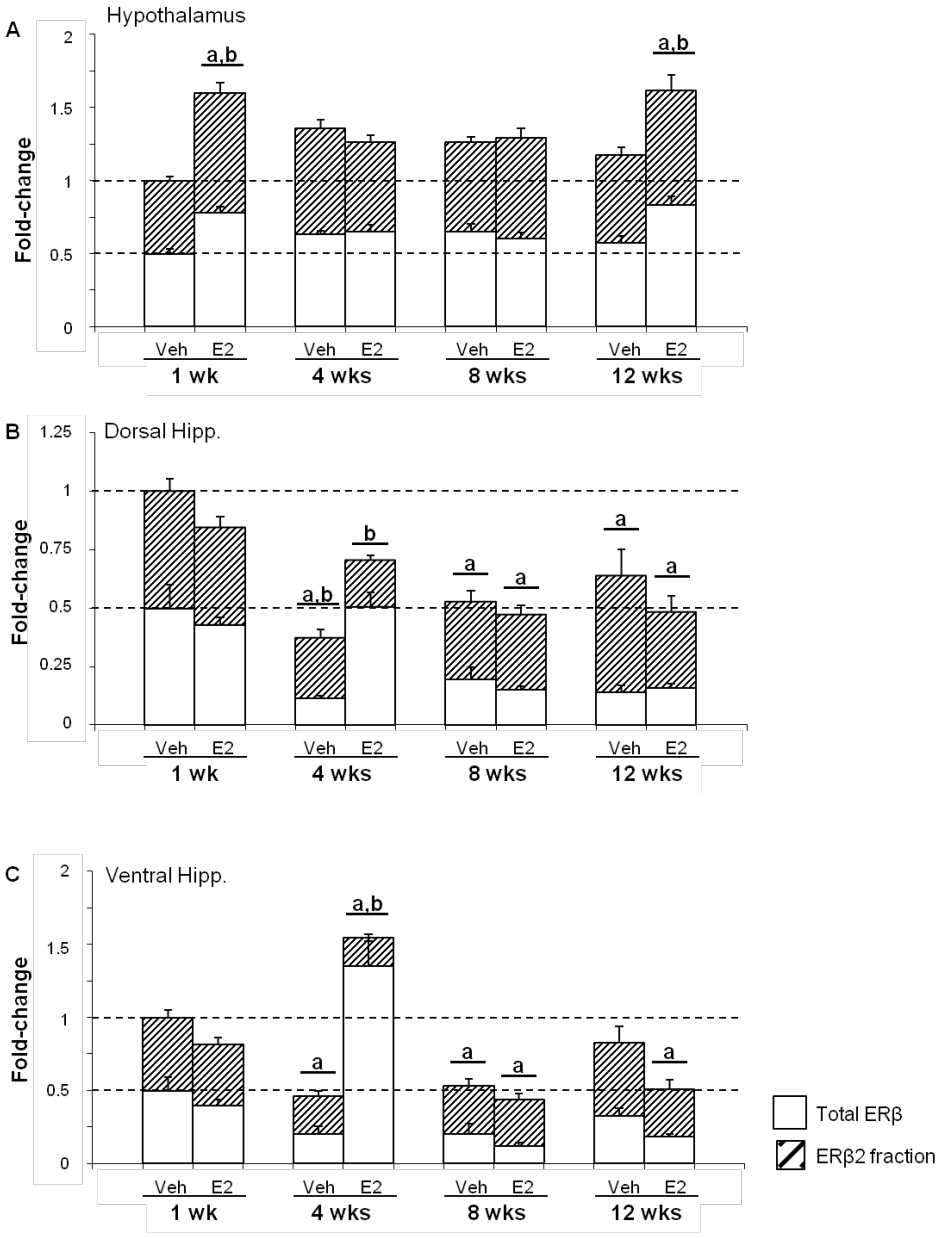

Figure 10. Expression of total ERβ and ERβ2 mRNA in the brain of aged (18-mo. old) female rats following varying periods of E2 deprivation. ERβ2 mRNA expression was measured in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1, 4, 8, and 12 weeks post-OVX (N = 6-10/age/treatment group). Animals were treated with either vehicle (safflower oil) or 2.5 µg E2 (dissolved in safflower oil). The letter "a" indicates a statistically significant difference ($p < 0.05$) in total ERβ and the letter "b" indicates a statistically significant difference ($p < 0.05$) in ERβ2 as determined by Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

ERβ expression with treatment mirrored the pattern observed for ERβ2 expression at 1 and 12 weeks deprivation (Fig. 10A). We observed a significant decrease in ERβ2 expression in both the dorsal and ventral hippocampus following E2 treatment at 4 weeks, but expression levels returned to baseline (i.e. one-week post OVX) beyond this time point (Fig. 10, B and C). Total ERβ expression, on the other hand, decreased after 4 weeks deprivation in both dorsal and ventral hippocampus, and remained lowly expressed through the remainder of the deprivation paradigm, whereas administration of E2 at 4 weeks in the ventral hippocampus resulted in a significant increase in total ERβ expression (Fig. 10C).

E2-induced increases in alternative ERβ splice variant expression is mediated primarily by ERβ

The effects mediated by E2 on ERβ alternative splicing could be mediated by either ERα or ERβ, as both are expressed in each of the brain regions tested [70]. To determine which E2 receptor mediated observed increases in ERβ2, acute administration of the ERβ-specific agonist diarylproprionitrile (DPN) or the ERα-specific agonist propyl pyrazole triol (PPT) was given to 18-mo. old female OVX rats. Our results demonstrated that DPN, but not PPT, significantly increased expression of ERβ2 (Fig. 11). Administration of E2, DPN or PPT significantly increased total ERβ as observed previously.

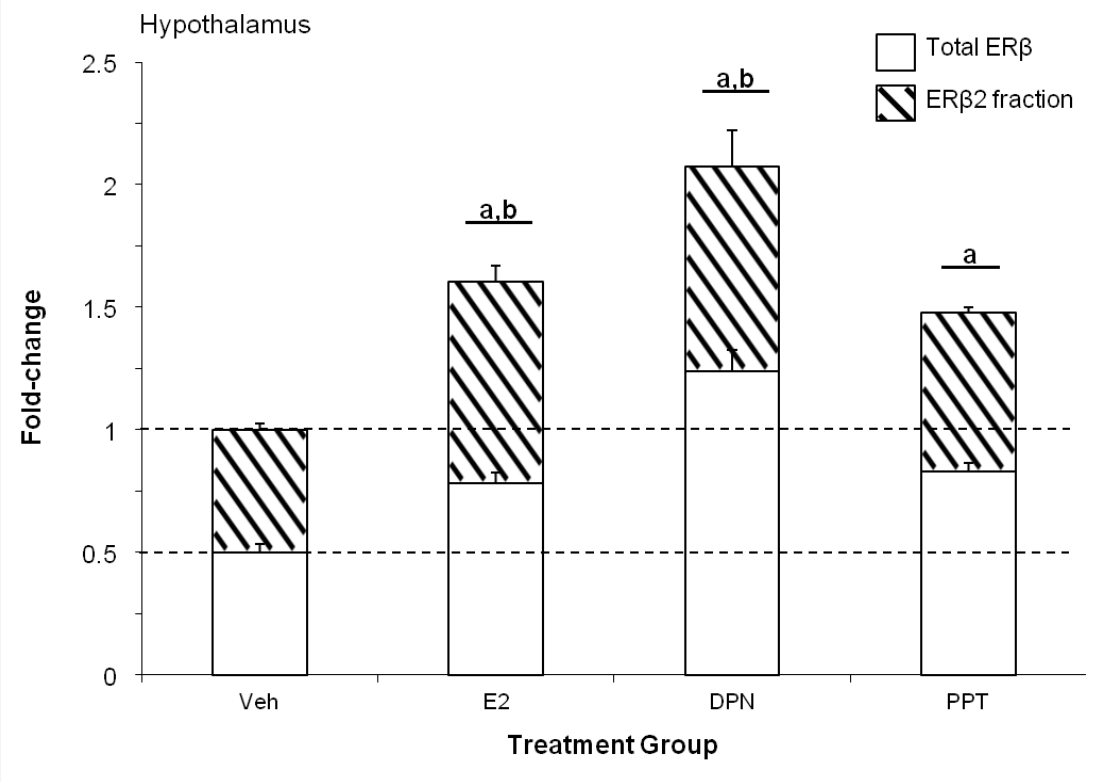

Figure 11. The ERβ-selective agonist DPN increases ERβ alternative splicing. (A) Total ERβ and ERβ2 (hatched region) mRNA expression was measured in the hypothalamus of aged female rats that were OVX and 1 week later treated with either vehicle, E2, 1mg/kg DPN, or 0.5 mg/kg PPT once/day for three consecutive days (N = 6-8/treatment group). The letter "a" indicates a statistically significant difference ($p < 0.05$) in total ERβ and the letter "b" indicates a statistically significant difference ($p < 0.05$) in ERβ2 as determined by Tukey's Honestly-Significant-Difference Test following one-way ANOVA. Data are expressed as mean ± SEM.

Altering splicing kinetics increases ERβ2 expression

The insertion of 54 base pairs (bp) that encode the rERβ2 splice variant are located within the intron between exons 5 and 6, which together encode the ligand-binding domain of ERβ (Fig. 2). Inhibition of topoisomerase I (TOP1) can slow down splicing kinetics, resulting in exon inclusion by allowing the spliceosome machinery more time to recognize weak splice sites in shorter intronic elements flanking the exon [166, 167], yet exons flanked by longer intronic sequences are generally excluded despite TOP1 inhibition. Interestingly, the putative sequence upstream of the ERβ2 insertion is much shorter than most intronic elements in the ERβ pre-mRNA transcript (841 bp compared to the next shortest sequence which is greater than 1K bp). To test if ERβ2 alternative splicing increases with slower splicing kinetics through TOP1 inhibition, we used hypothalamic-derived neuronal cells (GT1-7). Cells were treated with the TOP1 inhibitor camptothecin (32 ng/μl) in the presence or absence of E2 following a period of steroid hormone deprivation. Our results also revealed a significant main effect of treatment ($F(3,12=3.544, p<0.05)$ on ERβ2 expression. Specifically, inhibition of TOP1 significantly increased ERβ2 expression and co-treatment with E2 blocked this effect (Fig. 12).

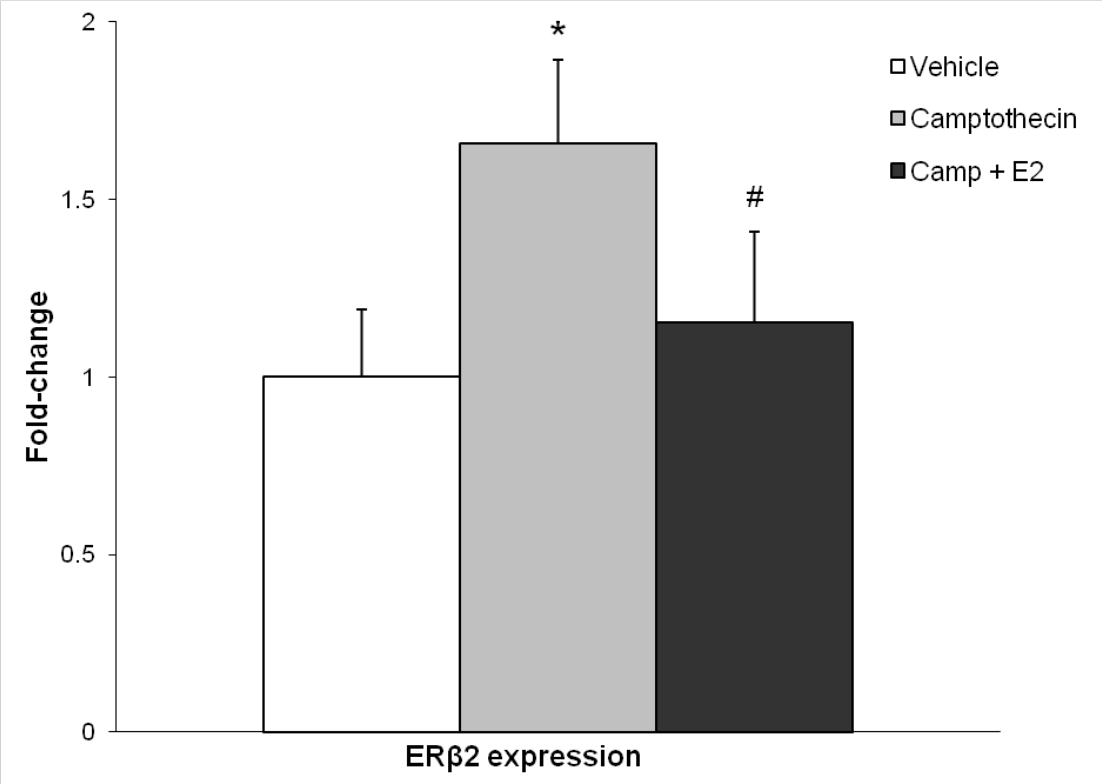

Figure 12. Camptothecin treatment in hypothalamic GT1-7 cells increases ERβ2 expression. ERβ2 mRNA expression was measured in the GT1-7 cell line following 6 hour treatment with 32 ng/ul camptothecin or camptothecin + 10 nM E2 or vehicle (DMSO) (N = 3). *, indicate statistically significant difference ($p < 0.05$) from 1 week vehicle-treated animals as determined by Tukey's Honestly-Significant-Difference Test following one-way ANOVA. Data are expressed as mean ± SEM.

Brain region-specific changes in RNA Polymerase II in aged female rats

We hypothesized that increased RNAPII expression and/or activity would be coincident with our observed increases in age-related alternative splicing of ERβ. Our results revealed a statistically significant interaction between age and treatment in the hypothalamus ($F(1,18)=8.317$, $p<0.01$) (Fig. 13A), but not in the dorsal or ventral hippocampus (Fig. 13B, C). Further, E2 treatment increased RNAPII mRNA expression in the 3-mo. animals, whereas neither age nor E2 affected RNAPII mRNA expression in the 18-mo. animals. A significant main effect of age on RNAPII expression was observed in the dorsal hippocampus ($F(1,18)=4.825$, $p<0.05$) (Fig. 13B), which was further increased by E2 treatment in aged, but not young, animals (Fig. 13B).

Aged animals subjected to our E2 deprivation paradigm showed a statistically significant main effect of E2 deprivation alone (i.e. vehicle treatment) and a main effect of subsequent E2 treatment on RNAPII expression in the hypothalamus ($F(3,40)=4.591$, $p<0.01$ & $F=(1,40)8.504$, $p<0.01$, respectively) and dorsal hippocampus ($F(3,44)=3.541$, $p<0.05$ & $F(1,44)=4.222$, $p<0.05$, respectively) (Fig. 13D, E). Conversely, in the ventral hippocampus there was a significant main effect of deprivation alone ($F(3,42)=6.316$, $p<0.001$), but not E2 treatment (Fig. 13F). In the dorsal hippocampus, E2 treatment significantly increased RNAPII expression at 1-week deprivation only (Fig. 13E). E2 treatment resulted in a significant decrease of RNAPII in the ventral hippocampus of animals deprived of E2 for 12 weeks (Fig. 13F) with or without E2 treatment.

Next, we measured RNAPII activity using western blot by probing for phosphorylated RNAPII (p-RNAPII) and comparing these levels to total RNAPII protein

expression in the animal paradigm described previously. The data revealed a significant main effect of deprivation in all three brain regions ($F(3,40)=3.634$, $p<0.05$, hypothalamus; $F(3,44)=4.467$, $p<0.01$, dorsal hippocampus; $F(3,42)=3.341$, $p<0.05$, ventral hippocampus) (Fig. 14). In the hypothalamus, there was a significant difference between the 1-week and 8-week vehicle-treated animals, as p-RNAPII expression was nearly undetectable at the 1-week time point for both vehicle- and E2-treated animals (Fig. 14A, B). The ventral hippocampus had a significant decrease in p-RNAPII expression following treatment with E2 at 12-weeks when compared to the vehicle-treated animals at the same time point (Fig. 14E, F).

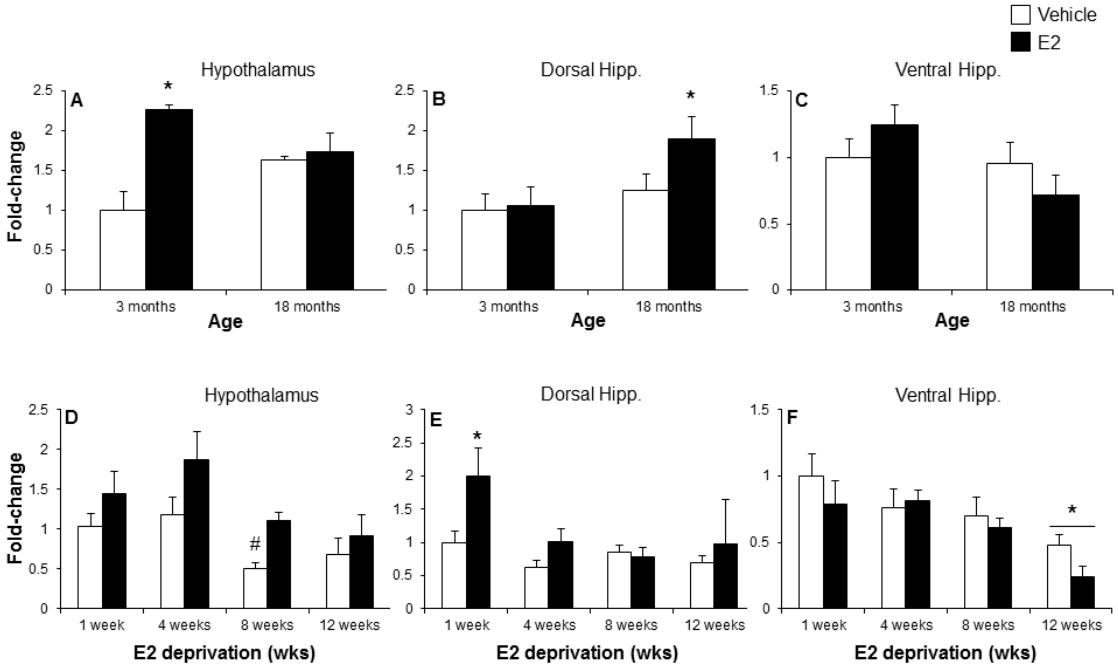

Figure 13. Effects of age and varying periods of E2 deprivation on RNA polymerase II mRNA expression in the brain of young and aged female rats. RNAPII expression was measured in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1 week post-OVX in 3 and 18-mo. old animals (N = 6/age/treatment group). RNAPII expression was also measured in the (D) hypothalamus, (E) dorsal hippocampus, and (F) ventral hippocampus at 1, 4, 8, and 12 weeks post-OVX (N = 6-10/age/treatment group). Animals were treated with either vehicle (safflower oil) or 2.5 µg E2 (dissolved in safflower oil). An * indicates a statistically significant difference ($p < 0.05$) between groups and # indicates a statistically significant within groups as determined by Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

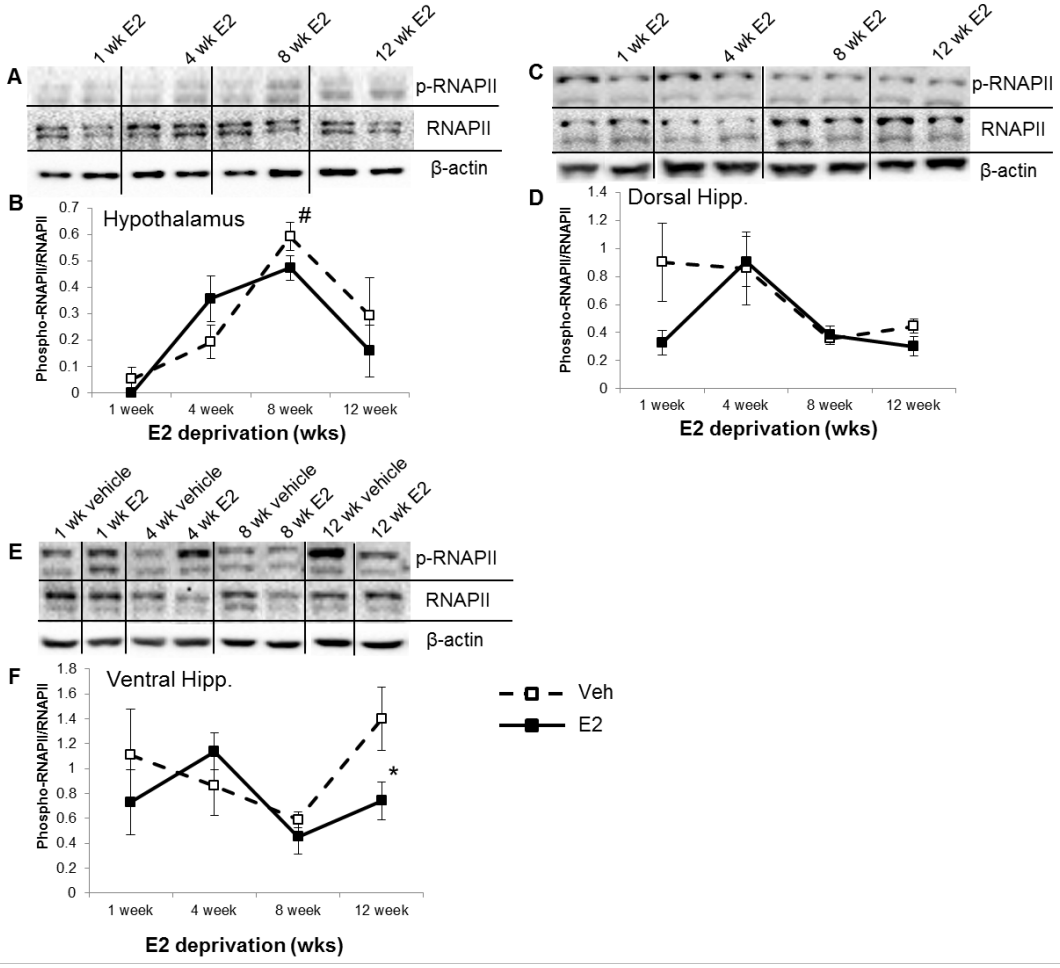

Figure 14. Effects of varying periods of E2 deprivation on RNA Polymerase II activity in aged female rats. Phosphorylated RNAPII and total RNAPII protein levels were measured by western blot in the (A, B) hypothalamus, (C, D) dorsal hippocampus, and (E, F) ventral hippocampus at 1, 4, 8, and 12 weeks post-OVX (N = 6-10/age/treatment group). Following E2 deprivation, animals were treated with either vehicle (safflower oil) or 2.5 µg E2 (dissolved in safflower oil). An * indicates a statistically significant difference ($p < 0.05$) between groups and # indicates a statistically significant within groups as determined by Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Representative blot images combined from several western blots used to quantify protein expression.

Summary

This study presents a detailed analysis of the alternatively spliced ERβ variant, ERβ2, in the hypothalamus, dorsal hippocampus, and ventral hippocampus with regards to aging and E2 deprivation in young animals, and in an aged surgically-induced menopausal animal model designed to mimic E2 replacement therapy occurring at varying time points following menopause. Our studies demonstrate the novel findings that age and E2 deprivation affect ERβ alternative splicing in a brain region-specific manner. The brain regions analyzed all express ERβ2, as previously shown through immunocytochemistry using an ERβ2-specific antibody, and E2 signaling is physiologically important in these regions for mediating stress responses or enhancing memory through neurogenesis and synaptic plasticity [79, 100, 101, 170, 171]. Importantly, we also report the first evidence for a putative molecular mechanism regulating alternative splicing of ERβ in the brain, although these data are cautiously interpreted and more mechanistic studies are required to validate a causal relationship. Specifically, these data reveal that the E2-induced alternative splicing of ERβ may be mediated by actions through its own receptor and through changes in splicing kinetics. Indeed, the inclusion of the ERβ2 insert that results in the dominant negative phenotype of the receptor can be increased by inhibiting TOP1, which suggests that the 18AA ERβ2 "insert" in the ligand binding domain might actually be considered an exon [133, 166, 167]. We also demonstrate a putative molecular mechanism for age-related changes in alternative splicing through our observations that age and E2 treatment alter RNAPII mRNA expression and activity. Age-dependent changes in RNAPII mRNA expression

levels and/or enzymatic activity might have implications on global alternative splicing patterns where E2 could play a key regulatory role. Taken together, these data contribute to our overall understanding of ERβ alternative splicing in the aging female brain.

