2014

The Molecular Components of Estrogen Receptor Beta (ERβ) Signaling in Neuronal Sytems

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THE MOLECULAR COMPONENTS OF ESTROGEN RECEPTOR BETA (ERβ) SIGNALING IN NEURONAL SYTEMS

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

PROGRAM IN INTEGRATIVE CELL BIOLOGY

BY

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CHICAGO, IL

MAY 2014

ACKNOWLEDGEMENTS

To my advisor/mentor/friend/confidant, Dr. Toni Pak: I could not have asked for a better mentor. You have given me all the tools that I needed to succeed in grad school and hopefully beyond. I've experienced first-hand how your advice and guidance has gone far beyond expectations of a graduate mentor. I look forward to the future, where I imagine we will sit together and relive great memories, discuss experiments and debate our craziest vortex-of-insanity ideas over a martini – or two. There aren't enough pages in this document for me to thank you for all of the insight, laughs and motivation you have given me.

To my dissertation committee: Dr. Clodia Osipo, Dr. Nancy Zeleznik-Le, Dr. Wendy Kartje, and of course, our prized male member, Dr. James Roberts: Thank you all for having faith in me and most of all for pushing me to the pinnacle of my intellect, for this has shown me that I'm capable of more than I thought. Each meeting, I felt more and more that you all were the perfect committee: Giving me valuable recommendations, reminding me what I need to do to succeed and most importantly – never letting me off too easily! The only problem with being the best possible combination of people for a committee is that I will certainly call on you in

the future for advice, letters and to read my grant proposals. I am beyond grateful for the support and guidance I have received from each of you.

To my lab mates, past and present: You all are the best and brightest and more importantly, some of my very best friends. The amazing dynamic of our group could never be replicated.

Finally, thank you to the ICB program, and the Physiology department: Dr. Le, thank you for convincing the rest of the CBNA admissions committee that I was a candidate worthy of acceptance into the program! Dr. DeTombe, thank you for allowing me to use your playground of equipment. Kay and Joe, thank you for your help with things I couldn't figure out on my own. Last and certainly not least, thank you to Kim Stubbs for every single thing you've done to help me, as an administrator, but moreso as my dear friend.

To Ken, Nova, Mom, Dad, FAS, Deanna, Pearl and TJ

Science is a way of thinking more than it is a body of work.

Carl Sagan

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AP-1	activator protein-1
AVP	arginine vasopressin
ATLAS	Adjuvant Tamoxifen Longer Against Shorter
BVA	biological variance analysis
BERKO	ERβ knockout mice
CEE	conjugated equine estrogens
CORT	corticosterone
CRH	corticotrophin releasing hormone
DIA	differential in-gel analysis
DHT	dihydrotestosterone
ERE	estrogen response element
E_2	17β-estradiol
ENO	alpha enolase
ERβ	estrogen receptor beta
ERα	estrogen receptor alpha
ET	estrogen therapy
GAPDH	glyceraldehyde-3-phosphate
GELS	gelsolin

HNRNP	heterogeneous nuclear riboprotein
HSP	heat shock protein
HT	hormone replacement therapy
KEEPS	Kronos Early Estrogen Prevention Study
LBD	ligand binding domain
LC-ESI- MS/MS	liquid chromatography-electrospray-tandem mass spectrometry
MBP	c-Myc binding protein
MeA	medial amygdala
MIRAGE	Multi-institutional Research in Alzheimer's Genetic Epidemiology
MPA	medroxyprogesterone acetate
PI3K	phosphoinositol-3-kinase
PTM	post-translational modification
PVN	paraventricular nucleus
SR	steroid receptor
SUMO	small ubiqutin like modifier
T	testosterone
VCP	valosin containing protein
WHI	Women's Health Initiative
2D-DIGE	two dimensional-differential gel electrophoresis
3β-diol	5α-androstane-3β, 17β -diol

ABSTRACT

With increasing life expectancy, women are now living upwards of 50 years without circulating estrogens, therefore, it is essential to investigate how the brain is changed by estrogen deprivation and also how aging influences these changes. The Women's Health Initiative (WHI) study spurred rigorous debate regarding estrogen therapy for postmenopausal women due to dichotomous effects of estrogens in menopausal and post-menopausal women. Meta-analyses of the WHI study revealed that after circulating estrogens are depleted for many years re-exposure may cause aberrant, negative health effects, indicating that there is an age-related 'switch' in estrogen signaling around menopause. These age-related effects of HT expose a gap in scientific knowledge as to how estrogen receptors, ERα and ERβ signal when the body is deprived of estrogen and under the natural context of aging. ERβ regulates a number of genes governing grievous symptoms menopausal symptoms such as anxiety, depression, and cognitive decline. Further, alternative splice variants derived from ERβ do not bind estrogens as well as ERβ1, and importantly, ERβ splice variants increase in the brain with age. I hypothesized that altered splice variant signaling contributes to a switch in estrogen signaling around the time of menopause. Herein, I demonstrate that human ERβ splice variants are constitutively active transcription factors, supporting my hypothesis. I also describe

another contribution to ERβ functions in the brain resulting from age and E2-dependent changes in protein:protein interactions with ERβ. This dissertation reveals 1) the varied transcriptional effects of ERβ alternative splice variants, 2) identification of novel ERβ protein interaction partners, 3) how these interactions and the expression of these proteins change as a factor of age and 4) the effects of changes in these interactions on gene transcription which could be part of the switch in molecular signaling of estrogens at the time of menopause.

CHAPTER I

STATEMENT OF THE PROBLEM

With perpetual advances in medical research throughout the last century, the average life expectancy for women in the United States has increased ~62% (from 51 to 81 years of age, 1910 -2010) and continues to climb. This phenomenon is creating a large population of elderly people and an unprecedented set of issues for geriatric care. Adding to this problem, the age at which women experience reproductive senescence is not increasing at the rate of life expectancy, creating a group of women that will spend one-third of their life in a post-menopausal state without high circulating levels of ovarian hormones.

The actions of ovarian hormones, thought to be primarily important for reproductive processes, are often overlooked in the aged population. However, the primary circulating estrogen, 17β-estradiol (E_2) has wide-spread effects on homeostasis, stress responses, bone regeneration, neuroprotection, cognition, cardiovascular disease and immune responses – all of which are concerns especially for aging women. Therefore the WHI conducted a large-scale, 15 year study that was aimed at directly investigating the impact of ovarian hormone replacement on a variety of health issues. The study was suspended prematurely in 2002, due to

negative health consequences that were arising as a direct result of the study. Participants that received combined treatment of conjugated equine estrogens (CEE, a mixture of estrogenic compounds) plus medroxyprogesterone acetate (MPA) experienced the most detrimental effects, however the CEE alone group experienced increased risk for stroke and blood clots, and no difference in myocardial infarction, colorectal cancer and breast cancer risk (Rossouw *et al.*, 2007; Rapp *et al.*, 2003). The only positive effect of estrogens in this study was a reduced risk for bone fracture. These results came as a complete surprise to the medical and basic science communities, and were soon dissected to understand the discrepancies between previous studies and the newest and largest study to evaluate hormone therapy.

Arguably, the greatest discovery from post-study analyses was a dichotomous effect of aging and/or length of estrogen deprivation. WHI participants were on average 63 years of age, approximately 10 years past the menopausal transition, with about 20% of participants over the age of 70. Secondary analysis of the data from this study suggests that early intervention is critical to the benefits of estrogen therapy (ET) as participants who were between 50-59 had a reduced