We used a surgically induced menopause and E2 replacement paradigm to address the hypothesis that aging and/or long periods of ovarian hormone deprivation alters the expression of alternatively spliced ERβ variants, which could provide a mechanistic explanation for decreased E2 efficacy in older women. Specifically, ERβ2 has been shown to antagonize the actions of ERβ1 and has a much lower binding affinity for E2; both of these factors could decrease the efficacy of E2 replacement therapy following menopause. We were able to achieve clinically relevant levels of E2 following administration in our female OVX rats that were similar to those observed in postmenopausal patients that received hormone replacement therapy [34, 172]. Interestingly, E2 administration had brain region-specific effects between the hypothalamus and hippocampus: treatment in the hypothalamus resulted in significant increases in ERβ alternative splicing, whereas treatment in the dorsal and ventral hippocampus resulted in significant decreases. The significant increases in the hypothalamus of ERβ2 following E2 treatment also corresponded with significant increases in total ERβ expression. Therefore, increased availability of ERβ pre-mRNA due to increased transcription could allow for increased alternative splicing through cotranscriptional events [165, 169]. These data suggest that any assessments of E2 efficacy will differ depending on the physiological endpoint measured, because each of these brain regions regulates distinct physiological functions. In particular, gene

expression within the hypothalamus has recently been shown to adapt to changes in circulating E2 levels, and the increase observed in ERβ2 expression, along with its ability to act in a ligand-independent manner, may underlie this neuronal adaptation to fluctuating circulating E2 levels throughout the lifespan [56, 100, 173]. Another important recent study investigated E2 deprivation of 9 mo. old animals given E2 treatment either 6 or 180 days (i.e. 15 mo. old) post-OVX using Sprague-Dawley rats. They demonstrated a decrease in E2 efficacy on hippocampal neurogenesis and neuroprotection that coincided with increased expression levels of ERβ2, suggesting that ERβ2 was responsible for the observed decreased E2 efficacy [79]. Our data suggest that aged Fisher344 rats this increase in ERβ2 does not persist in the hippocampus, but instead becomes much more evident in the hypothalamus, a brain region with direct anatomical connections to the hippocampus. Wang and colleagues also showed that E2 treatment blocked the observed increase in ERβ2 expression in animals that were deprived of E2 for only 6 days, but E2 was ineffective following 180 days post-OVX [79]. Our results are consistent with these, as we also did not observe a significant effect of E2 following longer periods of E2 deprivation (>8 weeks). Collectively, these data and those from previous studies suggest that ERβ2 is the predominant variant expressed in the hippocampus and hypothalamus as the time of E2 deprivation lengthens [19, 174, 175]. Future studies into specific hypothalamic nuclei and hippocampal subgroups may further reveal finite brain-region specific changes occurring during E2 deprivation.

We have shown that E2 can regulate ERβ alternative splicing in a brain region-specific manner, however whether E2 was acting through ERα or ERβ was unclear.

Previous studies have shown age-related changes in the expression levels of ERα within these specific brain regions, whereas there are conflicting reports regarding the expression of ERβ [70, 176, 177]. These data led us to predict that E2 effects in aged animals would most likely be mediated by ERβ or ERα. Indeed, treatment with the ERβ-selective agonist DPN resulted in a significant increase of ERβ alternative splicing, whereas treatment with the ERα-selective agonist PPT had no effect on splice variant expression however, it may be possible that ERα may be mediating increases in total ERβ in coordination with ERβ, as shown in the PPT-treated animals. It is possible that the lack of a PPT effect is reflective of altered expression levels of ERα in aged animals; therefore we are unable exclude the possibility that ERα could mediate E2-induced alternative splicing in younger animals.

To our knowledge, no studies to date have investigated the mechanisms regulating ERβ alternative splicing in the brain or any other tissue. In these studies, we investigated two potential mechanisms to explain age-related changes in alternative splicing of ERβ. First, taking advantage of a novel compound called camptothecin, a TOP1 inhibitor, we tested the idea that the weak splice site near the short, 54bp intronic sequence of ERβ2 would increase if transcription kinetics were slowed. Camptothecin has been used previously in several studies to investigate how the rate of transcription can affect alternative splicing [167, 178]. Therefore, we treated hypothalamic-derived GT1-7 cells, which express ERβ2, with camptothecin and observed a statistically significant increase in ERβ2 expression. Contrary to what we predicted based on the data obtained *in vivo* in our aged animals, concomitant E2 and camptothecin treatment attenuated the observed

increase in ERβ alternative splicing. These data suggest that E2 might be able to overcome RNAPII stalling, whether by upregulating expression of RNAPII to compensate for the stalled enzymes or increasing activation of other RNAPII present via phosphorylation, however further experiments are required to validate this mechanism.

The kinetics of gene transcription is largely dependent on the activity of RNAPII. Given that a slower rate of transcription promoted increased inclusion of a unique sequence, thereby increasing expression of ERβ2, we tested if RNAPII mRNA expression was affected either by age, loss of circulating E2, or both. Our results demonstrated that RNAPII mRNA increased with age in the hypothalamus, which correlated with a decrease in ERβ2 expression that was previously observed. Moreover, E2 treatment also increased RNAPII expression in an age and tissue-specific manner, but expression of this enzyme may require distinct splicing factors, which may or may not be altered by age, E2, and brain region. One putative candidate is the CNS-specific splicing factor Nova1, as it has been previously shown to decrease in the brain with age [149], however no studies to date have made a direct link between ERβ and any splicing factor. Investigation of each deprivation time point in which these changes occur could provide more insight into which splicing factors could be involved, and our observed correlative results may also provide insight into mechanistic explanations for the timing hypothesis of hormone replacement therapy efficacy that need to be further investigated.

Our model, along with the previous studies outlined in this manuscript, raise important limitations to consider. The model we used to study menopause and the timing hypothesis differ in some aspects with other similar studies [79, 173]. Importantly,

rodents do not undergo a menopausal transition that is similar to humans and, therefore, most studies have relied on using a surgically-induced menopause model, or a chemically-induced delayed depletion of ovarian follicles. Chemically induced ovarian failure, as reported previously, is a valuable model to study the menopausal transition, however in these studies we chose the surgical model in order to precisely measure the length of time following complete ovarian E2 deprivation [179]. In addition, there is some debate regarding the correct age in rodents to begin manipulations so that they will provide a good comparison to the human menopausal condition and yield generalizable results. These discrepancies are likely related to strain-specific differences in rats and this is a very important consideration when comparing various reports in the literature. For instance, Sprague-Dawley rats have an average cycle cessation at 8-12 months, whereas the Fisher344 rat strain undergoes cycle cessation at 16-18 months [180, 181]. Also, the predominant postcyclic vaginal state in Sprague-Dawley rats is persistent estrous, resulting in high estrogen levels. Fisher344 by contrast typically exhibit repeated pseudopregnancy with low estrogen, but higher progesterone [182]. Utilizing previously reported data on aging in Fischer 344 rats, we were able to correlate an approximate age of our animals with a human age (an 18-mo. old rat equals a 55 year old human [180-183]. Interestingly, a recent study by Yin and colleagues, also using the surgically-induced menopause rat model, demonstrated that changes in the brain occur well before the onset of menopause and that hypothalamic neuronal networks are highly adaptable to fluctuations in circulating E2 levels [173]. We propose that this adaptability could be the

result of altered alternative ERβ splicing and subsequent changes in the ratio of alternatively spliced ERβ isoforms.

CHAPTER IV

AGING AND LOSS OF CIRCULATING 17B-ESTRADIOL ALTERS THE EXPRESSION OF NOVA1, A REGULATOR OF ERβ ALTERNATIVE SPLICING

Introduction

Alternative splicing contributes to increased biodiversity of proteins that can be translated from the limited set of genes within eukaryotes. It is estimated that over 95% of multi-exonic genes are alternatively spliced [116]. At the center of this, RNA-binding proteins (RBPs) fundamentally regulate post-transcriptional alternative splicing. These *trans*-acting factors bind to *cis*-sequences contained within the pre-mRNA transcript to coordinate and facilitate alternative splicing events. RNA binding proteins not only regulate pre-mRNA splicing, but also coordinate 5' end capping, cleavage, polyadenylation, nuclear mRNA export, localization, translation, and degradation of mRNA [164]. These critical RNA binding proteins can also be tissue-specific, which is evidenced by the disproportionately high number of alternative splicing events that occur in the brain [118, 184].

Recent evidence has suggested that alternative splicing increases in the brain with age [149]. These studies found that the CNS-specific splicing factor Nova1 decreased with age while alternative splicing events in several proteins increased concomitantly [149]. Increases in alternative splicing events can have negative implications on disease

and neurodegeneration due to variant function in the brain [185, 186]. Nova1 is a RNA-binding protein that binds YCAY elements coded in pre-mRNA transcripts to enhance or block exon exclusion in a location-dependent manner [143, 145]. Nova1, like many RNA binding proteins, work in concert with hnRNPs, SR proteins, and other factors to regulate splicing [187].

To further compound the effects of aging is the menopausal transition and the associated decline in circulating estrogens, more specifically 17β-estradiol (E2), the major estrogen produced by the ovaries. E2 mediates its actions through two important nuclear steroid receptors in the brain called estrogen receptor (ER) α and ERβ. In the classical model of nuclear receptor action, these ERs bind ligand, dimerize, and translocate to the nucleus, where they mediate E2-regulated gene transcription [43]. Our laboratory has recently shown that age and increased lengths of E2 deprivation can alter the mRNA expression of alternatively spliced ERβ in a brain region-specific manner [96]. Those studies showed age-related increases in ERβ2 mRNA expression, which is a dominant negative form of the receptor that is unable to bind E2 efficiently due to a spliced insert within the ligand-binding domain (LBD). Others have also observed an increase in ERβ2 protein levels following prolonged periods of E2 deprivation [79]. The efficacy of hormone replacement therapy (HT) may depend on the expression of these splice variants due to their inability to mediate normal E2 signaling in the cell [46, 56, 100, 158]. Interestingly, the ERβ pre-mRNA transcript contains several Nova1 RBP consensus sequences upstream of the ERβ2 insert that may promote exon inclusion or

exclusion based on their location (Figure 6). Therefore, it is possible that Nova1 can regulate ERβ alternative splicing through RBP interactions post-transcriptionally.

The purpose of this study was to determine how aging and E2 deprivation, as occurs during menopause, affects Nova1 in the female brain. We hypothesized that Nova1 expression would decrease in a brain region-specific manner due to age-related changes in neuronal function and E2 deprivation-related changes in neuroprotection. Further, we predicted that acute E2 treatment would abrogate the effects of age and prolonged E2 deprivation on Nova1 expression. To test this hypothesis, female Fisher344 rats were ovariectomized (OVX) at 3 and 18 months of age to study the effects of aging and administered acute E2 treatment after 1 week. Next, aged animals (18 mo.) were OVX and administered acute E2 treatment at varying times post-OVX (1-12 weeks OVX) to assess the effects of prolonged E2 deprivation on Nova1 expression (Fig. 7). We also quantified the expression of ERβ following Nova1 overexpression, and determined if there was a direct interaction with ERβ pre-mRNA in hypothalamic-derived neuronal cells to determine whether Nova1 regulates ERβ alternative splicing. Our data revealed that Nova1 expression was altered by age and prolonged periods of E2 deprivation in a brain region-specific manner. Further, we provide evidence that increased Nova1 expression decreased alternative splicing of ERβ and that Nova1 directly interacts with ERβ pre-mRNA transcripts. Taken together, our results suggest a putative molecular mechanism regulating the alternative splicing of ERβ.

Briefly, 3 and 18 month old animals underwent OVX and subjected to our E2 deprivation paradigm and then treated with vehicle or E2 (Fig. 7). Circulating E2

concentration were reported in serum to determine levels of hormone from prior studies [188]. A separate group of 18 mo. animals were treated with ERα and ERβ-selective agonists. To determine the effects of aging and E2 deprivation on Nova1 expression, Nova1 mRNA and protein expression measured in the hypothalamus, dorsal hippocampus, and ventral hippocampus of these animals. A set of intact animals were used to compare expression levels of Nova1 in the hypothalamus. An *in vitro* set of studies were conducted in cell lines overexpressing Nova1 and/or ERβ2 in order to assess effects of Nova1 on ERβ alternative splicing.

Results

Nova1 expression changes in the aging brain

No changes were detected in Nova1 mRNA expression in the hypothalamus of ovarian intact animals at any age (3, 18, 19, 20, 21 mo. old, Fig. 15). By contrast, comparison of the 3-mo. and 18-mo. OVX animals treated with vehicle or E2 revealed a significant main effect of treatment on Nova1 mRNA expression in the hypothalamus ($F(1,22)=11.708$, $p<0.005$), dorsal hippocampus ($F(1,21)=5.892$, $p<0.05$) and ventral hippocampus ($F(1,21)=14.064$, $p<0.005$) (Fig. 16). In the hypothalamus and dorsal hippocampus, E2 treatment at 3-mo. significantly increased Nova1 expression when compared to 18-mo. vehicle- or E2-treated animals (Fig. 16A,B). Conversely, E2 treatment resulted in decreased Nova1 expression in the ventral hippocampus at 3-mo. when compared to vehicle-treated animals at the same age (Fig. 16C). Nova1 expression remained significantly low in the ventral hippocampus at 18-mo when compared to 3-mo

vehicle-treated animals. These data are consistent with previously observed age-related decreases in Nova1 expression with age.

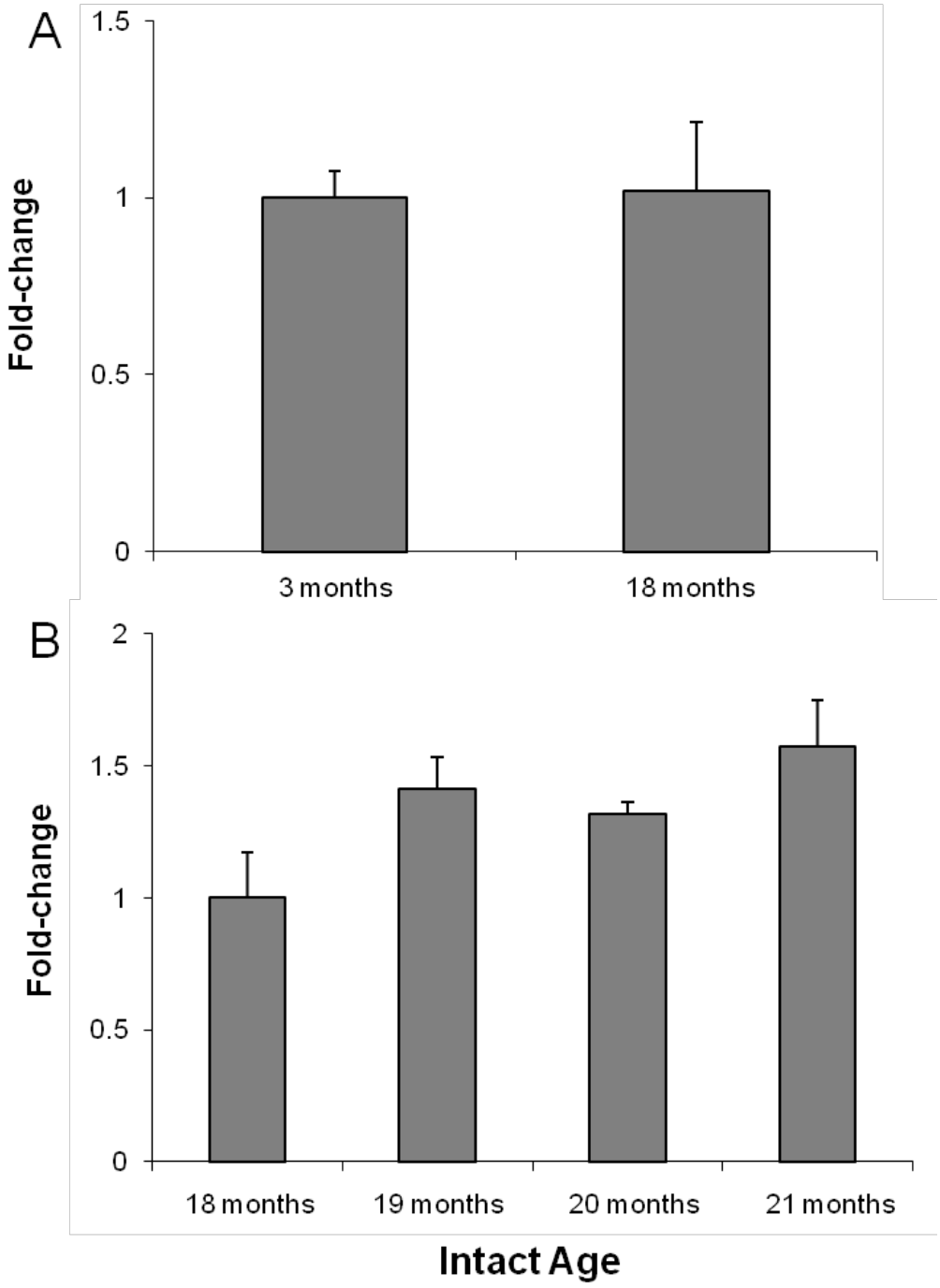

Figure 15. Expression of Nova1 mRNA in intact (non-OVX) animals at 3, 18, 19, 20, & 21 months old. Nova1 mRNA expression was measured in (A) 3 & 18-mo. old animals and (B) 18, 19, 20, & 21-mo. old (N=6/age). No statistically significant differences were observed. Data are expressed as mean ± SEM.

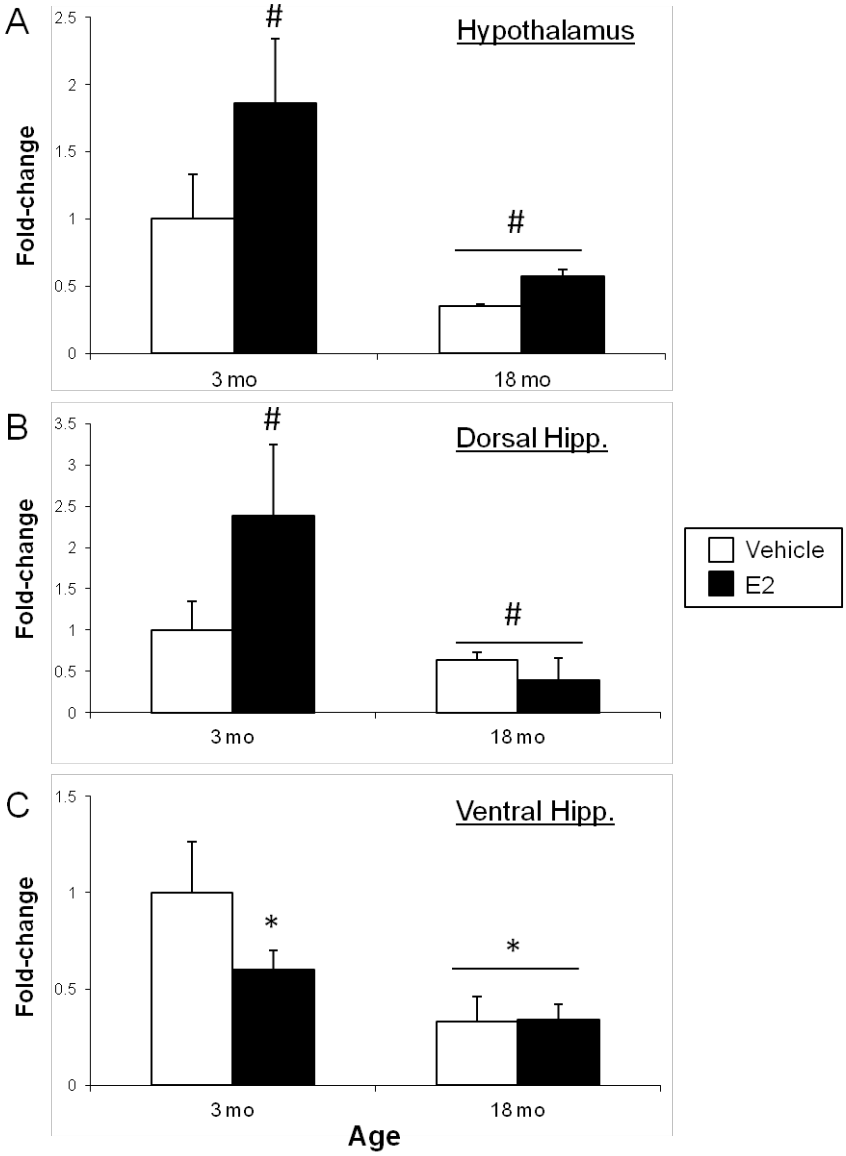

Figure 16. Comparison of Nova1 mRNA expression in the young and aged female rat brain. Nova1 expression was measured in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1 week post-OVX in 3 and 18-mo. old female rats (N = 6/age/treatment group). Animals were OVX and 1 week later treated with either vehicle (safflower oil) or 2.5 µg E2 (dissolved in safflower oil). Asterisk '*' indicates a statistically significant difference ($p < 0.05$) compared to 1 week vehicle and hashtag '#' indicates a statistically significant difference ($p < 0.05$) between groups by Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

Expression of Nova1 is altered by prolonged E2 deprivation in a brain-region specific manner in aged female rats

To elucidate the effects of diminished E2 levels on Nova1 expression in the aged female brain, 18-mo. old animals were OVX then deprived of E2 for increasingly longer periods of time before acute treatment with vehicle or 2.5 ug/kg E2 once/day for 3 consecutive days (Figure 7). A significant interaction between length of deprivation (vehicle-treated) and E2 treatment was observed via two-way ANOVA on Nova1 mRNA expression in the hypothalamus ($F(3,41)=52.407$, $p<0.001$) (Fig. 17A), demonstrating the the expression of Nova1 depends on length of E2 deprivation. There was also a significant main effect of E2 deprivation time on Nova1 expression in the ventral hippocampus ($F(3,42)=4.496$, $p<0.01$), but not the dorsal hippocampus (Fig. 17C).

Acute E2-treatment in the hypothalamus significantly increased Nova1 expression 8-fold in at 4 weeks post-OVX and 13-fold at 8 weeks post-OVX when compared to animals treated with vehicle 1 week post-OVX (Fig. 17A). Deprivation of E2 also significantly increased Nova1 expression in this brain region at 8 weeks. In the dorsal hippocampus, E2 deprivation significantly decreased Nova1 expression at 4 and 8 weeks post-OVX and acute treatment with E2 at these time points abrogated these effects (Fig. 17B). In the ventral hippocampus, E2 deprivation resulted in a significant increase at 12 weeks post-OVX, but subsequent acute E2 treatment decreased Nova1 expression at this time point. There were no changes detected in Nova1 mRNA expression in ovarian intact animals at these age groups (Fig. 15B).

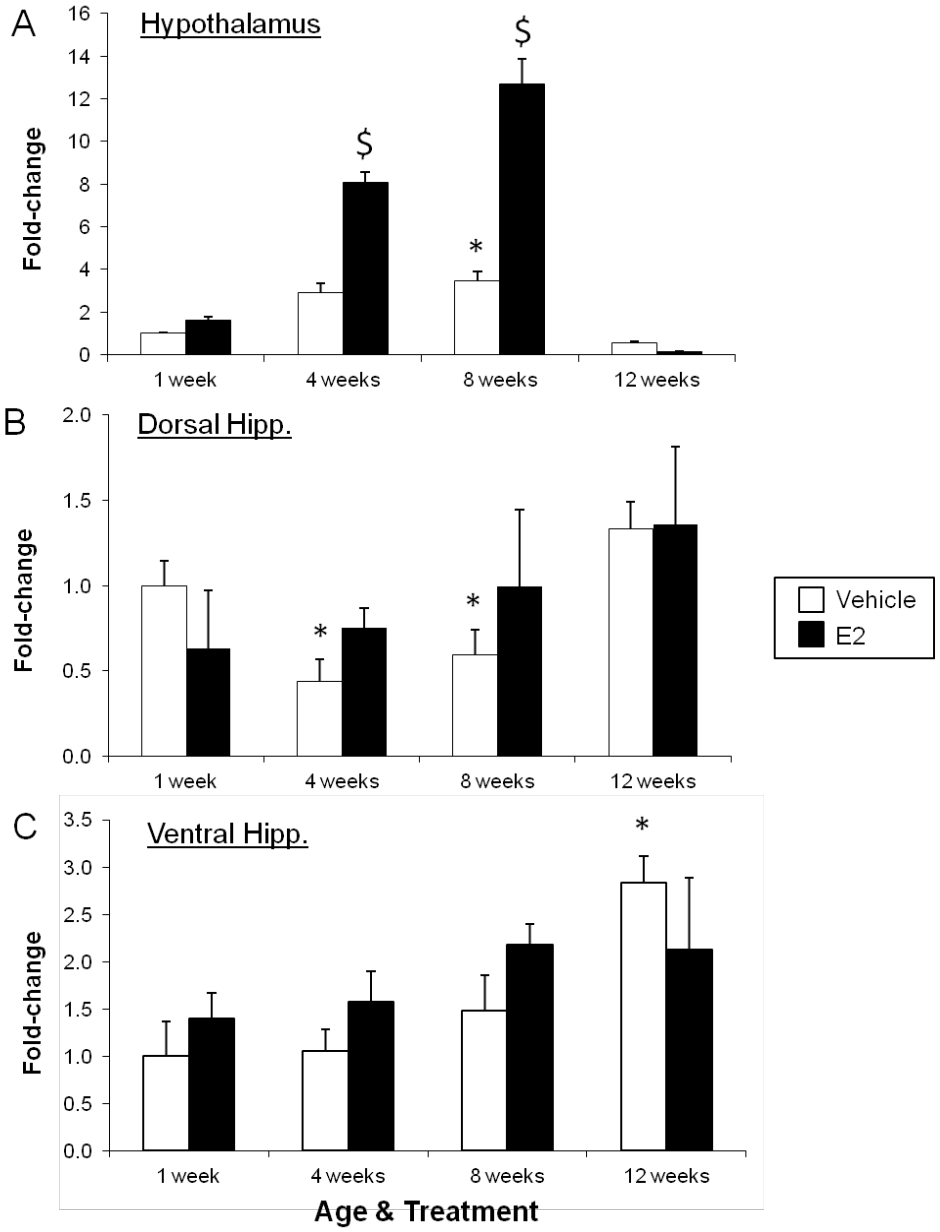

Figure 17. Expression of Nova1 mRNA in the brain of aged (18-mo. old) female rats following varying periods of E2 deprivation. Nova1 mRNA expression was measured in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1, 4, 8, and 12 weeks post-OVX (N = 6-10/age/treatment group). Animals were treated with either vehicle (safflower oil) or 2.5 µg E2 (dissolved in safflower oil). Asterisk '*' indicates a statistically significant difference ($p < 0.05$) compared to 1 week vehicle and '$' indicates a greater statistically significant difference ($p < 0.001$) compared to 1 week vehicle as determined by Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

Detection of Nova1 protein in these brain regions revealed a significant main effect of E2 deprivation time in the hypothalamus ($F(3,41)=15.125$, $p<0.001$) (Fig. 18A) and ventral hippocampus ($F(3,42)=55.5$, $p<0.001$) (Fig. 18C), but not in the dorsal hippocampus (Fig. 18B). Nova1 protein expression significantly increased at 4 weeks in both vehicle- and E2-treated animals in the hypothalamus (Fig. 18A). Nova1 significantly increased in the ventral hippocampus at 8 weeks post-OVX in both treatment groups, and remained elevated up to 12 weeks deprivation (Fig. 18C).

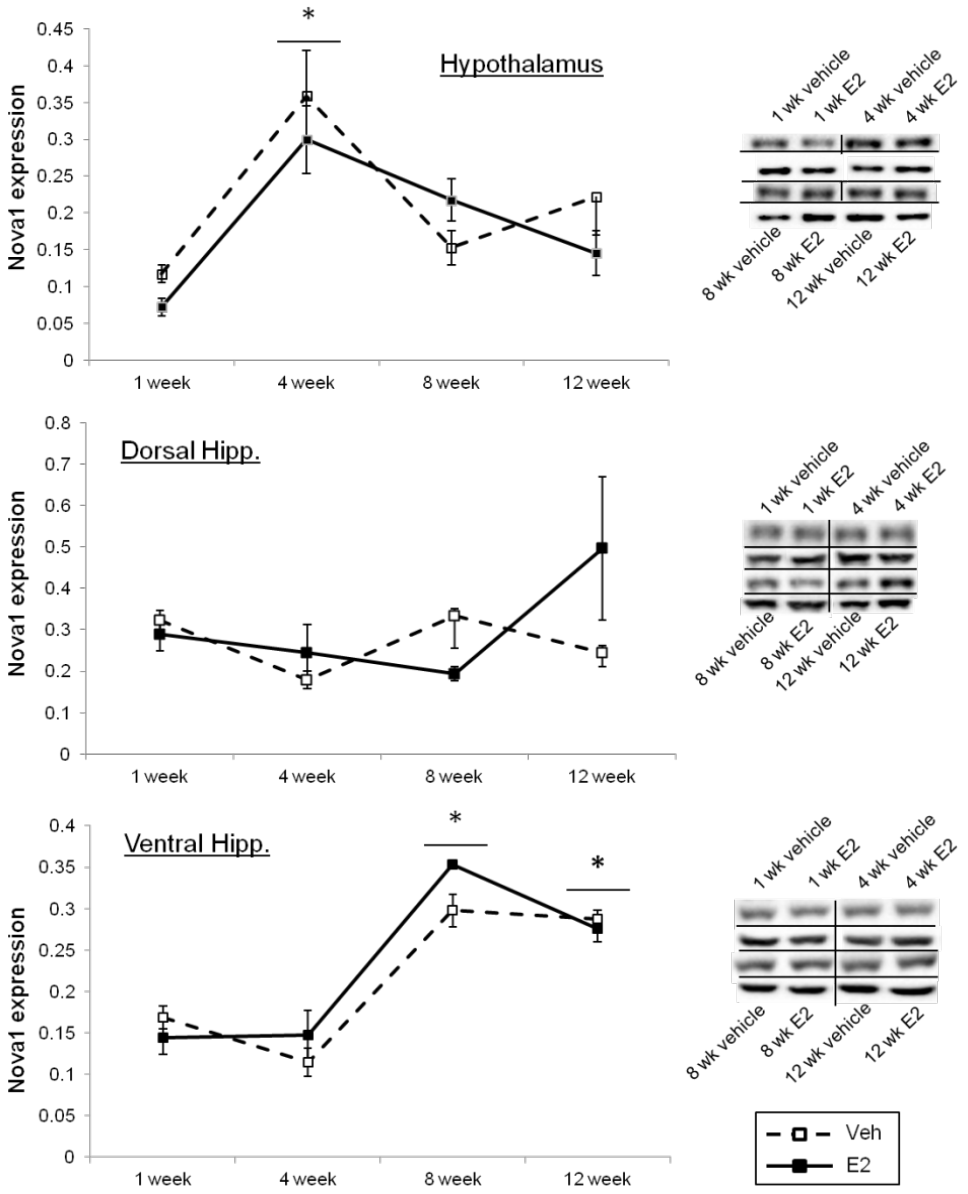

Figure 18. Expression of Nova1 protein in the brain of aged (18-mo. old) female rats following varying periods of E2 deprivation. Nova1 and β-actin protein levels were measured by western blot in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1, 4, 8, and 12 weeks post-OVX (N = 6-10/age/treatment group). Following E2 deprivation, animals were treated with either vehicle (safflower oil) or 2.5 µg E2 (dissolved in safflower oil). Asterisk '*' indicates a statistically significant difference ($p < 0.05$) between groups as determined by Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Nova1 protein levels were quantified after normalization to β-actin from the same sample. Representative blot images combined from several gels utilized to perform densitometry quantification of western blots.

E2-induced increases in Nova1 expression may be mediated primarily by ER homodimers

The effects mediated by E2 on ERβ Nova1 could be mediated by either ERα or ERβ, as both are expressed in each of the brain regions tested [70]. To determine which E2 receptor mediated observed increases in Nova1, acute administration of the ERβ-specific agonist diarylproprionitrile (DPN) or the ERα-specific agonist propyl pyrazole triol (PPT) was given to 18-mo. old female rats 1-week following OVX. Our results demonstrated that both DPN and PPT significantly increased expression of Nova1 in the hypothalamus, yet the effects of PPT on Nova1 expression increased 3.5-fold compared to 2.25-fold in DPN-treated animals (Fig. 19). Administration of E2 had no effect at this time point as shown previously (Figure 17A).

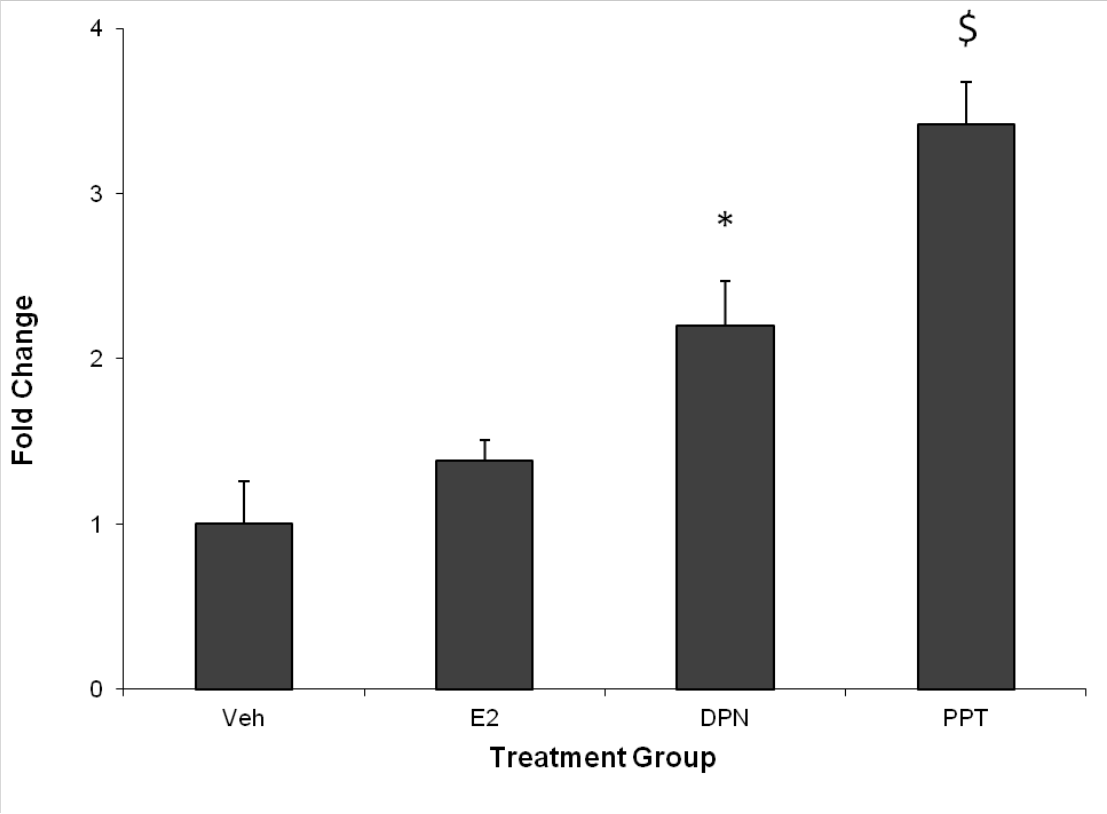

Figure 19. ERα- and ERβ-selective agonists increase Nova1 expression. Nova1 mRNA expression was measured in the hypothalamus of aged female rats that were OVX and 1 week later treated with either vehicle, E2, 1mg/kg DPN, or 0.5 mg/kg PPT once/day for three consecutive days (N = 6-8/treatment group). Asterisk '*' indicates a statistically significant difference ($p < 0.05$) and '$' indicates a greater statistically significant difference ($p < 0.001$) compared to 1 week vehicle as determined by Tukey's Honestly-Significant-Difference Test. Data are expressed as mean ± SEM.

Overexpression of Nova1 decreases ERβ splice variant expression through direct binding of ERβ mRNA

Nova1 regulates exon splicing through differential binding of the pre-mRNA transcript. Nova1 promotes exon exclusion if it binds upstream of an alternatively spliced exon, but it can also promote inclusion of exons if it binds downstream of the exon [143, 145]. The naturally occurring rat ERβ2 splice variant contains an insert in its ligand-binding domain (LBD), which in turn affects its affinity for ligand. This insert is located between exons 5 and 6 in the ERβ pre-mRNA transcript. The upstream exon 5 has a cluster of Nova1 binding sites located within its nucleotide sequence (Fig. 6). Similar to Nova1, we have previously demonstrated that ERβ2 alternative splicing increases in a brain-region specific manner following E2 deprivation and treatment in the aged rat brain [96]. Therefore, in order to determine if Nova1 expression affects ERβ2 expression, Nova1 was transfected in a dose-dependent manner (0.01μg - 2μg plasmid DNA) *in vitro* in the IVB rat hypothalamic cell line that endogenously expresses ERβ. The results demonstrated that overexpression of Nova1 increased total ERβ expression in a dose-dependent manner and was statistically significant compared to empty vector at a plasmid concentration of 0.2 μg/well (Fig. 20A). However, overexpression of Nova1 at all concentrations resulted in a significant decrease in ERβ2 expression when compared to pcDNA empty vector (Fig. 20B).

To assess the effects of E2 on ERβ alternative splicing in the presence of Nova1, IVB cells were transfected with 0.25 μg Nova1, then treated with 100 nM E2 or vehicle

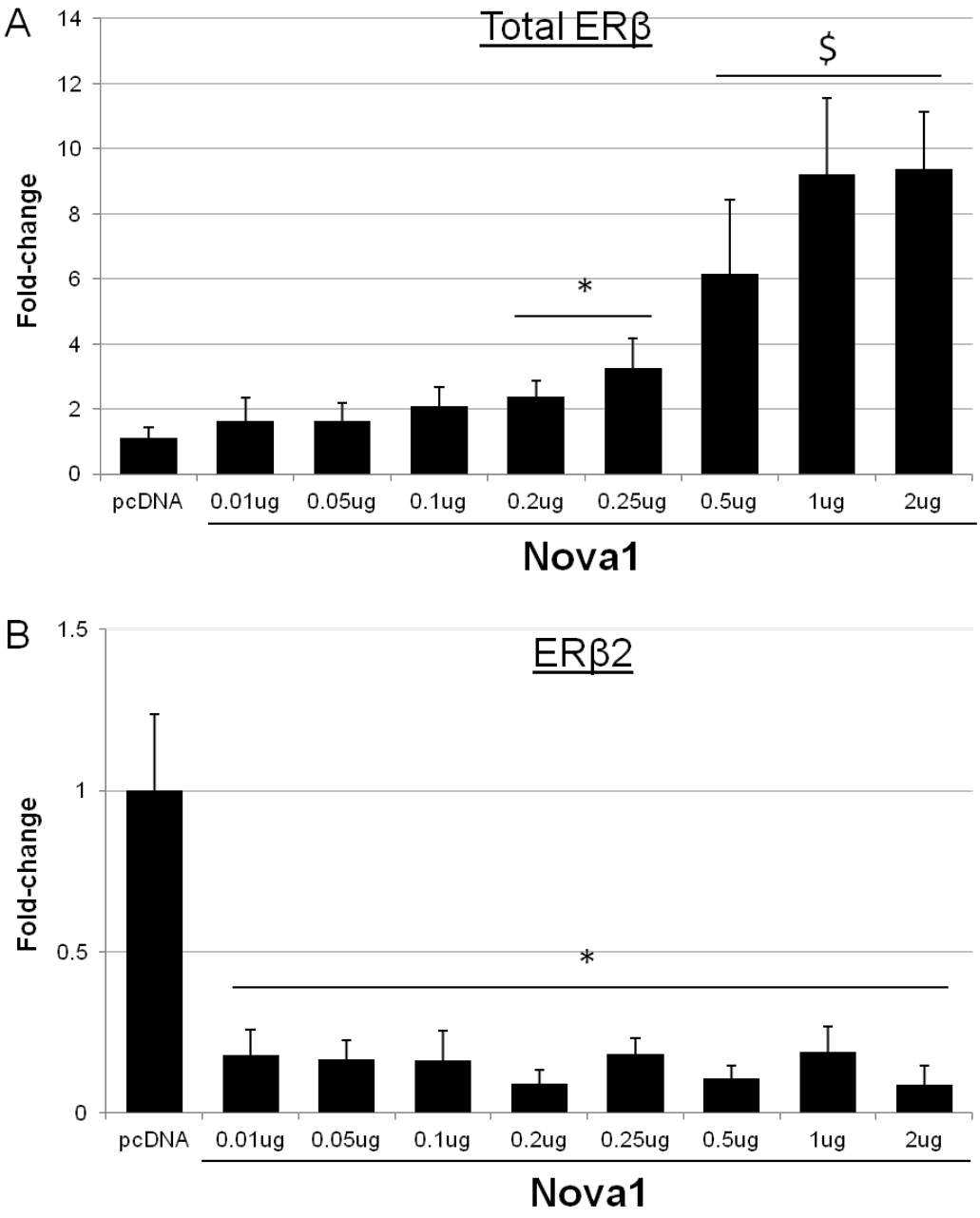

Figure 20. ERβ alternative splicing decreases with Nova1 overexpression. (A) Total ERβ and (B) ERβ2 mRNA expression was measured in hypothalamic-derived IVB cells transfected with Nova1 eGFP plasmid in a dose-dependent (0.01-2ug DNA) (N=3-4/amount transfected). pcDNA was used as empty vector. Asterisk '*' indicates a statistically significant difference ($p < 0.05$) and the '$' indicates a greater statistically significant difference ($p < 0.001$) compared to empty vector using t-test.

(alcohol). There was a significant main effect of treatment on ERβ2 expression in IVB cells ($F(1,8)=8.624$, $p<0.05$) (Fig. 21B). E2 treatment resulted in a significant decrease in ERβ2 expression pcDNA overexpressing cells when compared to vehicle-treated cells. Both vehicle- and E2-treated Nova1 cells had significantly low expression of ERβ2 when compared to vehicle-treated pcDNA cells. E2 treatment decreased total ERβ expression in Nova1 transfected IVB cells to levels that were no longer significant when compared to Nova1 vehicle-treated IVB cells (Figure 21A).

Because Nova1 overexpression had an effect on ERβ splice variant expression, we hypothesized that Nova1 directly interacts with ERβ mRNA. Nova1- and ERβ-null HEK cells were co-transfected with Nova1 and ERβ2 for RNA immunoprecipitation (RIP) analysis. Nova1 was also co-transfected with pcDNA as a negative control. ERβ2 mRNA was enriched in the Nova1-bound RIP between 40-50 fold when compared to rabbit IgG control (Fig. 22), demonstrating a direct interaction between Nova1 and ERβ mRNA transcript.

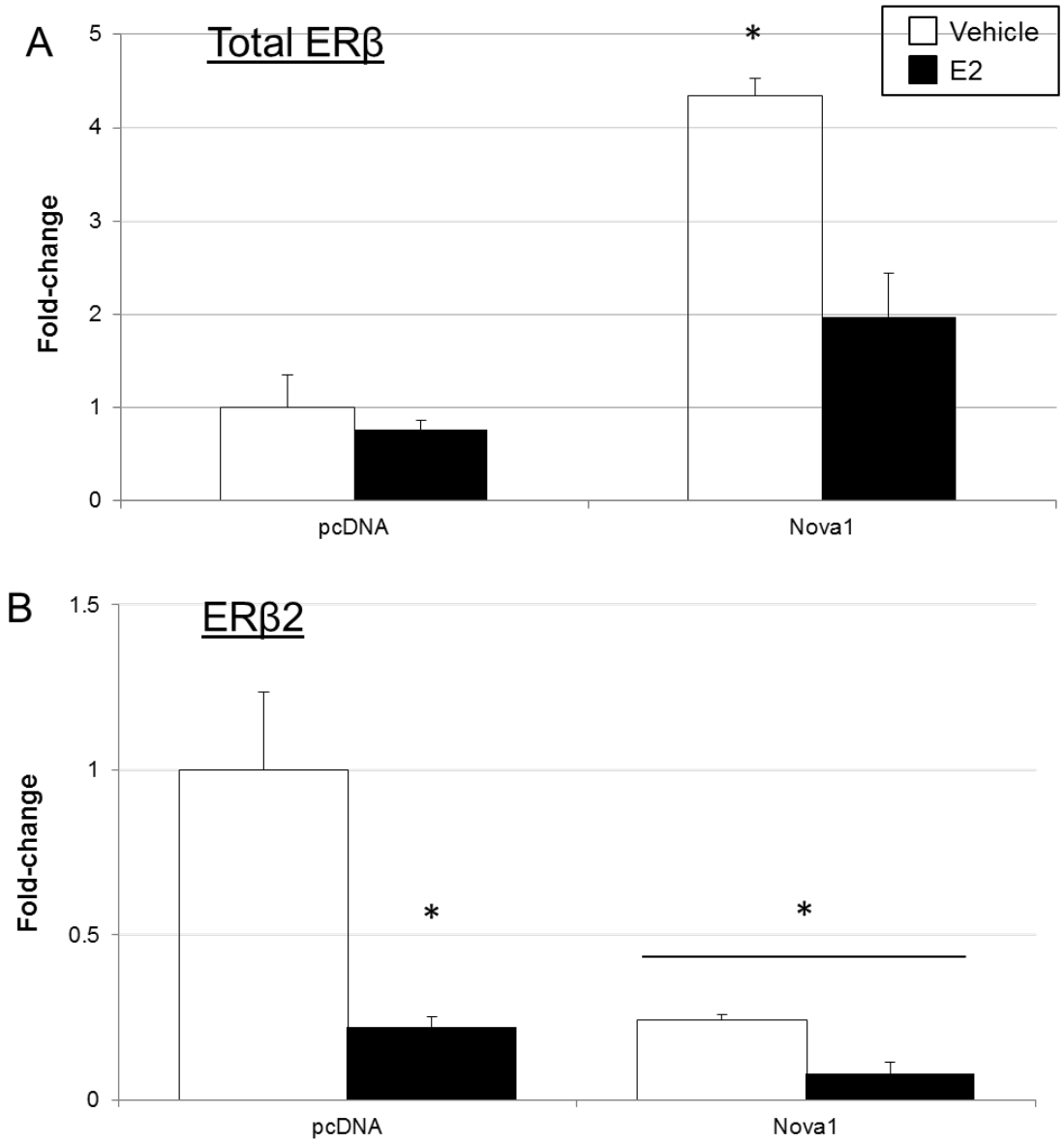

Figure 21. ERβ alternative splicing decreases with E2 treatment in the presence of Nova1. (A) Total ERβ and (B) ERβ2 mRNA expression was measured in hypothalamic-derived IVB cells transfected with 0.25µg Nova1 eGFP (N = 3). pcDNA was used as empty vector. Asterisk '*' indicates a statistically significant difference ($p < 0.05$) compared to vehicle-treated pcDNA expressing cells as determined by two-way ANOVA (ERβ2) and t-test (total ERβ).

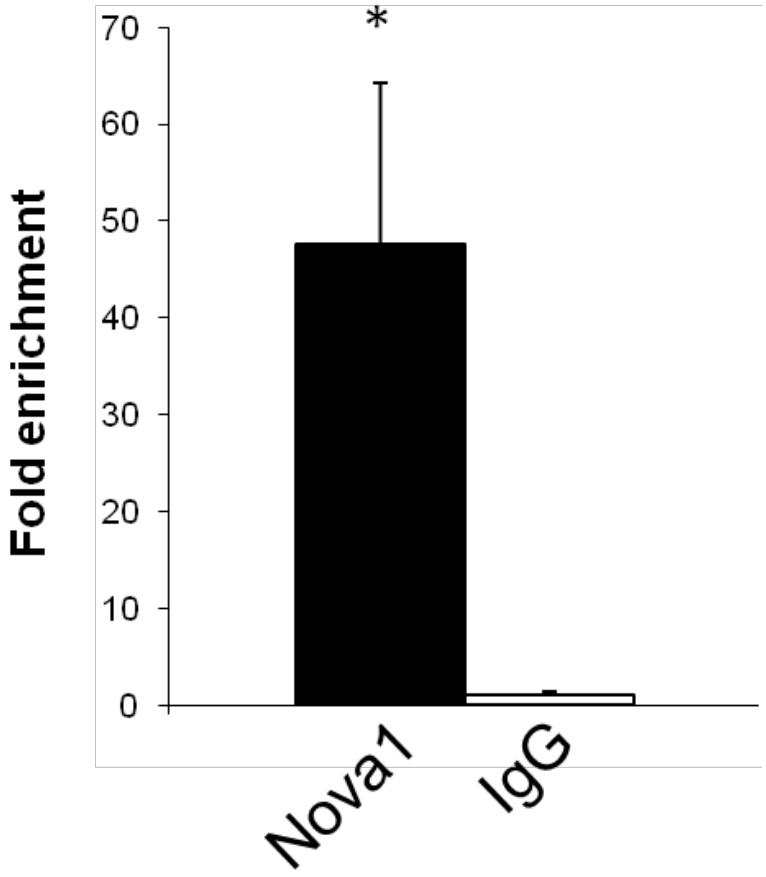

Figure 22. Nova1 directly interacts with ERβ mRNA. ERβ mRNA expression was measured via qRT-PCR following RNA immunoprecipitation with anti-Nova1 antibody or rabbit IgG in HEK overexpressing Nova1 and ERβ2 (N=3/RIP). Asterisk '*' indicates statistically significant difference compared to IgG isotype control ($p<0.05$) by t-test. Data are expressed as mean fold-enrichment ± SEM.

Discussion

The Nova1 splicing factor is an important RBP responsible for regulating the alternative splicing of many different transcripts in the complex environment of the brain. Our studies present novel findings that age and E2 deprivation affect Nova1 expression in a brain-region specific manner, supporting the idea that changes in Nova1 have important consequences for alternative gene splicing postmenopause. Specifically, these experiments show detailed analyses of the effects of aging on Nova1 expression in the hypothalamus, dorsal hippocampus, and ventral hippocampus as compared between young and aged female rats. Further, analysis of Nova1 expression was also conducted in aged animals that underwent our surgically-induced menopausal model designed to mimic HT occurring at increasingly longer time points following menopause. The brain regions tested all express Nova1, as previously observed by immunohistochemistry with a Nova1 specific antibody, and also express important Nova1-regulated proteins [128, 138, 142, 189]. E2 signaling is physiologically important in these regions, and the actions of E2 may work in conjunction with Nova1 in neurogenesis and synaptic plasticity [142, 147, 155, 170, 171, 190, 191]. The E2 deprivation-related changes on Nova1 might further compound the effects of aging in the female brain as previously demonstrated in the aged male brain [149]. Importantly, we also demonstrate that Nova1 can directly interact with ERβ mRNA transcripts, providing a mechanism by which alternative splicing of ERβ is regulated by Nova1. Further evidence of this mechanism is reported through our Nova1 overexpression studies and their impact on ERβ splice variant

expression. Taken together, these data contribute to our overall understanding of Nova1 and alternative splicing in the aging female brain.

In order to address our hypothesis that aging and/or loss of E2 over long periods of time alters the expression of Nova1, we used a surgically-induced menopause model and E2 replacement paradigm. We were able to achieve clinically relevant levels of E2 following administration in our female OVX rats that were similarly reported in postmenopausal women receiving hormone replacement therapy [34, 96, 172, 188]. Nova1 had been shown to decrease with age in the brain, but these studies were only conducted in the human male cortex. Our results show similar decreases in Nova1 expression with age in all three brain regions studied. Interestingly, the effects of E2 in young animals differed between brain regions: treatment in the hypothalamus and dorsal hippocampus significantly increased Nova1 expression compared to 18-mo., whereas treatment in the ventral hippocampus significantly decreased Nova1 to levels observed at 18-mo. The loss of Nova1 might be a consequence of the aging brain where neurons are subjected to increases in oxidative stress [192], perturbed energy homeostasis [193], DNA damage [194], and accumulation of misfolded/aggregated proteins [195]. The 18-mo. old animals in our studies equate to an approximate age of 55 human years old [96]. However, the advanced ages in previous Nova1 studies were conducted in men between 82-86 years old. Therefore, these data suggest that Nova1 expression can potentially decline in the brain at a younger age.

It is also possible that there are some sex-specific differences in Nova1 expression. In the hypothalamus and ventral hippocampus, Nova1 expression increased

with longer periods of E2 deprivation (8 and 12 weeks, respectively). This was not true for the dorsal hippocampus, which we detected significant decreases in Nova1 expression after 4 and 8 weeks deprivation. Interestingly, treatment with E2 attenuated these observed changes in the hippocampus, but not in the hypothalamus, where E2 potentiated the significant increase in Nova1 expression nearly 3-fold. Several deprivation time points correlated with Nova1 protein expression, however, not all points that were significantly altered at the mRNA level corresponded with protein expression. These data suggest Nova1 expression changes in a brain region specific manner with longer periods of ovarian hormone deprivation, which may further alter splicing events associated with neurodegeneration and disease [149, 192, 194]. Specifically in the hypothalamus, it appears that Nova1 transcription becomes increasingly sensitive to E2 treatment up to 8 weeks deprivation, but these effects do not persist with longer deprivation. This could occur due to ER-specific expression changes in these regions that occur during ovarian hormone deprivation [96]. Early E2 replacement (1 week) did not alter Nova1 expression at the mRNA or protein level, which could maintain persistent expression to prevent further accumulation of alternatively spliced disease variants in specific brain regions like the dorsal hippocampus.

Changes in the expression of the nuclear receptors responsible for mediating the effects of E2 may also play a pivotal role in Nova1 expression. Our lab previously demonstrated that ERβ expression and alternative splicing is altered with age and E2 deprivation in a brain-region specific manner while others have shown similar effects on ERα expression with age and neurodegeneration [96, 176, 177, 196-198]. Treatment with

the ERα-selective agonist PPT and ERβ-selective agonist resulted in a significant increase in Nova1 expression, whereas E2 had no effect. Increases in Nova1 may be mediated by ERα and ERβ independently of each other through homodimerization of the receptors acting within the Nova1 promoter, whereas heterodimerization may have inhibitory effects on Nova1 transcription [46]. There are both half estrogen response elements (EREs) and AP-1 sites predicted within the Nova1 promoter region via ALGGEN PROMO online prediction software (Table 1). ERs act at these two distinct sites in different manners, more specifically at AP-1 sites where ERα enhances gene transcription and ERβ inhibits transcription [52, 199]. Therefore, activation of ERα may upregulate Nova1, while activation of ERβ may inhibit expression of a factor or miRNA that downregulates Nova1 expression, such as miR-181b-5p [148]. If heterodimerized, ERα has been shown to be the dominant partner in ERα/ERβ interactions, whereby ERβ is no longer able to inhibit Nova1 downregulation [200]. Future studies would need to be performed to determine the exact mechanism by which ERs regulate Nova1.

ERβ pre-mRNA nucleotide sequence contains many RBP sites that may regulate its alternative splicing. Among these RBP sites are several Nova1 consensus sites clustered upstream of the ERβ2 insert. While high throughput studies of Nova1 on pre-mRNA transcripts did not detect an interaction between ERβ and Nova1, it may be possible that it was undetectable due to the use of whole brain that may wash out more intricate interactions [140]. We observed correlations with Nova1 expression and ERβ alternative splicing from our previous studies; therefore we decided to determine if

Response element	location from transcriptional start site
ERE half-site	-633
AP-1	-1187
	-1656

Table 1. Location of response elements within the Nova1 promoter. Elements were predicted with ALGGEN PROMO predictor software (http://alggen.lsi.upc.es/) with search ERE and AP-1 search parameters. 2kbp of Nova1 promoter were analyzed based on sequence provided by NCBI database (http://www.ncbi.nlm.nih.gov/gene/4857).

Nova1 regulated ERβ alternative splicing. First, we transfected Nova1 in a dose-dependent manner in hypothalamic-derived IVB cells and observed concomitant significant increases in total ERβ expression. ERβ2 expression significantly decreased with Nova1 overexpression when compared to pcDNA even at the lowest dose of Nova1 DNA (0.01μg). These data suggest that Nova1 is mediating increased exon exclusion of the ERβ2 insert, which results in significantly more wild-type ERβ1 expression. These effects could counteract splicing silencers like hnRNPs that mediate exon inclusion events that result in transcripts that are targeted for mRNA nonsense-mediated decay, therefore increasing transcript numbers to be translated. A similar effect was observed on dopamine D2 receptors, where Nova1 expression counteracted the effects of hnRNP that increases the expression of an alternatively spliced dopamine receptor [139]. This increase coincides with decreased expression of the short form of the dopamine receptor that is mediated through hnRNP interactions. Interestingly, treatment of cells with E2 in the absence of Nova1 being overexpressed significantly decreased ERβ2 expression to levels similar to Nova1 overexpression. Furthermore, treatment of Nova1 expressing cells with E2 further decreased ERβ2 expression when compared to vehicle-treatment with Nova1 expression, although not significantly. These results show that Nova1 and E2 are acting on ERβ alternative splicing with the same outcome: decreased ERβ2 expression. Interestingly, E2 treatment decreased total ERβ expression in Nova1 expressing cells to levels that were no longer significant when compared to vehicle-treated Nova1 cells. E2 treatment had no effect on Nova1 overexpression. Therefore, E2 could be affecting overall availability of ERβ mRNA transcripts that acts independently of Nova1

overexpression due to differences between total ERβ and ERβ2 expressed. E2 has been shown to downregulate ERβ expression in a dose-dependent manner [201]. Thus the ability of Nova1 to regulate ERβ alternative splicing may depend on the availability of the pre-mRNA.

RIP experiments revealed a direct interaction between Nova1 and ERβ mRNA. Interactions with the ERβ pre-mRNA within exon 5 may mediate the exon exclusion event of the ERβ2 insert. These findings are supported by previous studies that show that Nova1 binding upstream of an alternatively spliced exon results in exclusion of this exon [145]. There are also Nova1 consensus sequences located within the intron upstream of exon 5, which may provide another binding site to mediate the exclusion of the ERβ2 insert. Also, we were unable to detect any predicted sequences downstream of the ERβ2 insert, meaning that Nova1 would likely not mediate exon inclusion, which is also supported by our findings.

Our model, along with the previous studies outlined in this manuscript, raise important limitations to consider. The studies by Tollervey and colleagues only studied Nova1 expression in the cortex of human male patients, whereas our studies were conducted in female rats in the hypothalamus, dorsal hippocampus, and ventral hippocampus. Therefore, more studies should be conducted to truly asses the expressional differences between species, brain regions, and possible sex differences. While these studies would be largely descriptive, it provides a gap in the literature to assess the mechanism of alternative splicing of ERβ in a more clinically relevant manner. These findings would also implicate possible global alternative splicing events that can

occur with age and loss of circulating E2. I was unable to detect a selected set Nova1-regulated genes in our studies (data not shown), however, a large scale detection in Nova1-regulated alternative splicing would provide insight into this possibility.

Another important aspect to consider is the presence of the Nova1 related RBP Nova2. Nova2 is generally opposite in expressional patterns in the brain when compared to Nova1, however these RBPs can overlap in expression in the hypothalamus [202]. More importantly, these two related proteins have highly related KH domains that both recognize YCAY consensus pre-mRNA sequences [203]. Therefore, we cannot rule out the possibility that Nova2 may act on ERβ in the same manner as Nova1. This may also explain some of the differences observed in ERβ alternative splicing between the hypothalamus and hippocampus as demonstrated in chapter III.

Overall, the results of the present study provide insight into the effects of aging and prolonged ovarian hormone deprivation on Nova1. We also provide a novel mechanism by which ERβ is alternatively spliced, and how observed changes in Nova1 expression may alter expression of not only ERβ splice variants but could also alter a variety of Nova1-regulated proteins in the brain. Consequences of these changes in expression may further explain the effects of reproductive senescence on cognitive function and memory, and Nova1 may be a novel therapy target in maintaining efficacy in hormone replacement therapies. Further investigation into ERβ:Nova1 interactions could reveal the exact sites on the ERβ pre-mRNA that Nova1 binds and mediates exclusion of the ERβ2 insert. Nova1 may be a potential therapeutic target for determining the effectiveness of E2-mediated HT.

CHAPTER V

AGING AND LOSS OF CIRCULATING 17B-ESTRADIOL ALTERS THE EXPRESSION OF SPLICING FACTORS IN THE FEMALE RAT BRAIN

Introduction

The complex regulatory system of post-transcriptional alternative splicing has many components. Apart from Nova1, there are a plethora of RNA-binding proteins (RBPs) that are expressed in the central nervous system, some uniquely expressed in neurons. These include NeuN and ELAV, two well-known RBPs that has also been used as neuronal markers [204, 205]. These RBPs form a core in ribonucleoprotein (RNP) complexes that mediate these mechanistic processes of binding consensus sequences on pre-mRNA transcripts. A recent review has amassed the number of RBPs expressed in the genome at over 1,500 [206].

While the primary focus of ERβ and the neuron-specific RBP Nova1 have led us to novel findings outlined in the previous chapters, there are other RBPs that are highly expressed in these same regions of interest in the brain, notably the hippocampus and hypothalamus. For instance, Rbfox1 has been shown to control neuronal excitation in the hippocampus [129]. RBPs not only mediate alternative splicing through direct interaction with pre-mRNA transcripts, but also mediate spliceosome/ribosome assembly. DEAD-Box RNA helicase Ddx17 (also known as p72) has been shown to be an important

component upstream and downstream in ER signaling pathways [207]. Ddx17 has also been shown to coordinate splicing events in cooperation with hnRNPH [208]. RBPs are also able to regulate each other's actions on alternative splicing, such as SR proteins opposing hnRNP interactions on splice site regulation [209]. Our laboratory has previously reported that ERβ can interact with hnRNPH1, and this interaction is enhanced in young (3 month old) female rats when compared with aged (18 month old) [210]. These studies did not assess the expression of hnRNPH1. Interestingly, Nova1 has been shown to counteract the effects of hnRNPs on dopamine D2 receptors, altering exon exclusion events [139]. Members of the CELF/BRUNOL family, Celf4 and Celf5, are important in brain development and maturation [131]. Celf4/5 are expressed within the CNS, and Celf4 is thought to be important for both neuronal and myocardial signaling [130].

The focus of this study was to determine the effects of aging and/or E2 deprivation on splicing factors that are highly expressed in the female brain. We hypothesized that splicing factor expression is altered in a region-specific manner, like Nova1, due to age- and E2-related changes that occur in neuronal gene expression. To test this hypothesis, we OVX Fisher344 rats at 3 and 18 months of age to study the effects of aging (1 week OVX) and E2 deprivation (1-12 weeks OVX) followed by acute E2 treatment in these animals (Fig. 7).

Results

HnRNPH1

Comparison of 3-mo. and 18-mo. animals treated with either vehicle or E2 revealed a significant main effect of aging and treatment on hnRNPH1 expression in both the hypothalamus ($F(1,22)=23.263$, $p<0.001$) and dorsal hippocampus ($F(1,21)=8.896$, $p<0.01$) (Fig. 23, A, B). A significant interaction between age and treatment was observed in the ventral hippocampus ($F(1,21)=12.971$, $p<0.05$) (Figure 23C), demonstrating that the expression of hnRNP-H1 depends on the age that E2 treatment is administered. The only significant increase in hnRNPH1 expression with age alone (ie. vehicle-treatment) occurred in the hypothalamus, there was a significant increase in hnRNPH1 expression in 18-mo. animals when compared to the 3-mo. old animals. Treatment with E2 potentiated this increase at 18 mo., resulting in highly significant hnRNPH1 expression. E2 had a significant effect on hnRNPH1 expression in 3-mo. old animals in both the dorsal hippocampus and ventral hippocampus, but not in 18-mo. animals.

To elucidate the effects of E2 deprivation on hnRNPH1 expression in the aged female brain, 18-mo. old animals were OVX then deprived of E2 for increasingly longer periods of time before treated with vehicle or 2.5 ug/kg E2 once/day for 3 consecutive days (Fig. 7). A significant interaction between length of deprivation and treatment was revealed in all three regions: hypothalamus ($F(3,41)=3.875$, $p<0.05$); dorsal hippocampus ($F(3,41)=4.955$, $p<0.05$); ventral hippocampus ($F(3,40)=3.527$, $p<0.05$) (Fig. 24). In the hypothalamus, there was a significant increase in hnRNPH1 expression following E2

treatment at both the 1- and 4-week post-OVX time points (Fig. 24A). E2 treatment resulted in a significant increase in hnRNPH1 expression at 8 weeks post-OVX in the dorsal hippocampus (Fig. 24B). The length of deprivation significantly increased hnRPH1 expression after 8 weeks when compared to the significant decrease in expression at 12 weeks post-OVX (Figure 24C). Acute E2 treatment also had varying effects on hnRNPH1 expression in the ventral hippocampus: E2 significantly increased expression at 4 weeks post-OVX when compared to the significant decrease in mRNA at 12 weeks.

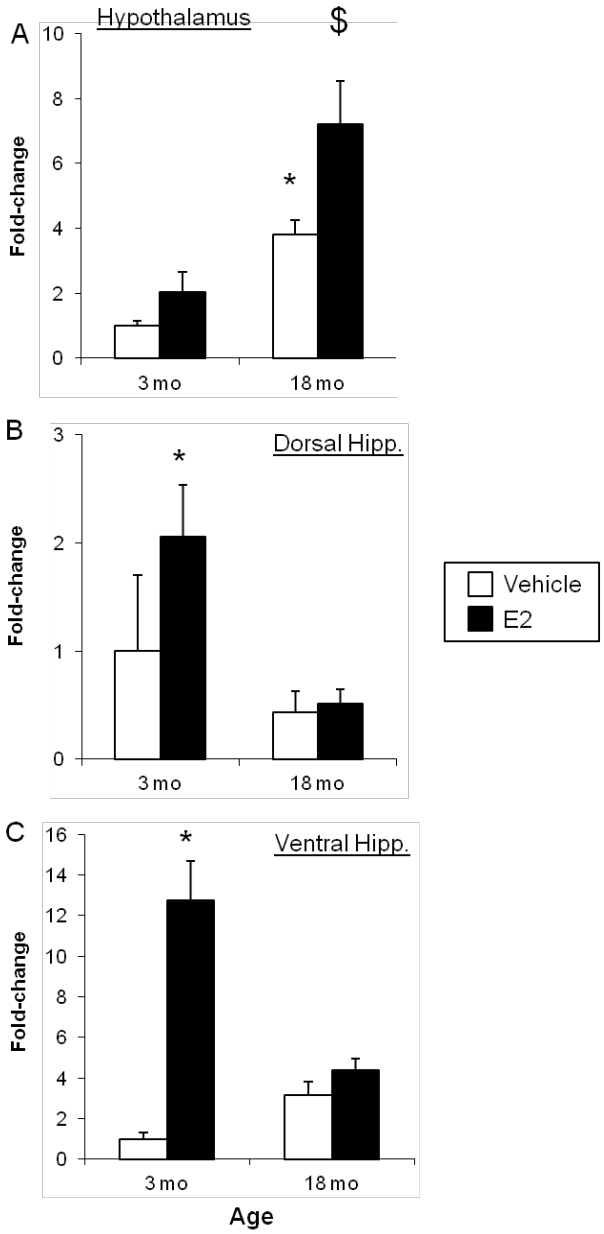

Figure 23. Comparison of hnRNPH1 mRNA expression in the young and aged female rat brain. hnRNPH1 expression was measured in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1 week post-OVX in 3 and 18-mo. old female rats (N = 6/age/treatment group). Animals were OVX and 1 week later treated with either vehicle (safflower oil) or 2.5 µg E2 (dissolved in safflower oil). Asterisk '*' indicates a statistically significant difference ($p < 0.05$) compared to 1 week vehicle and '$' indicates a greater statistically significant difference ($p < 0.001$) by as determined Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

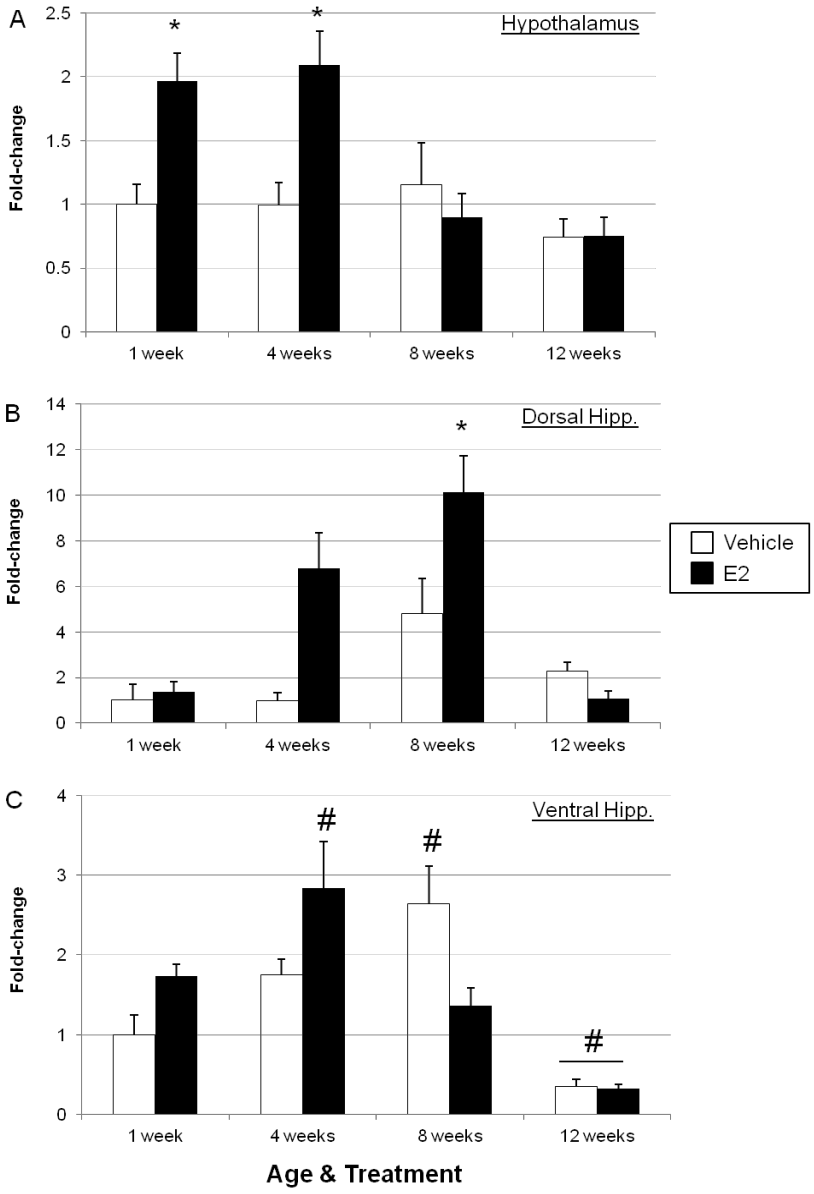

Figure 24. Expression of hnRNPH1 mRNA in the brain of aged (18-mo. old) female rats following varying periods of E2 deprivation. HnRNPH1 mRNA expression was measured in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1, 4, 8, and 12 weeks post-OVX (N = 6-10/age/treatment group). Animals were treated with either vehicle (safflower oil) or 2.5 µg E2 (dissolved in safflower oil). Asterisk '*' indicates a statistically significant difference ($p < 0.05$) compared to 1 week vehicle and '#' indicates statistically significant difference between groups ($p<0.05$) as determined by Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

Rbfox1

Comparison of Rbfox1 mRNA expression between 3-mo. and 18-mo. animals revealed a significant main effect of treatment in the ventral hippocampus ($F(1,21)=4.032$, $p<0.05$) (Fig. 25C) but not in the hypothalamus or dorsal hippocampus (Fig. 25A,B). However, there were significant effects of E2 treatment in all three brain regions measured in 3-mo. old animals.

E2 deprivation in aged animals revealed a significant main effect of treatment in the hypothalamus ($F(3,37)=5.343$, $p<0.05$) on Rbfox1 mRNA expression (Fig. 26A). Also observed were significant main effects of length of E2 deprivation on Rbfox1 mRNA expression in the dorsal hippocampus ($F(3,41)=7.007$, $p<0.01$) and ventral hippocampus ($F(3,40)=6.120$, $p<0.01$) (Fig. 26B,C). Acute E2 treatment significantly increased expression of Rbfox1 1-week post-OVX in the hypothalamus and at 4 weeks in the ventral hippocampus. Rbfox1 expression was significantly increased in the dorsal hippocampus after 8 weeks deprivation in both vehicle- and E2-treated animals. This increase was no longer observed in the ventral hippocampus at 12 weeks E2 deprivation (post-OVX).

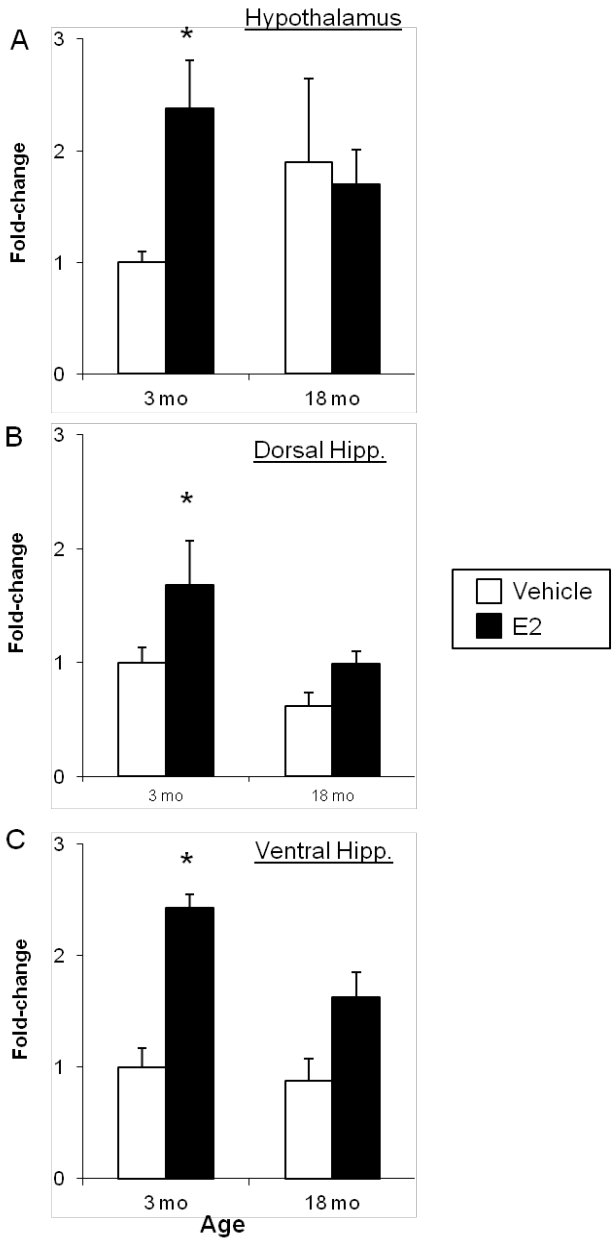

Figure 25. Comparison of Rbfox1 mRNA expression in the young and aged female rat brain. Rbfox1 expression was measured in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1 week post-OVX in 3 and 18-mo. old female rats (N = 6/age/treatment group). Animals were OVX and 1 week later treated with either vehicle (safflower oil) or 2.5 µg E2 (dissolved in safflower oil). Asterisk '*' indicates a statistically significant difference ($p < 0.05$) compared to 1 week as determined by Tukey's Honestly-Significant-Difference Test following two-way ANOVA in the ventral hippocampus or by t-test in the hypothalamus and dorsal hippocampus. Data are expressed as mean ± SEM.

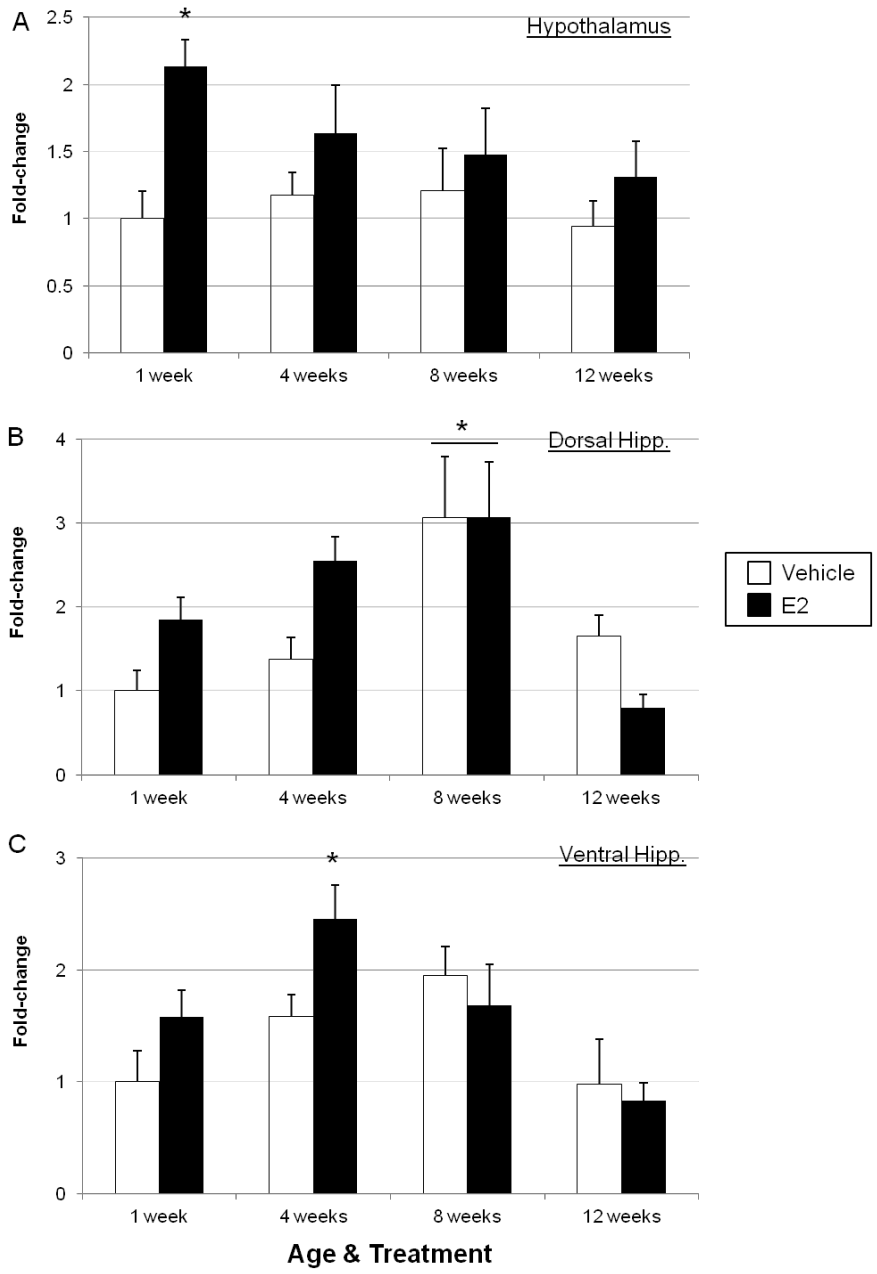

Figure 26. Expression of Rbfox1 mRNA in the brain of aged (18-mo. old) female rats following varying periods of E2 deprivation. Rbfox1 mRNA expression was measured in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1, 4, 8, and 12 weeks post-OVX (N = 6-10/age/treatment group). Animals were treated with either vehicle (safflower oil) or 2.5 μg E2 (dissolved in safflower oil). Asterisk '*' indicates a statistically significant difference ($p < 0.05$) compared to 1 week vehicle as determined by Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

Ddx17

Analysis of Ddx17 expression comparing 3-mo. and 18-mo. old animals reported significant main effects of aging in the hypothalamus ($F(1,21)=56.752$, $p<0.001$) and dorsal hippocampus ($F(1,20)=10.05$, $p<0.01$) (Fig. 27A,B). A significant main effect of treatment on Ddx17 expression was also observed in the dorsal hippocampus ($F(1,20)=4.933$, $p<0.05$). There were no significant effects observed in the ventral hippocampus (Fig. 27C). Overall expression of Ddx17 was significantly elevated in the 18-mo hypothalamus, and the same was also true in the dorsal hippocampus with E2 treatment-only.

The loss of E2 had a significant main effect in both the dorsal hippocampus ($F(3,40)=62.896$, $p<0.001$) and ventral hippocampus ($F(3,39)=8.915$, $p<0.01$) on Ddx17 expression (Fig. 28B,C). There were no significant effects observed in the hypothalamus with increasing deprivation time or E2 treatment (Fig. 28A). E2 deprivation significantly increased Ddx17 levels after 4 weeks deprivation, but the effect was lost with longer periods of deprivation. E2 treatment significantly increased Ddx17 expression at 4 weeks deprivation in the dorsal hippocampus, with overall expression remaining significantly increased in both treatment groups at 8 weeks deprivation. These effects are lost at the 12 week time point.

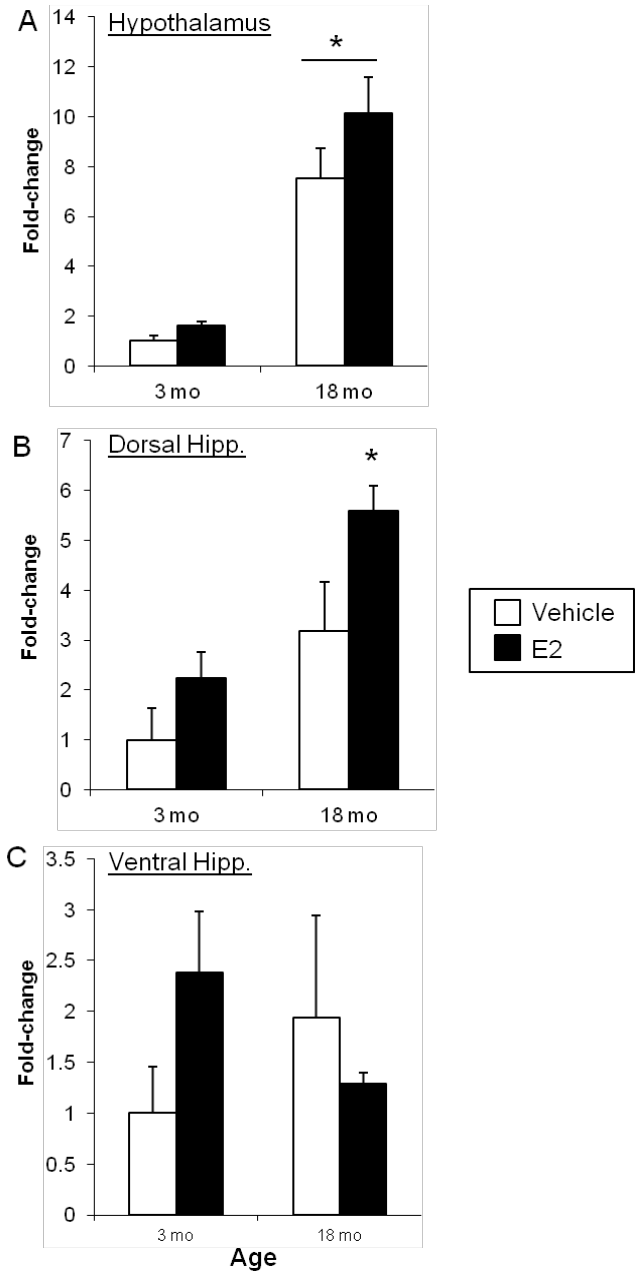

Figure 27. Comparison of Ddx17 mRNA expression in the young and aged female rat brain. Ddx17 expression was measured in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1 week post-OVX in 3 and 18-mo. old female rats (N = 6/age/treatment group). Animals were OVX and 1 week later treated with either vehicle (safflower oil) or 2.5 µg E2 (dissolved in safflower oil). Asterisk '*' indicates a statistically significant difference ($p < 0.05$) compared to 1 week as determined by Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

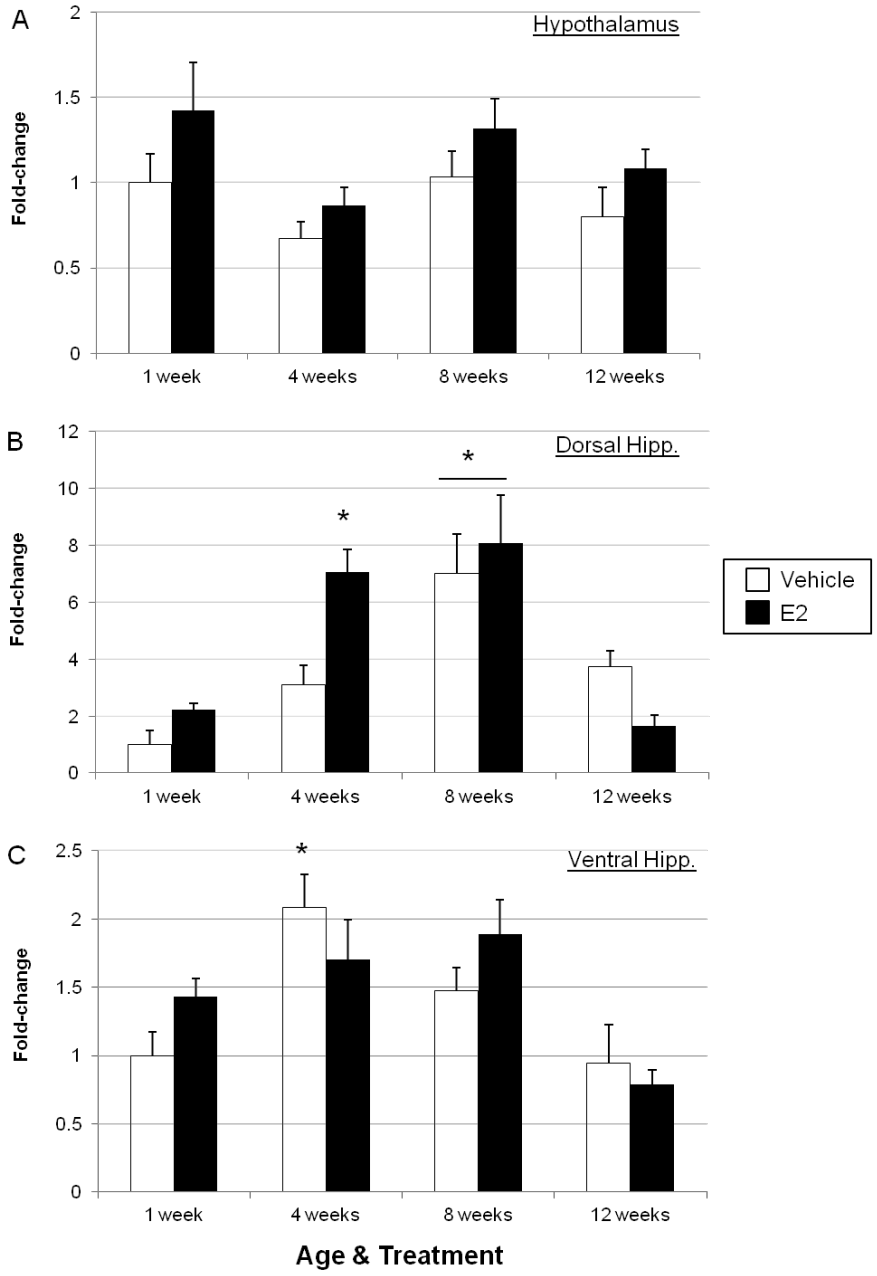

Figure 28. Expression of Ddx17 mRNA in the brain of aged (18-mo. old) female rats following varying periods of E2 deprivation. Ddx17 mRNA expression was measured in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1, 4, 8, and 12 weeks post-OVX (N = 6-10/age/treatment group). Animals were treated with either vehicle (safflower oil) or 2.5 μg E2 (dissolved in safflower oil). Asterisk '*' indicates a statistically significant difference ($p < 0.05$) compared to 1 week vehicle as determined by Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

Celf4 and Celf5

Comparisons of the CELF/BRUNOL factors Celf4 and Celf5 between 3-mo. and 18-mo. old animals revealed significant main effects of aging on expression of Celf4 ($F(1,21)=17.43$, $p<0.001$) and Celf5 ($F(1,21)=16.266$, $p<0.001$) in the hypothalamus (Figures 29A & 30A). There was also a significant interaction between aging and treatment on Celf4 expression in the ventral hippocampus ($F(1,20)=6.534$, $p<0.05$) (Figure 29C). Expression of Celf4 and Celf5 were significantly increased in the hypothalamus with E2 treatment in the 18-mo. females, although vehicle treatment also significantly increased Celf5 expression at this age (Figs 29A & 30A). In the dorsal hippocampus, there was a significant increase in Celf5 from E2 treatment in 3-mo. old animals (Figure 30B). In the ventral hippocampus, there was a significant increase in Celf4 expression following E2 treatment in 3-mo. animals, whereas Celf5 expression significantly increased in 18-mo. animals (Figures 29C & 30C).

In female rats deprived of ovarian hormones, there was a significant interaction between timing and treatment on Celf5 expression in the dorsal hippocampus ($F(3,40)=18.589$, $p<0.001$) and ventral hippocampus ($F(3,39)=5.048$, $p<0.01$) (Figure 32B,C). A significant interaction also occurred in the ventral hippocampus on Celf4 expression ($F(3,39)=6.723$, $p<0.001$) (Figure 31C). A significant main effect of deprivation was observed in the dorsal hippocampus ($F(3,40)=6.499$, $p<0.001$) on Celf4 expression (Figure 31B). While no significant interactions or effects were observed in the hypothalamus on either Celf4 or Celf5, there was a significant increase in Celf5 expression at 4 weeks deprivation following E2 treatment (Figure 32A). This same E2

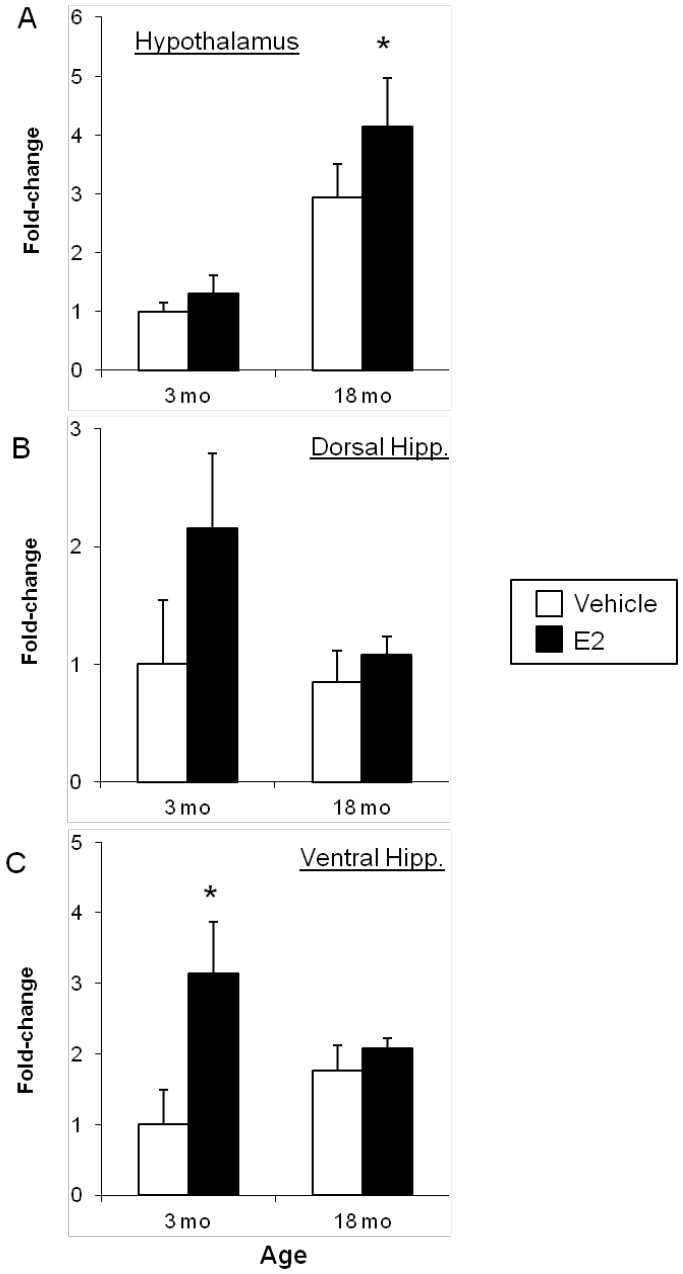

Figure 29. Comparison of Celf4 mRNA expression in the young and aged female rat brain. Celf4 expression was measured in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1 week post-OVX in 3 and 18-mo. old female rats (N = 6/age/treatment group). Animals were OVX and 1 week later treated with either vehicle (safflower oil) or 2.5 µg E2 (dissolved in safflower oil). Asterisk '*' indicates a statistically significant difference ($p < 0.05$) compared to 1 week vehicle as determined Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

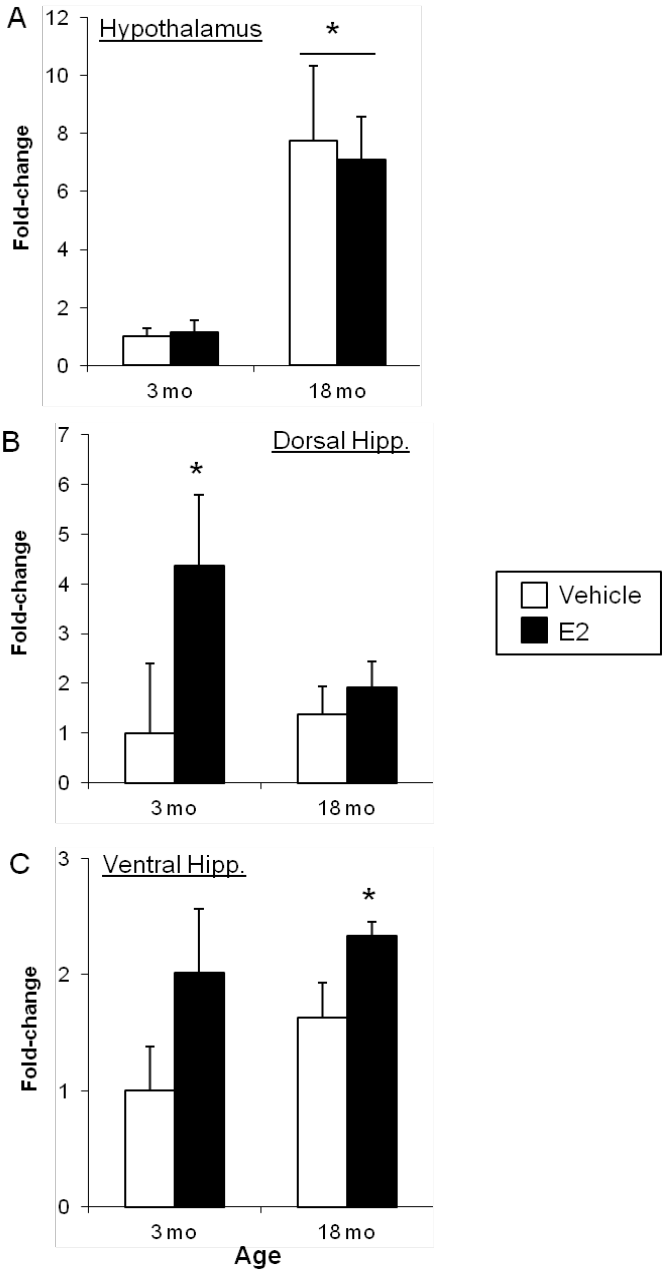

Figure 30. Comparison of Celf5 mRNA expression in the young and aged female rat brain. Celf5 expression was measured in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1 week post-OVX in 3 and 18-mo. old female rats (N = 6/age/treatment group). Animals were OVX and 1 week later treated with either vehicle (safflower oil) or 2.5 µg E2 (dissolved in safflower oil). Asterisk '*' indicates a statistically significant difference ($p < 0.05$) compared to 1 week vehicle as determined Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

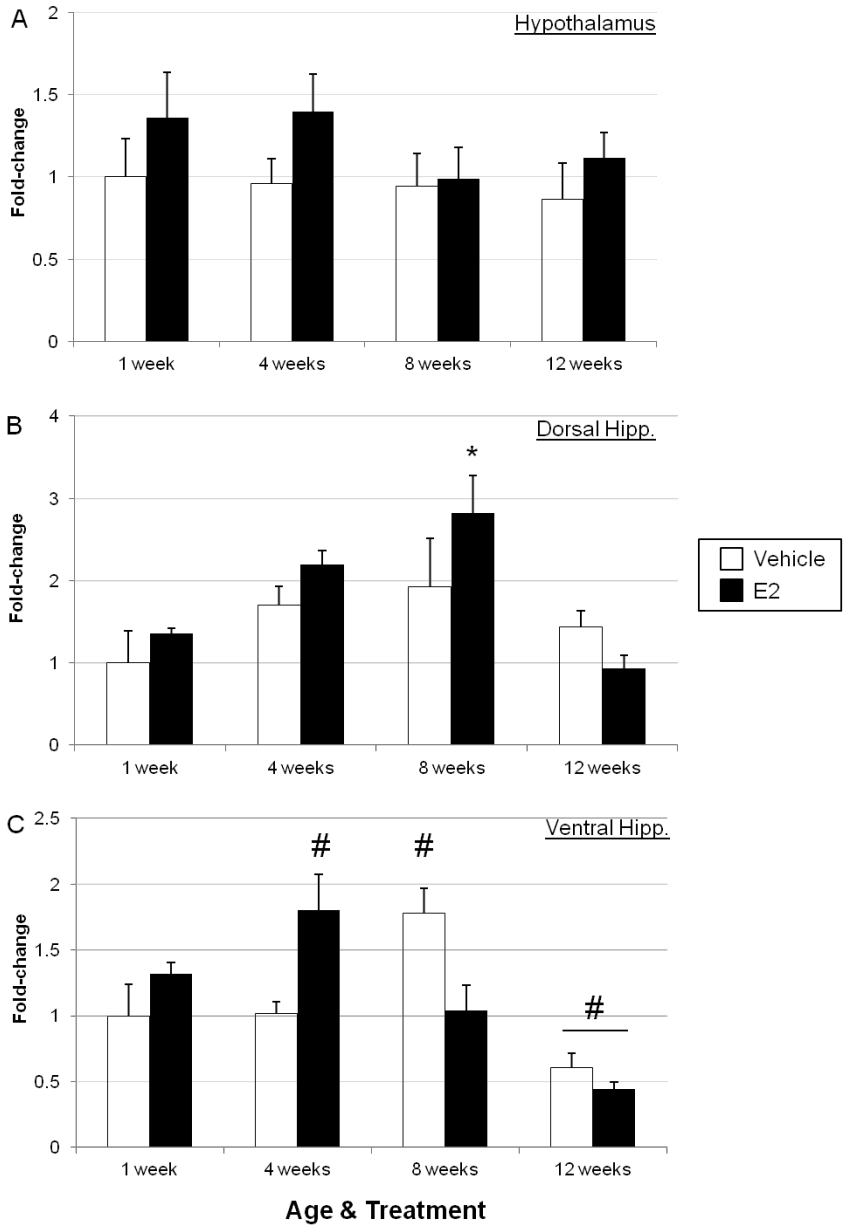

Figure 31. Expression of Celf4 mRNA in the brain of aged (18-mo. old) female rats following varying periods of E2 deprivation. Celf4 mRNA expression was measured in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1, 4, 8, and 12 weeks post-OVX (N = 6-10/age/treatment group). Animals were treated with either vehicle (safflower oil) or 2.5 µg E2 (dissolved in safflower oil). Asterisk '*' indicates a statistically significant difference ($p < 0.05$) compared to 1 week vehicle and '#' indicates statistically significant difference between groups ($p<0.05$) as determined by Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

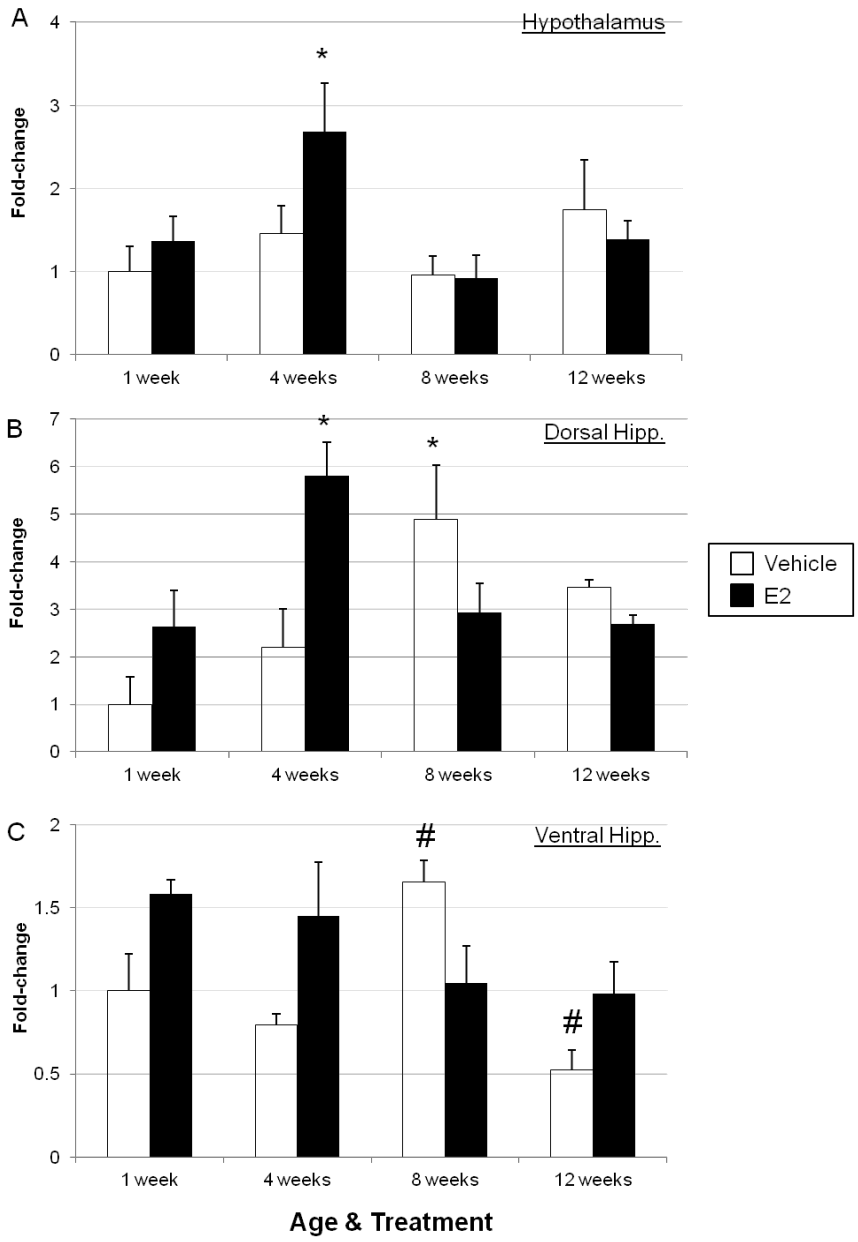

Figure 32. Expression of Celf5 mRNA in the brain of aged (18-mo. old) female rats following varying periods of E2 deprivation. Celf5 mRNA expression was measured in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1, 4, 8, and 12 weeks post-OVX (N = 6-10/age/treatment group). Animals were treated with either vehicle (safflower oil) or 2.5 µg E2 (dissolved in safflower oil). Asterisk '*' indicates a statistically significant difference ($p < 0.05$) compared to 1 week vehicle and '#' indicates statistically significant difference between groups ($p<0.05$) as determined by Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

effect at 4 weeks was also observed in the dorsal hippocampus; however, there was a significant increase with deprivation after 8 weeks, where E2 treatment at this time point attenuated this increase (Figure 32B). Celf4 and Celf5 expression in the ventral hippocampus had no significant differences when compared with 1 week vehicle-treated animals, but there significant differences in later deprivation time points (Figure 31C & 32C).

Summary

The results from these studies demonstrate that aging and loss of E2 in the aged female brain affect RBPs that are integral for coordinating a variety of alternative splicing events. These data provide further evidence that these physiological changes affect a broader set of RBPs in a region-specific manner beside the neuron-specific Nova1 splicing factor studied in chapter IV. Taken together, these data suggest that alternative splicing events may be altered in tissues where these RBPs are expressed, contributing to the decline in health that is associated with aging and reproductive senescence. These data also provide further targets to study the effects on ERβ alternative splicing that may also occur in brain and other organ systems.

HnRNPH1 is a critical RBP that acts on splice site silencers (SSS), whether intronic or exonic [187, 209]. This RBP works in coordination with Ddx17 to regulate splicing programs during cell differentiation [208]. These two factors also orchestrate transcription, and have both been implicated in E2 signaling through protein:protein interactions [207, 210]. The effects of aging and E2 deprivation on hnRNPH1 expression

demonstrated in these studies may in turn affect the interaction of this RBP with ERβ as observed previously by our laboratory. Our data demonstrate that this RBP is altered primarily by E2, and loss of E2 over time appears to no longer maintain expression of hnRNPH1 in these brain regions. If alternative splicing increases with age, the increase in hnRNPH1 may be due to a compensatory mechanism to limit the number of alternative splicing events that can occur as our data suggest in the hypothalamus. E2 potentiated this increase even further, which may imply further benefits of E2 treatment in aged individuals. The brain region specific changes were apparent when comparing the effects of age on hnRNPH1 in the hippocampus, where it significantly increased in 3 mo. old animals but not 18 mo. old. E2 has been shown to upregulate cell proliferation in the hippocampus, however this effect is lost with longer periods of hormone deprivation [79]. While there is a significant effect of E2 in later stages of our hormone deprivation in the hippocampus, the effects of hnRNHP1 on these processes may rely on other factors present, like Ddx17. As stated previously, hnRNPH1 is also important for cell differentiation, and the pool of neural progenitor cells decreases significantly with age in the hippocampus [211]. This may be why E2 treatment significantly increased expression of hnRNPH1 in both the dorsal and ventral hippocampus in 3 mo. old animals as an important mediator of cell proliferation where these progenitor pools are still high. However, loss of hnRNPH1, like in the ventral hippocampus with E2 deprivation, and its possible recruitment to the transcriptional complex by ERβ may alter expression of splice variants that rely on recognition of splice site silencing through hnRNPH1 [208]. These interactions may be further altered by the expressional differences in ERβ1 and ERβ2

previously outlined in chapter III that occur in the brain during aging and loss of E2. Further studies on ERβ interaction with hnRNPH1 may reveal more on the dependency of these two factors for expression of ER-regulated gene expression and/or alternative splicing events.

Ddx17 is an important regulator of ER signaling, mainly through control of the expression of key regulators of ER activity [207]. These include kinases important for phosphorylation of ERs like cyclin-dependent kinase 2 (CDK2) and mitogen-activated protein kinase (MAPK) 1. Ddx17 also acts downstream as a required factor for transcriptional and splicing regulation of ER-regulated genes like nuclear receptor co-repressor 2 (NCOR2). Like hnRNPH1, Ddx17 expression drastically increases with age in the hypothalamus, therefore it is possible that the expression of this RBP is regulated under similar mechanisms as hnRNPH1. There are also similar expression patterns between these two RBPs in the hippocampus, which may echo the previous statements about cell differentiation with hnRNPH1 in this brain region. However, the responsiveness to E2 is lost in the advanced deprivation time periods in all brain regions. These effects may impact ER regulation of transcription and splicing of E2 target genes, such as glycogen synthase kinase-3 (GSK3)β. Further studies on Ddx17 expression and the impact on steroid hormone signaling warrants further investigation due to the master regulator status of Ddx17.

Interestingly, Rbfox1 expression was not altered as much by age in the ventral hippocampus, but drastically increased in the dorsal hippocampus and hypothalamus. Brain region specificity on Rbfox1 expression was also observed in the E2 deprivation

paradigm, where deprivation had affects in the hippocampus, but not the hypothalamus. Rbfox1's role in development and on synaptic transmission in the brain make this an RBP to which altered expression could have implications on behavior and memory [129]. Rbfox1 can also regulate hnRNPH1 and Ddx17 expression, therefore impacting expression of a whole set of alternatively spliced genes regulated by these RBPs [212]. Rbfox1, hnRNPH1, and Ddx17 expression change in a relatively consistent manner with E2 deprivation, however there appear to be greater differences when comparing these RBPs in our aging model. These differences may be dependent on another set of factors that interact with Rbfox1 and/or these other RBPs to regulate their expression that could mirror their importance to brain development.

The CELF/BRUNOL factors had the least amount of significant differences between aging and E2 deprivation in the brain regions studied when compared with the other RBPs covered in our studies. These data reveal that both Celf4 and Celf5 are altered greatly in the hippocampus when compared with the hypothalamus with regards to hormone deprivation, however, the largest differences with aging occurred in the hypothalamus when compared with the hippocampus. Celf4 is an important regulator of synaptic plasticity in a similar manner as Rbfox1, therefore loss of this factor could contribute greatly to alternative splicing events that affect brain physiology and behavior [130, 131]. While there is little known about Celf5 other than its neuron-specific expression, it could also mediate important alternative splicing events due to it homologous nature to Celf4. It is interesting that its expression did not follow the same expressional profile pattern as Celf4 in the dorsal hippocampus with age, but it appears

that the other regions examined had similar differences between these two highly related factors.

These factors studied in this chapter, may have far reaching consequences due to altered expression patterns, all which provide possible mechanisms for the negative effects associated with aging and diminished levels of E2 in the aged female brain. However, these studies are limited to their descriptive nature, and follow up studies on the implications of these RBPs being altered by age and/or E2 deprivation may provide more insight on alternative splicing outcomes in the brain.

CHAPTER VI

FINAL DISCUSSION

Summary of Key Findings

The outcomes from the WHI studies brought to light an important observation that there is a critical window in which HT is beneficial. After this period of time, the negative effects of HT present adverse health outcomes in postmenopausal women. If the efficacy of E2 is no longer valid, then it is becomes important to understand why. While there are many different avenues to take, understanding the receptors by which this ovarian hormone mediates its actions should be of utmost importance. There is evidence that alternative splicing events increase in the brain with age. The impact of ERβ splice variants are becoming more apparent through both human and rat studies. Therefore, the goals of this project were to examine the mechanism by which alternative splicing of ERβ occurs in the aged female brain. In chapter III, the data show that both aging and loss of E2 contribute to altered expression of ERβ splice variants. Chapter IV revealed that the expression of Nova1, an important neuronal RBP for regulating a multitude of alternative splicing events, is also affected by loss of E2 in the aged female brain. The studies done in chapter V continue upon these changes that can occur in alternative splicing, as a number of other RBPs are affected by age- and E2-related changes. However, the novel findings that Nova1 may regulate ERβ alternative splicing through interactions with the

ERβ mRNA shown in chapter IV may provide the most integral target to study in the regulation of ERβ alternative splicing in the aging female brain. Taken together, the data presented in this dissertation provide novel mechanisms for age-related changes in ERβ alternative splicing, and demonstrate how loss of E2 can potentially affect global alternative splicing events that may potentiate these effects in postmenopausal women who seek HT.

Menopause and ERβ Alternative Splicing

The findings presented in chapter III make significant contributions toward understanding the how age-related changes can affect the alternative splicing of ERβ. Also presented in this chapter are the significant findings of how E2 deprivation can affect the alternative splicing of ERβ. These two contributing factors occur in a brain region specific manner. Therefore, the rate at which the brain is altered due to increased age and loss of E2 may be significantly different between brain regions, and that the predominant receptor expressed in these regions is the dominant negative ERβ2 splice variant. ERβ2 is considered a dominant negative splice variant due to its decreased affinity for ligand and opposing effects on ER-mediated signaling [102, 201, 213]. Ultimately, the expression of this receptor may contribute to the reduced efficacy through reduced ligand-binding affinity associated with HT, and the differential expression of this splice variant receptor between brain regions may shift the critical window of HT between regions.

These findings presented in the context of a window of time are very appropriate. The window is 'open' widest during a woman's early pre-pubertal life up to pre-perimenopause. E2 signaling is maintaining homeostatic environments in both the brain and throughout the body as ovarian hormone productions remains consistent. As E2 production begins to slow down and becomes acyclic, the critical window begins to close. At this point in time, ER alternative splicing events may begin, shifting dominance from the wild-type ERβ to the dominant negative ERβ2. These mechanisms must also take into the account the age of the individual at which this occurs, as aging contributes to increases in alternative splicing [149]. E2-regulated gene expression of RBPs most likely sets these early alternative splicing changes in motion. There can also be subtle fine-tunings of these changes that can also begin altering alternative gene transcripts through mRNA stability [214].

E2 is also known to regulate non-genomic effects within cells through the GPER, mediating secondary messengers that affect cell metabolism [63, 69]. Increases in oxidative stress due to aging can affect these processes, and altered expression of ERs, whether NR or GPCR, can influence these actions. There have also been several GPER alternative splice variants that differ in the 5’ untranslated region (UTR), although little has been studied about these variants. However, changes to the 5’ UTR can affect the open reading frame of the mRNA to be translated, thereby regulating the expression of the protein [215]. Expressional differences in GPER could have negative implications on E2 gene regulation, especially in activating kinases important for E2 signaling like Erk2 [61].

When menopause occurs, the diminished levels of E2 circulating in the body may accelerate the mechanisms that increase alternative splicing, which we know can be compounded by aging. Leading in to early postmenopause, the window is still open but is beginning to close at a steady rate. Studies on alternative splicing and aging have provided a basis to what could be occurring during this natural process [149, 216]. Our observations of not only altered expression of RBPs due to age but also due to E2 brings to light the reality of sex-specific aging. It is entirely possible that these changes could be a compensatory mechanism to acclimate to the changing hormonal environment, which may be why there is a hypersensitivity to E2 treatment on ERβ and certain RBP mRNA expression within the different brain regions. However, these changes may come at a cost to brain processes, which can begin impacting cognitive function, where memory deficits and mood disorders begin to manifest [15, 16]. The persistent low levels of circulating E2 and age-related effects on alternative splicing could in turn be becoming the molecular steady state within neurons, and these cells begin working with what they have, although a much better prognosis would be for continuous E2 circulating throughout the body. Altered expression of ERβ splice variant expression was observed with aging in the hypothalamus in aged rats, to which we equate based on previous estrous cycle and survival studies to a 50-56 year old woman who is in the menopausal transition to early postmenopause [181]. While there was not a significant change in ERβ expression in the hippocampus, there was no longer an effect of E2 on its expression. This may have to do with changes in RBPs that regulate the alternative splicing of ERβ that are now altered in this brain region, as will be discussed later.

Eventually, the window will 'close', and the benefits of HT will be lost. However, the concept of a critical window by which HT is beneficial must be reexamined. These data present the idea that there isn't just one window: there are multiple windows of time in which HT is beneficial. These windows are open in conjunction with each brain region, and one window closing does not mean all of them are closing. For example, the efficacy of E2 on hypothalamic processes may be longer than the efficacy of E2 on hippocampal process; therefore, the critical window for HT is open much longer in the hypothalamus than in the hippocampus.

In the context of the hippocampus, ERβ expression changes rapidly during the early deprivation stages. This brain region is the epicenter of memory, and many studies in postmenopausal women have shown a decline in memory [20, 162, 174]. Our findings in chapter III show that ERβ2 splice variant becomes the dominant form of the receptor in this region of the brain, therefore HT may no longer benefit this brain region. These data further corroborate similar findings in middle-aged rats who underwent prolonged E2 deprivation [79]. Increased ERβ2 expression may contribute to the cognitive deficits in this brain region, as E2 has been shown to increase hippocampal neurogenesis and improve memory through spine density and synaptic plasticity [83, 85, 217]. In both the dorsal and ventral hippocampus after 4 weeks deprivation there was a significant decrease in total ERβ expression. Following E2 treatment, total ERβ expression significantly increased, while the ERβ2 fraction maintained a consistent expression level compared to untreated animals at this time point. The effect of E2 treatment on ERβ expression in the hippocampus is gone after 8 weeks, and never recovers. This 4 week

time points signals a critical period in the hippocampus where there may still be responsiveness to E2 and this time point is still within that critical window of time for a postmenopausal women undergoing HT (19-mo. old rat = human 53-59 years of age) as determined in chapter III. After 8 weeks of ovarian hormone deprivation, the efficacy of E2 could be lost, as has been observed in postmenopausal women within the latter of this age range (20-mo. rat = 56-62.5 year old human). Observations in animals who underwent prolonged E2 deprivation displayed decreased hippocampal neurogenesis as ERβ2 expression increased [79]. These studies also showed that animals with increased ERβ2 expression exhibited more depressive-like behaviors, providing another link to the effects of ERβ alternative splicing in the hypothalamus.

E2 deprivation did not have the same effect in the hypothalamus, where there is still a complimentary amount of both ERβ isoforms with prolonged deprivation (up to 12 weeks). These region specific differences must rely on a multitude of conditions that could be changed in the hippocampus but not in the hypothalamus, such as the presence of brain-region specific RBPs. However, this region is linked to mood disorders and homeostasis, so these behavioral changes could be related in part to the age-related changes in ERβ expression and not to deprivation. Therefore, HT may still provide benefits to the hypothalamus in later stages of postmenopause, but due to the expressional profile of ERβ2 in the hippocampus, the hypothalamic-associated benefits may have negative consequences in the hippocampus.

RNA Polymerase II and Splicing Kinetics

Expression of RNAPII and its activity have been studied little in the context of aging and E2 deprivation. A study done in the 1980's observed that E2 administration following OVX in female rats significantly increased the activity of RNAPII 12-18 hours after E2 treatment [218]. I hypothesized that E2 would not only have an immediate effect on RNAPII, but a long lasting effect due to reproductive senescence. It was also hypothesized that RNAPII expression may be altered in a brain-region specific manner due to aging and loss of circulating E2. In our studies conducted in chapter III, we found that RNAPII expression and activity was indeed altered by aging and E2 deprivation in a brain region-specific manner.

While there isn't a direct link between E2 and RNAPII expression/activity, E2 may act either directly or indirectly to have the effects observed in the brain. ERβ is a well-known transcription factor that interacts with a variety of coregulatory elements that in turn interact with RNAPII to initiate transcription [219]. Through altered expression of ERβ and its splice variants, the availability of ERβ and/or its transcriptional activity (ERβ splice variants can also act in a ligand-independent manner) could influence RNAPII at E2-regulated genes. Therefore, the activity of RNAPII would increase or decrease based upon the expression of ERβ. However, these E2-regulated genes could be important proteins that interact directly with RNAPII, resulting in decreased association of RNAPII with integral transcription factors. These findings could broaden the implications of loss of E2 on not only ERβ expression and its alternative splicing, but on global gene expression and global alternative splicing.

These studies began as a means to tease out a mechanism of why ERβ alternative splicing changes in the brain with age and E2 deprivation. While RNAPII expression and activity may play a large role in this mechanism, RNAPII is known to also interact with a host of RBPs via its C-terminal region. These interactions rely on the phosphorylation of this domain, which is interesting in that phosphorylation of RNAPII C-terminal region also affects the rate of transcription [166]. Hyper-phosphorylation of RNAPII can slow down transcription, yet phosphorylation is required for this enzyme to be active. This slowing of RNAPII could allow factors attached to the C-terminal domain to recognize RBP motifs to mediate splicing events, and in some case, alternative splicing. E2 can regulate the kinase CDK9 that phosphorylates RNAPII in ERα+ breast cancer cell lines, although this has not been studied in the context of the brain [220]. Therefore, it is possible that E2 could also be regulating the rate at which RNAPII transcribes RNA and also alternative splicing events through RBP recruitment.

A novel compound called camptothecin can inhibit TOPI through competitive-binding [178]. Inhibiting the activity of TOPI stalls RNAPII on the DNA, resulting in slowed transcriptional kinetics. The evidence that transcription and splicing are coupled processes have been demonstrated with the interaction of RNAPII and RBPs as stated previously, and have been studied extensively the past decade [165, 168, 169]. Therefore, stalling RNAPII allows time for RBPs to mediate alternative splicing events whose probability of occurring is reduced due to the location of *cis* RBP binding sites or weak splice sites [166]. Another aspect to this phenomenon is that the size of the intron between alternatively spliced exons can also influence these events. Shorter intronic

elements result in increased alternative exon inclusion following camptothecin treatment [167]. The intronic element upstream of the ERβ2 insert is about 900bp, and contains weak splice sites at the appropriate 5' and 3' sites that can be recognized by spliceosome components [117]. We observed a significant increase in ERβ2 expression following TOPI inhibition in the ERβ-expression GT1-7 hypothalamic cell line. However, E2 treatment in coordination with camptothecin eliminated this effect. In the context of breast cancer, E2 can overcome RNAPII transcriptional stalling by recruiting CDK9 to the transcriptional complex [221]. While there is no evidence that stalled RNAPII is hypophosphorylated, the association of ERs with kinases and other transcriptional regulatory components may counteract these effects *in vitro*. Slowing of transcription *in vivo* would most likely have detrimental effects on cell survival. Cells treated with camptothecin for more than 24 hours or with higher doses undergo apoptotic cell death [222]. However, these observations provide novel findings about the ERβ mRNA transcripts and how its structure may influence its alternative splicing as well as global alternative splicing through its subsequent function.

Nova1 expression changes in a brain region-specific manner

Prior to these studies, Nova1 has not been shown to associate or regulate the alternative splicing of ERβ. While this was the ultimate goal of these studies, there were several more gaps in the literature that would lead us to these findings. Namely, Nova1 was shown previously to be downregulated by the synthetic GC dexamethasone *in vitro* in a hypothalamic cell line [147]. These findings appear to be the only demonstration by

which steroid hormones were used to study regulation of Nova1 expression. Recent *in vivo* studies demonstrated that Nova1 decreased with age in male cortical samples [149]. It should be noted that these studies did not include female subjects. Scanning the promoter of Nova1 contains an ERE half site as well as several AP-1 sites upstream of the transcription start site. These findings along with our previous observations of aging and ovarian hormone deprivation on ERβ alternative splicing led us to target Nova1 as a potential RBP that is regulated not only by E2 but also by aging in females. This in turn may provide more evidence for the role of Nova1 in the alternative splicing of ERβ. The findings in chapter IV revealed that Nova1 decreased with age in female OVX rats, and that E2 deprivation and subsequent treatment altered the expression of this RBP. E2 was unable to increase Nova1 expression in 18 month animals to levels observed at 3 months in the studied brain region. These brain region-specific Nova1 decreases may occur due to the synergy of diminished E2 levels and increased cortisol levels that can accumulate over time [223]. These increases in cortisol result in an increase GC production, and GCs can act in opposing manners to E2 on gene expression [224].

However, Nova1 expression in the dorsal hippocampus and hypothalamus was responsive to E2 treatment in the younger animals, where treatment resulted in a significant upregulation of Nova1. We also observed significant increases in Nova1 expression following treatment with the ERα-selective agonist PPT in 18 month old animals. ERα expression has been shown to decrease with age in a brain region specific manner [70, 225]. Therefore these brain region specific changes in Nova1 expression

with loss of circulating E2 may be due to combinatorial loss of ERα and ERβ expression with age in the hypothalamus, but may rely on other factors in the hippocampus.

The loss of E2 over extended periods of time had an interesting effect on Nova1 expression at both the mRNA and protein level. Nova1 significantly increased with longer periods of deprivation in the hypothalamus and ventral hippocampus. However, these increases occurred at different time points in the deprivation paradigm, and Nova1 expression in the hypothalamus becomes nearly undetectable after 12 weeks. This could be due to mRNA stability, as well as protein stability and turnover within this environment. Even more surprising was the effect of E2 in the hypothalamus: Nova1 mRNA expression increased nearly 13-fold when compared to 1 week treated animals. The hypersensitivity to E2 on Nova1 expression may be a consequence of the deprivation paradigm: an influx of circulating E2 to a deprived system may signal to the cell to quickly upregulate primed genes. RNAPII can be preloaded at the promoter of E2-regulated genes ready to initiate transcription [226]. Ligand-bound ERs activate RNAPII at these sites to rapidly induce transcription, and a majority of E2-regulated genes were found to have RNAPII preloaded at many of these promoter sites. There are also rapid non-genomic effects of GPER that could also prime the cells to rapidly upregulate expression of Nova1 during the 3 day regiment of E2 treatment. However, the region-specific differences may be dependent upon the expressional levels of ERs and transcription factors needed to recruit RNAPII.

The altered expression of Nova1 due to aging and E2 deprivation can have a variety of implications on alternative splicing events. Nova1 is critical in the expression of several receptors in the brain, including dopamine receptors. Knockdown studies of Nova1 revealed aberrations in dopamine D2 receptor alternative splicing, resulting in increased expression of the short form of the receptor [139]. While this form of the receptor is important for presynaptic functions like neuronal firing and dopamine release, loss of Nova1 expression decreases expression of the long form of the D2 receptor, which is important for postsynaptic processes [96, 124]. Mouse models of D2 long receptor knockouts have deficits in motivated behaviors and learning [124, 227]. These findings were echoed in studies involving D2 expression in the hippocampus on learning and working memory [228]. Therefore the altered expression of Nova1 may affect the relative expression of these two forms of the receptor that may affect downstream process associated with cognitive function in aged individuals deprived of circulating E2.

Nova1 regulation of ERβ alternative splicing

Up to this point, our findings demonstrated that aging and E2 deprivation alter ERβ alternative splicing and Nova1 expression. These observations led us to pursue the possibility that Nova1 may play a regulatory role in the alternative splicing of ERβ, whether directly or indirectly. Several Nova1 consensus sequences were found when scanning the ERβ pre-mRNA transcript, although a cluster of sequences were found directly upstream of the ERβ2 insert within exon 5 (Figure 6). The canonical ERβ2 54bp insert is located within the intron between exons 5 and 6. This YCAY consensus cluster

is located appropriately upstream of the insert that would result in Nova1 promoting exon exclusion, or loss of the ERβ2 insert. I hypothesized that increased expression of Nova1 would decrease ERβ2 expression, and that Nova1 could mediate this effect by directly binding to ERβ pre-mRNA.

Our findings confirm that Nova1 directly binds to ERβ mRNA, and we also provide evidence that Nova1 overexpression significantly decreases ERβ2 expression. The model proposed in Figure 33 shows how Nova1 enhances exclusion of the ERβ2 insert through excision of the entire intron containing the 54bp. Nova1 recognizes the YCAY sequence cluster located within exon 5. This may also be coordinated by Celf4/5 RBPs as discussed later in this chapter. Upon binding, Nova1 enhances the U1 snRNP binding to the 5' splice site. U2AF locates the branch point to assist the binding of U2 to this site, forming the initial A/E complex. This branch point must be located downstream of the 54bp insert, and protein:protein interaction through Nova1 on other spliceosome components must guide the U2AF to this point. There have not been many studies that have determined the interactions between Nova1 and the spliceosome machinery, however there is evidence of Nova1 interactions with the splicing regulator TIAR [135]. This regulator can form a complex with U1 snRNP, therefore facilitating U2 snRNP binding to the correct branch point.

An equally interesting possibility is that Nova1 could be competing with other splicing factors that may enhance the inclusion of the ERβ2 insert. HnRNPH1 can bind to splice site silencers to inhibit the actions of Nova1, as observed with another hnRNP called hnRNP M on dopamine D2 splicing regulation [139]. It may be that Nova1

binding to these consensus sequences within the exon 5 may block binding of an equally important splicing factor, such as SRSF2, that may enhance exon inclusion, which would lead to a significant increase in ERβ2 expression. SRSF2 binds ESEs to enhance exon inclusion events through recruitment of U2AF to the branch point of the upstream intron to be excluded. Using ESEFinder 3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi), there is an SRSF2 site located within the intron upstream of the ERβ2 insert. Binding of U2 to this branch point would most likely result in excision of the intronic element upstream of the ERβ2 insert, therefore including this insert in the mature mRNA transcript. SRSF2 may also be working in conjunction with hnRNPH1 to mediate ERβ2 inclusion as shown in Figure 34. Future studies on other possible RBP interactions with ERβ pre-mRNA will likely yield the finer details on ERβ alternative splicing regulation.

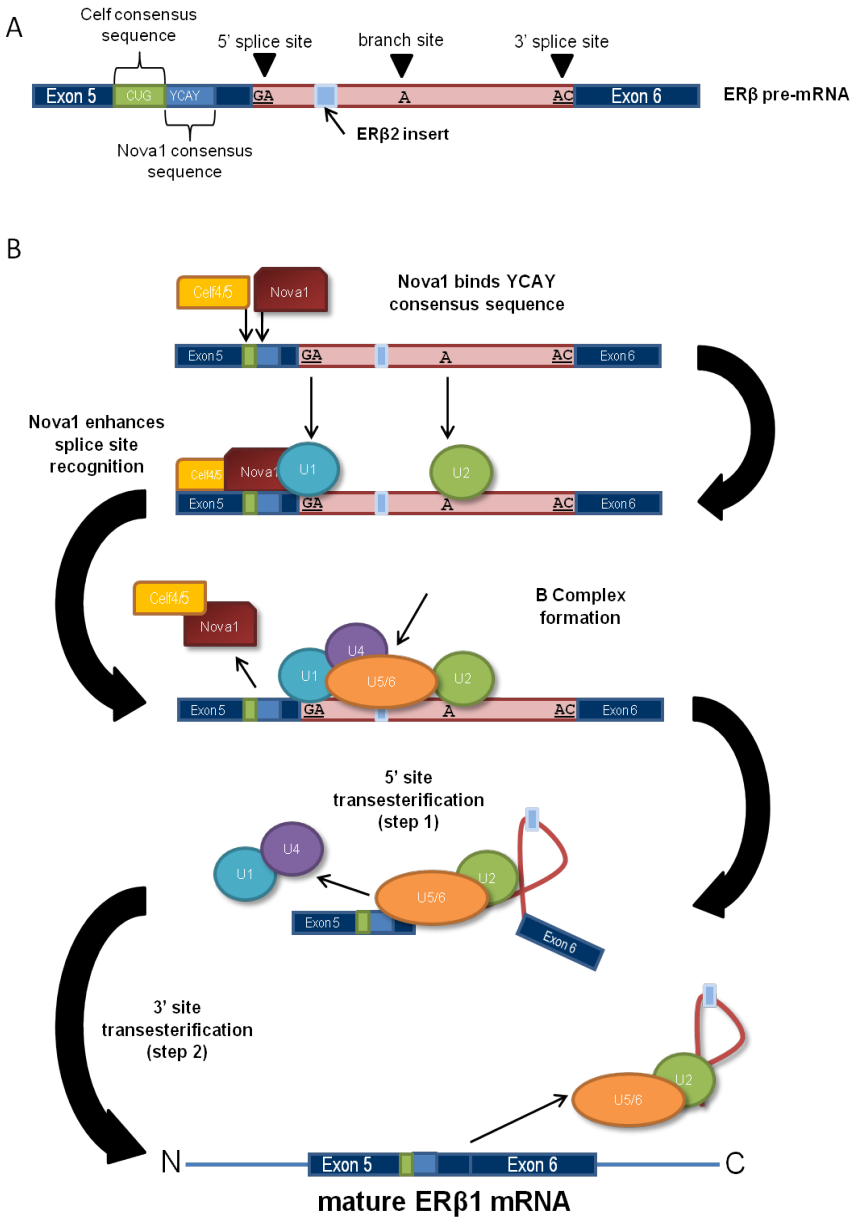

Figure 33. Nova1 promotes exclusion of the 54bp ERβ2 insert. Nova1 binds to YCAY consensus sequences located in exon 5. Celf4/5 coordinate Nova1 binding. Upon binding, Nova1 enhances assembly of the U1 snRNP at the splice site located directly downstream within the intron. The U2 snRNP recognizes the intron branchpoint, binds, and assists in guiding the U4/U5/U6 trimer to form the core spliceosome B complex. Nova1, U1 and U4 snRNPs dissociate, and the remaining complex (C complex) becomes catalytically active to excise the ERβ2 containing intron through successive transesterification reactions. The final step, after the lariot formation, ligates the coding exons together to form the ERβ1 transcript.

RNA-binding proteins

The nature of our E2 deprivation model gives us the ability to study the effects of diminished ovarian hormones on a variety of targets. Due to the fact that we saw changes in the RBP Nova1, we sought to investigate how aging and loss of E2 might affect other integral RBPs. 15 different RBPs were analyzed from all 3 brain regions to determine which factors were highly expressed. We were able to determine that 5 out of these 15 were highly expressed in all 3 brain regions. These factors were hnRNPH1, Ddx17, Rbfox1, Celf4, and Celf5. We hypothesized that aging and loss of E2 would alter expression of these RBPs in a brain region specific manner. In chapter V, we demonstrated that all 5 of these factors change in expression with age and increasingly longer periods of E2 deprivation.

Changes in the expression of these RBPs can amount to varying effects within the brain, mediated primarily by the alternative splicing patterns that they regulate. HnRNPH1, Rbfox1, Celf4, and Celf5 are important RBPs that regulate alternative splicing exon choices. The inclusion or exclusion of an exon can have immediate effects upon the function of the protein, as evidenced by previous discussions on ERβ2. A prominent feature of aging is the accumulation of proteins that may or may not be functional [229]. Aberrant splicing of these transcripts might lead to accumulation of mutant proteins [230]. Increased accumulation of alternatively spliced proteins that undergo misfolding events, as evidenced by Tollervey and colleagues, include 11 disease related proteins associated with neurodegeneration [149, 216].

While we didn't test the effects of these RBPs on the alternative splicing of ERβ, it is possible that altered expression of these factors could directly or indirectly affect this process. We know that hnRNPH1 is important for binding splicing site silencers, and hnRNPs have been previously shown to have opposing effects on Nova1-regulated alternative splicing events. Therefore it is possible that hnRNPH1 could mediate inclusion of the ERβ2 insert in cooperation with the previously mentioned SRSF2 (Fig. 34). Immediately downstream of the ERβ2 insert is a consensus sequence that hnRNPH1 could theoretically bind, impairing assembly of the spliceosome to assemble at this point to excise the insert-containing intron [62]. Rbfox1 can have similar effects by blocking binding of splicing factor 1 (Sf1) to alternative exon branch points [231]. This could result in alternative exon inclusion or exclusion events which may also be possible due to several predicted Rbfox1 binding sites upstream and downstream of the ERβ2 insert [62]. However, as displayed in Figure 35, an Rbfox1 site upstream of the ERβ2 insert would result in its exclusion due to blockage of Sf1 binding (Figure 35B). If Sf1 is able to bind to its consensus site, it mediates binding of the U2 snRNP to the branch point, leading to RNA splicing of this intronic element, leaving the ERβ2 insert intact (Figure 35C). Due to the importance of this factor in neuronal development, it is most likely that maintenance of ERβ2 expression is important for mature neuron differentiation and proper E2 signaling [212]. Lastly, Rbfox1 is an important regulator of hnRNPH1 expression, making its opposing actions on ERβ2 alternative splicing more succinct.

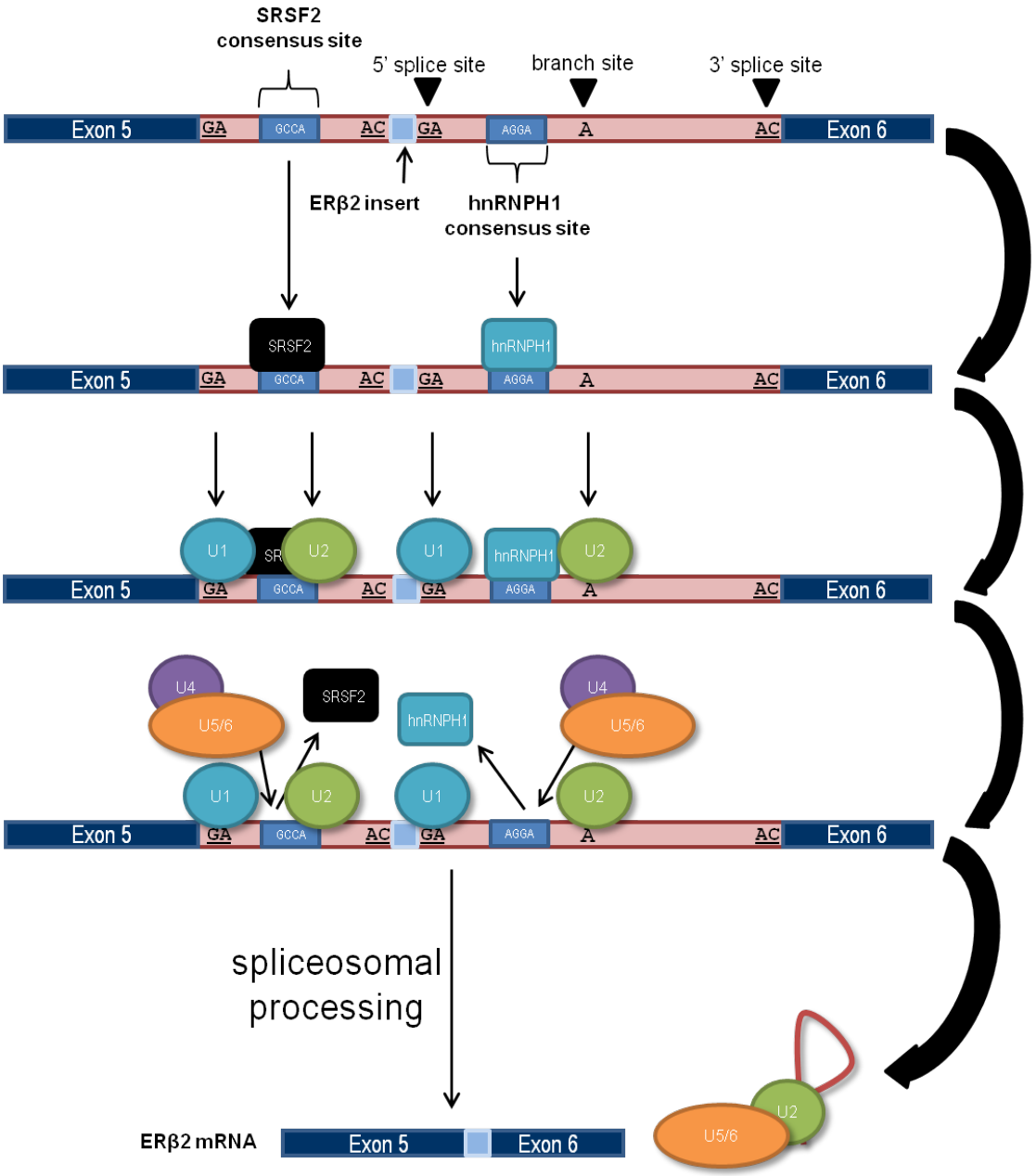

Figure 34. hnRNPH1 and SRSF2 enhance ERβ2 expression. The consensus site for hnRNPH1 is located downstream of the ERβ2 exon, whereas the SRSF2 site is upstream. Binding of these RBPs to the pre-mRNA sequence of ERβ results in the alternative splicing of ERβ to ERβ2 through enhanced spliceosomal assembly on the intronic elements flanking the insert. SRSF2 mediates the removal of the upstream intron while hnRNPH1 mediates the removal of the downstream intron, thus including the ERβ2 insert.

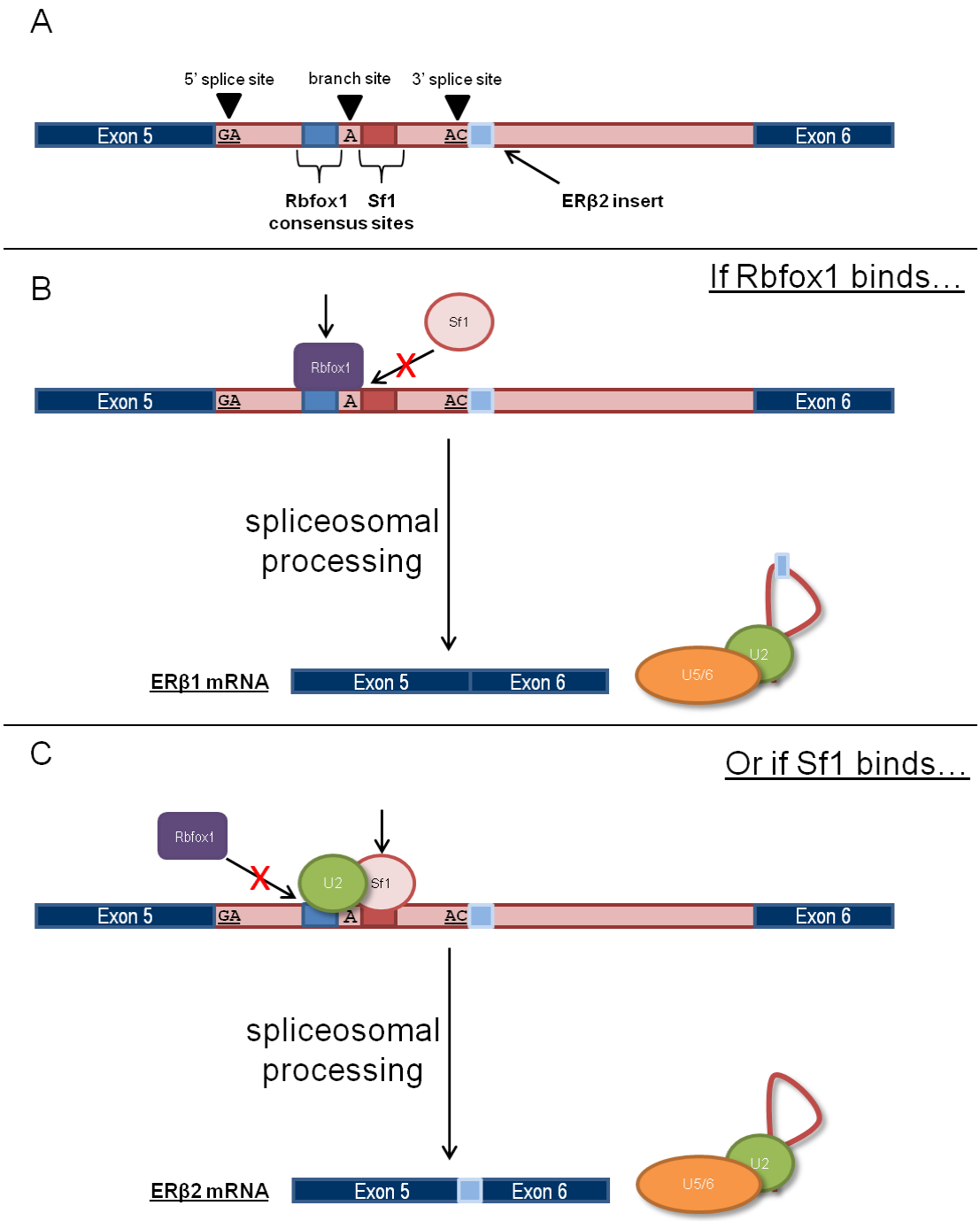

Figure 35. The possible role of Rbfox1 on ERβ alternative splicing. (A) ERβ pre-mRNA transcript contains Rbfox1 and Sf1 consensus sites upstream of the ERβ2 insert. (B) If Rbfox1 binds to its site, it blocks the ability of Sf1 to bind to the alternative branch point, therefore mediating exclusion of the alternative ERβ2 exon. (C) If Sf1 is able to bind its site, it enhances U2 snRNPs recognition of this branch point through U2AF, thereby including the ERβ2 exon within the mature transcript.

Ddx17 is an important regulator of E2 signaling and gene regulation [207, 208, 232]. Like hnRNPH1, Ddx17 is also regulated by Rbfox1, and our data shows similar expressional patterns for both RBPs within our E2 deprivation [212]. Increases in ERβ2 expression can impact E2 signaling, and changes in Ddx17 expression could compound these effects. A subset of E2-regulated genes undergo RNA splicing under the control of Ddx17 and knockdown studies of Ddx17 in MCF-7 cells resulted in decreased alternative exon skipping events in GSK3β, an important kinase that phosphorylates and stabilizes ERs [207]. The alternative exon in the GSK3β2 isoform encodes a 13 amino acid sequence within the catalytic domain which reduces its kinase activity [233]. Interestingly, our findings observed only significant increases in Ddx17 expression, which most likely would result in significant increases in expression of the more catalytically active GSK3β1 isoform. GSK3β1 is important for maximizing sumoylation of ERβ by small ubiqutin like modifier (SUMO)-1 to prevent protein degradation by competing for ubiquitin acceptor sites [234]. Therefore, increased Ddx17 expression through E2 treatment may be an attempt by the cell to protect the expression of ERβ protein from ubiquitination via SUMO-1 in order to maintain ERβ expression similar to the premenopausal state. SUMO-1 can also dictate ERβ transcriptional inhibition by altering estrogen-responsive target promoter occupancy and gene expression, although this has only been observed in breast cancer cells [234]. These findings did show that ERβ protein can accumulate within the nucleus while not affecting ERβ-regulated gene transcription, which could be a way the cell compensates for the loss in E2 is to sequester

as much ERβ as possible for either ligand-independent transcription or ligand-dependent transcription as soon as E2 is available.

Celf4 and Celf5 bind CUG repeat motifs within pre-mRNA transcripts, mediating alternative splicing events [131, 235]. These RBPs are primarily expressed in the nervous system and deficiency in Celf4 has been linked with aberrant excitatory neurotransmission that can lead to seizures, a similar observation in Rbfox1-null mice [129, 130]. CUG motifs are scattered throughout the ERβ pre-mRNA transcript, and could be responsible for many RNA splicing events, including ERβ2. Interestingly, Celf4 sites have been shown to be enriched in Nova1-regulated exons [143]. Therefore, Celf4 and possibly Celf5 could be facilitating ERβ2 exclusion through coordination of Nova1, as there is CUG motif within exon 5 (Figure 33).

RNA-Binding protein	If RBP expression increases
Nova1	ERβ2 decreases
hnRNPH1	ERβ2 increases
Rbfox1	ERβ2 decreases
Celf4/5	ERβ2 decreases
Ddx17	Total ERβ decreases

Table 2. Summary of proposed actions of RNA-binding proteins on ERβ alternative splicing.

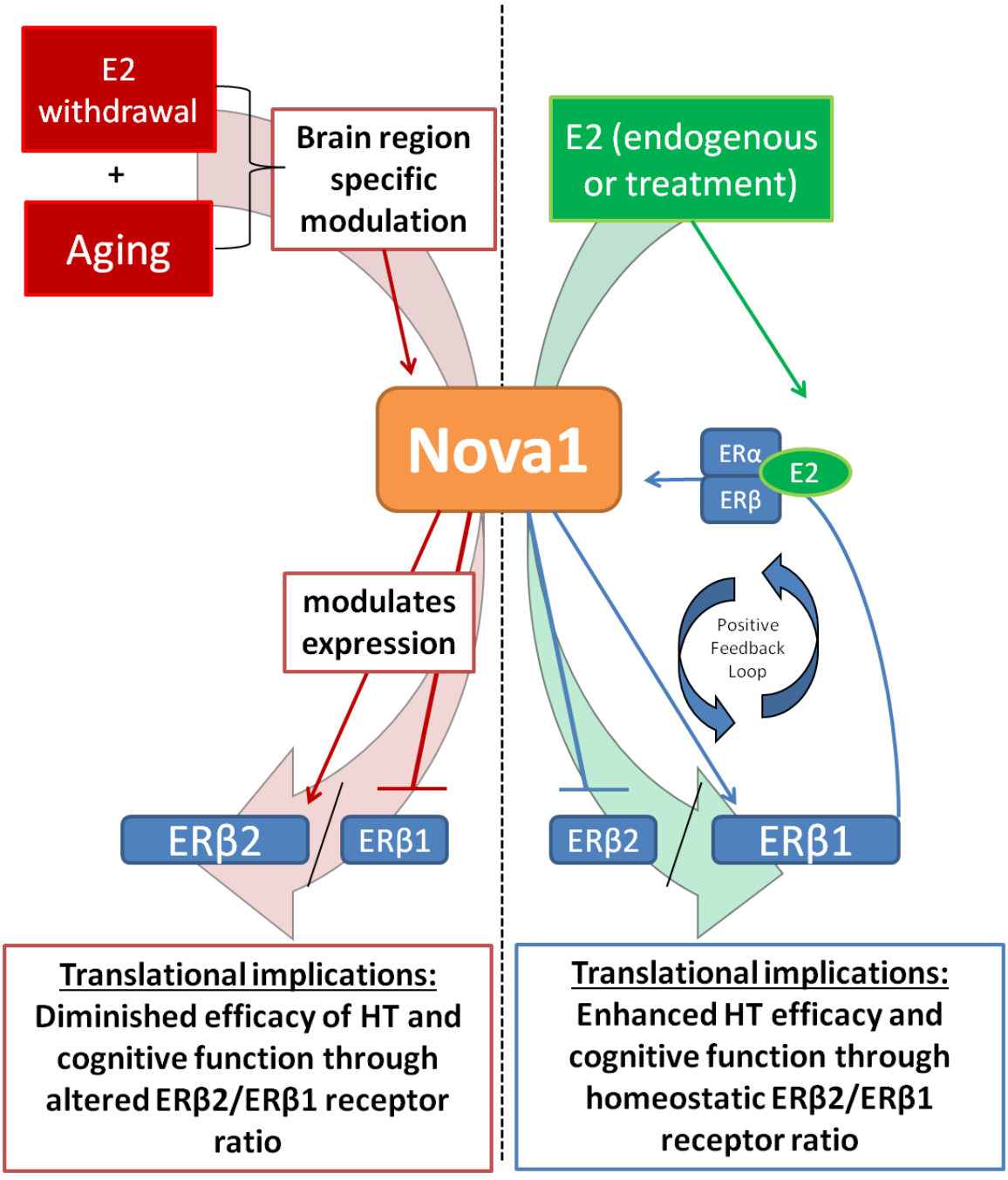

Figure 36. Proposed model from results of aging, E2, ERβ, and Nova1 studies.

Final Thoughts

There may be an answer to the potential issue arising from increased alternative splicing of ERβ in the hippocampus with longer periods of ovarian hormone deprivation: the *trans*-acting factors that act upon the pre-mRNA transcripts of ERβ. Splicing factors like Nova1 are potential therapeutic targets. The findings from chapter IV demonstrate the effects of aging and E2 deprivation on Nova1 expression, and as predicted these changes occur in a region-specific manner. These studies support the hypothesis of my dissertation, and these data provide novel findings that contribute to the field of neuroendocrinology that could be applied to other important studies of aging in the female brain.

CHAPTER VII

GENERAL METHODS

Ethics Statement

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Loyola University Chicago, permit number 2009018.

Animals and deprivation paradigm

Female Fischer 344 rats (3 mo. and 18 mo. of age) were obtained from the NIH aging colony (Taconic, Germantown, NY, USA). In this strain of rat, these ages equate to young adult (3 months) and postmenopausal (18 months) ages as related to humans based on calculations from rodent survival curves, reduced proestrous cycles and estradiol concentrations, and increased cycle durations in 18+ month old Fischer 344 rats [180-183]. Importantly, Fischer 344 female rats had an average lifespan of 26-29 months of age, therefore an 18-month old rat would therefore have lived 62-69% of its life expectancy. By contrast, women in the U.S. have an average life expectancy of 81.2 years (NCHS data brief, no 178, 2014). Therefore, a 55 year old woman is at 67% of her total life expectancy, which is within the same range (62-69%) as an 18 month old Fisher 344 rat.

Experiment #1: Comparison of ERβ2 and splicing factor expression in young (3 mo.) and aged (18 mo.) female rats. Animals were bilaterally ovariectomized (OVX) under vaporized isoflurane anesthesia, as previously described [210]. One week (7 d.) following OVX, animals were administered a subcutaneous injection of vehicle (safflower oil) or 2.5 µg/kg E2 (Sigma, Cat. No. E8875) (N=6-10/treatment group) based on previous studies [210]. Treatments were administered once/day x 3 consecutive days. Animals were humanely euthanized 24 hours after the last treatment, trunk blood was collected, and brains were rapidly removed and flash frozen for tissue processing.

Experiment #2: Effects of varying lengths of E2 deprivation on ERβ2, splicing factors, and RNAPII expression, and RNAPII activity in aged female rats. Aged (18 mo.) female rats were OVX as described above and then separated into 4 groups that would undergo increasingly longer periods of time without E2: 1, 4, 8, and 12 weeks (N = 16-20/deprivation group; Figure. 2) [6, 7, 181]. After the assigned E2 deprivation period, animals received a subcutaneous injection of either vehicle (safflower oil) or 2.5 µg/kg E2 (Sigma, Cat. No. E8875) (N=6-10/treatment group). Treatments were administered once/day x 3 consecutive days. Animals were humanely euthanized 24 hours after the last treatment, trunk blood was collected, and brains were rapidly removed and flash frozen for tissue processing. Net changes in animal weights are reported prior to OVX and 24 hours after final treatment in Table 3.

Groups of ovarian intact animals at all ages 3, 18, 19, 20, and 21 months of age were used to compare to OVX animals (N = 6/age group).

	pre-op weight (g)		weight at sacrifice (g)	
Treatment	**vehicle**	**E2**	**vehicle**	**E2**
1 week	285.4 ± 4.8	278.5 ± 6.8	280.8 ± 4.7	274.0 ± 6.8
4 weeks	268.5 ± 5.4	273.8 ± 9	277.8 ± 3.9	282.0 ± 8.9
8 weeks	272.0 ± 3.6	272.8 ± 5.8	298.2 ± 7.3	301.2 ± 4.8
12 weeks	280.5 ± 7.5	285.9 ± 6.7	312.7 ± 6.5	318.3 ± 4.2

Table 3. Body weights (g) of Fischer 344 rats before ovariectomy procedure and after final treatment. There were no statistically significant differences in body weight between treatment groups.

Experiment #3: Effects of ERβ and ERα selective agonists on ERβ2 and Nova1 expression in aged (18 mo.) female rats. Aged (18 mo.) female rats were OVX as described above. One week (7 d.) following OVX, animals were administered a subcutaneous injection of vehicle (safflower oil), 2.5 μg/kg E2 (Sigma, Cat. No. E8875), 1 mg/kg diarylproprionitrile (DPN, Tocris, Cat. No. 1494), or 0.5 mg/kg propyl pyrazole triol (PPT, Tocris, Cat. No. 1426) (N=6-10/treatment group) These doses were based on previous studies demonstrative selective activation of their respective receptors [236, 237]. Treatments were administered once/day x 3 consecutive days. Animals were humanely euthanized 24 hours after the last treatment, trunk blood was collected, and brains were rapidly removed and flash frozen for tissue processing.

E2 concentration assay and vaginal cytology

Trunk blood was collected and centrifuged at 4500 RPM for 8 mins at 4°C. The plasma samples first underwent a liquid-liquid extraction using diethyl ether to eliminate interfering compounds in the plasma as previously described [238]. Following diethyl ether extraction, samples were reconstituted using sample buffer contained in the 17β-estradiol high sensitivity ELISA kit (Enzo Life Sciences, #AD 901 174), which was used to determine concentration of circulating E2 within intact (N = 6/age), vehicle and E2-treated (N = 6/age/treatment) animals per manufacturer's specifications. Absorbance was measured on a BioTek Synergy HT plate reader. The sensitivity of the assay 14.0 pg/ml. Interassay and intrassay coefficients of variance (CV) were 4.6% and 3.8%, respectively. Cross reactivity with other endogenous estrogens were 17.8%, estrone and 0.9% estriol.

18-mo. animals prior to OVX had low circulating E2 levels (35.0 ± 7.1 pg/ml, N = 6). These low circulating E2 levels corresponded with consistent diestrous-like vaginal cytology as assessed daily for 2 weeks prior to sacrifice (Figure 3A). Circulating E2 levels remained low in 18-mo. old animals treated with vehicle 1-week post-OVX (23.2 ± 2.7 pg/ml, N = 6). E2 treatment elevated levels in OVX animals (56.5 ± 6.3 pg/ml, N = 6), which is within physiological range of women who received hormone replacement therapy during postmenopause (17-75 pg/ml) [34, 172]. Treatment with E2 increased circulating levels consistently within this range throughout the deprivation paradigm (Fig. 3B). E2-treated 3-month old OVX animals (66.7 ± 8.2 pg/ml, N = 8) had levels similar to diestrous intact animals at the same age (69.4 ± 15.2 pg/ml, N = 6).

Quantitative RT-PCR

Flash frozen brains were sectioned at 200 μm on a freezing microtome and regions of interest were microdissected utilizing 0.75 mm Palkovit's brain punch tool (Stoelting Co., Wood Dale, IL). The hypothalamus (-0.8 mm to -3.8 mm relative to bregma), dorsal hippocampus (−2.30 to −4.16 mm relative to bregma), and ventral hippocampus (4.30 to 6.04 mm relative to bregma) were all microdissected for RNA and protein isolation. Brains were split sagittally for tissue collection where one hemisphere was used for RNA isolation and the other for protein isolation in a non-biased manner. RNA isolation was performed on sonicated tissue samples using Trizol reagent (Invitrogen, #15596-026) according to the manufacturer’s specifications. All RNA samples were quantified using Nanodrop spectrophotometry and analyzed for quality by

visualization of the RNA on 1.0% agarose gel. cDNA was reverse transcribed from 1.0 μg RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368814) according to manufacturer's specifications. qRT-PCR for ERβ was performed with TaqMan Gene Expression MasterMix (Applied Biosystems, #4369016) and TaqMan custom FAM-probes that are specific for total ERβ and ERβ2 splice variants on an Eppendorf Realplex4. HPRT was used as a reference gene to check loading and normalize data, and was measured using TaqMan Gene Expression Assay Rn01527840_m1. Total ERβ was measured by TaqMan Gene Expression Assay Rn00562610_m1. The following custom probes sequences were designed and used to detect rat ERβ splice variants: *ERβ2 – TCCTCAGAAGACCCTCAC*, *ERβ1d3 & ERβ2d3 – ATTCAAGGATCCAGGAGA*, *ERβ1d4 – GTCAAGTGTGGCTTTGTG*. The following primer sequences were used to detect the custom probes listed previously: *ERβ2 forward – AGCCTGTTGGACCAAGT, reverse – GCACTCTTCATCTGCGCAAC*; *ERβd3 & ERβd4 forward – GAGAGACACTGAAGAGGAAGC, reverse – TCACGGAACCTTGACGTCGTC.*

qRT-PCR for RNAPII, Nova1, hnRNPH1, Rbfox1, Ddx17, Celf4, Celf5, and HPRT were performed with Fast Start Universal SYBR Green Master Mix (Roche, Cat. No. 04913914001) according to manufacturer's specifications. Primer sequences were designed and produced (Integrated DNA Technologies, Coralville, IA) in the sequences outlined in Table 4. Data was analyzed by delta delta ct method as described previously [239]. Briefly, the cycle detected for the gene of interest is subtracted from the cycle detected for a housekeeping gene, which in this case is HPRT, resulting in the △Ct

value. The average △Ct of the reference group (such as our 3 month old vehicle-treated animals) is subtracted from △Ct of the gene of interest at the data point in question (ie. 18 month old E2-treated) which derives the △△Ct value. Fold change is determined by taking $2^{-\triangle\triangle Ct}$. This Melting curves were performed after qRT-PCR to ensure products of interest were correctly quantified.

Gene of interest	Direction of sequence	Primer sequence (5' to 3')
RNA Polymerase II	Forward	*GCTGGACCTACTGGCATGTT*
	Reverse	*ACCATAGGCTGGAGTTGCAC*
Nova1	Forward	TTACCCAGGTACTACTGAGCG
	Reverse	CCCATCAGGTTTCTGGGA
HPRT	Forward	AGCAGTACAGCCCCAAAATGG
	Reverse	TGCGCTCATCTTAGGCTTTGT
hnRNPH1	Forward	ACTTCCAGGGGAGGAGTACG
	Reverse	GCCTCATCCTCTCAAAGCCA
Rbfox1	Forward	TGCCCGTAAGATCGGTTGGA
	Reverse	ATGTTGTCAACCTGTCCCCC
Ddx17	Forward	GCAACCTGAAGCAGGCTAGA
	Reverse	GGTGCCTTGAGCATAGGTGT
Celf4	Forward	CACCCTTACCCAGCACAGAG
	Reverse	GGGGCAGATGGTAGATGAGC
Celf5	Forward	CAGCGTGAAGGAGTTTGGAGA
	Reverse	GGGAGGAGGGTTGAATGACG

Table 4. Primer sequences for genes of interest.

Cell culture experiments

ERβ-positive hypothalamic-derived cells (GT1-7) were used for *in vitro* experiments (generously provided by Dr. Pamela Mellon, University of San Diego, La Jolla, CA) on ERβ2 expression in camptothecin treated studies [240]. Cells were maintained in Dulbecco's Minimum Essential Medium (DMEM) 50:50 F12 media containing glucose, L-glutamine, sodium pyruvate and 10% fetal bovine serum (FBS). Cells were grown to confluency (70-80%) and media replaced with DMEM 50:50 F12 containing 10% dextran-charcoal stripped FBS for steroid free conditions 48 hours prior to experiments. Cells were treated with either vehicle (DMSO, Fisher Scientific, #D128), 32 ng/µl camptothecin (Sigma, #C9911) alone, or camptothecin plus 100 nM E2 for 6 hours (N=3 plates/treatment group). All cell line experiments were done in triplicate to ensure reproducibility. Cells were then collected for RNA isolation, cDNA synthesis, and qRT-PCR as described above.

ERβ-positive hypothalamic-derived cells (IVB) and human embryonic kidney cells (HEK) were used for all *in vitro* Nova1 overexpression and RNA immunoprecipitation (RIP) experiments (generously provided by John Kaskow, University of Cincinnati and Ed Campbell, Loyola University Chicago, respectively). Cells were maintained in Dulbecco's Minimum Essential Medium (DMEM) media containing glucose, L-glutamine, sodium pyruvate and 10% fetal bovine serum (FBS). IVB cells were grown to confluency (70-80%) and were transfected with GFP-tagged Nova1 in a dose-dependent manner (0.01 - 0.2ug DNA). pcDNA3Nova1 eGFP was a gift from Nicolas Charlet-Berguerand (Addgene, Plasmid # 61275). PcDNA3 empty vector

was used as a negative control for the dose-dependent experiments. For RIP experiments, HEK cells were grown to confluency, then transfected with 3ug Nova1 eGFP and 3ug ERβ2 plasmid. PcDNA3 empty vector was used as a negative control. All transfections utilized Continuum transfection reagent (Gemini Bio-Products, #400-700). All cell line experiments were done in triplicate to ensure reproducibility. Cells were then collected 24-48 hours later for RNA isolation, cDNA synthesis, and qRT-PCR as described above.

RNA immunoprecipitation (RIP)

RIP experiments were performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (EMD Millipore, #17-700) following manufacturer's specifications. Briefly, HEK cells overexpressing Nova1 and ERβ2 were washed and isolated with 10ml ice-cold PBS, then centrifuged at 1500RPM for 5 minutes. An equal volume of RNA Lysis Buffer was used to resuspend pellet, then incubated on ice for 5 minutes in order to lyse the cells. Magnetic beads were pre-incubated with 2.5ug Nova1 antibody and 2.5ug rabbit IgG (EMD Millipore, Cat. No. PP64B) (negative control). Antibody-coated beads were resuspended and then incubated with cell lysates rotating overnight at 4°C. Supernatant was removed by centrifugation at 14K RPM for 30 min. and beads were washed 6 times with RIP wash buffer. Following proteinase K treatment at 55°C for 30 min., supernatant was removed from magnetic beads and placed in a new tube for RNA isolation and cDNA synthesis. RIP analysis used the -2 delta delta CT values for determining enrichment fold-change relative to IgG controls as previously described [241].

Protein isolation and western blotting

Hypothalamic, dorsal hippocampal, and ventral hippocampal tissue isolated from aged female rat brains were placed in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, #78510) supplemented with 7x Protease and Phosphatase Inhibitor cocktail (Thermo Fisher Scientific, #88668). Tissue was sonicated and insoluble material including DNA was pelleted and excluded from the soluble portion of the extracts. 10 µg protein was reduced in 4X laemmli buffer (Bio-Rad, #161-0747) at 95°C for 5 min. and run on 10% SDS-PAGE gel. RNAPII expression and phosphorylation were blotted with N-20 (sc-899, Santa Cruz Biotechnology) and 8A7 (sc-13583, Santa Cruz Biotechnology) antibodies, respectively (Table 5). These antibodies detect the largest subunit of RNAPII that binds DNA and conveys catalytic activity along with the second largest subunit that forms the active center of the RNAPII enzyme [242, 243]. Nova1 protein expression was detected with anti-Nova1 (Upstate/Millipore, Cat. No. #07-637) antibody that targets the N-terminus and has been used previously [128]. Blots were imaged using a ChemiDoc (Bio-Rad, Hercules, CA, USA) and quantified. Both RNAPII bands (2 largest subunits) and Nova1 were quantified together using Image Lab 3.0 Software (Bio-Rad, Hercules, CA, USA). Phosphorylated RNAPII (p-RNAPII), RNAPII, and Nova1 expression were both normalized to β-actin expression with anti-β-actin (13E5) antibody (Cell Signaling, Cat. No. #4970). A description of antibodies and amounts used can be found in Table 5. The ratio p-RNAPII/RNAPII to determine activity of RNAPII relative to its expression [244].

Peptide/protein target	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
RNAPII	Pol II (N-20)	Santa Cruz Biotech (sc-899)	Rabbit polyclonal	1:1000
p-RNAPII	p-Pol II (8A7)	Santa Cruz Biotech (sc-13583)	Mouse monoclonal	1:500
Nova1	anti-Nova1	Upstate/Millipore (07-637)	Rabbit polyclonal	1:500
β-Actin	β-Actin (13E5)	Cell Signaling (4970)	Rabbit monoclonal	1:1000

Table 5. Antibody table. RNAPII expression and phosphorylation were blotted with N-20 (sc-899, Santa Cruz Biotechnology) and 8A7 (sc-13583, Santa Cruz Biotechnology) antibodies, respectively. These antibodies detect the largest subunit of RNAPII that binds DNA and conveys catalytic activity along with the second largest subunit that forms the active center of the RNAPII enzyme. Nova1 expression was blotted with rabbit anti-Nova1 antibody (07-637, Upstate/Millipore). Blots were imaged using a ChemiDoc (Bio-Rad, Hercules, CA, USA) and quantified. Both RNAPII bands (2 largest subunits) Nova1, and β-actin were quantified together using Image Lab 3.0 Software (Bio-Rad, Hercules, CA, USA). Phosphorylated RNAPII (p-RNAPII) and RNAPII expression were both normalized to β-actin expression and then calculated as a ratio (p-RNAPII/RNAPII) to determine activity of RNAPII relative to its expression. Nova1 expression was normalized to β-actin expression.

Statistical Analysis

Significant interactions were assessed by two-way ANOVA with age x treatment as factors (Experiment 1: young vs. aged), or time x treatment (Experiment 2: E2 deprivation paradigm) using Systat 13 software (Systat Software Inc, San Jose, CA, USA), followed by Tukey's Honestly-Significant-Difference post hoc analysis to determine significant differences among groups where $p<0.05$. Different letters and/or symbols denote statistically significant differences between groups. A separate Tukey's Honestly-Significant-Difference post hoc test was utilized within groups that showed a statistically significant main effect of age and/or treatment. A one-way ANOVA followed by Tukey's Honestly-Significant-Difference post hoc analysis was conducted to determine significant difference ($p<0.05$) between selective ER agonists (DPN and PPT; Exp. #3) where treatment was the main effect in the hypothalamus of 18-mo. animals and *in vitro* studies. A paired t-test was used where noted if significant main effects or interactions were not observed. All data are presented as mean +/- SEM.

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Vita

Cody Shults was born in Binghamton, New York to Eric and Kim Shults, and raised in Homosassa Springs, Florida. Before attending Loyola University Chicago, he attended the University of South Florida, Tampa, where he earned a Bachelor of Science in Biology in 2009. At South Florida, Cody was awarded multiple scholarships and also participated in the Undergraduate Research Program.

Cody joined the Integrative Cell Biology Program and joined the laboratory of Toni R. Pak, PhD. Cody has several publications, as both primary and contributing author, and has given talks at both local and national scientific meetings. Cody won second place in the Graduate Student Oral Presentation Competition at the Chicago Society for Neuroscience Annual Meeting in 2014.

In June 2014, Cody married Jill Shults, PhD, who has just recently completed her PhD in the same program at Loyola. Cody and Jill live in Chicago, Illinois.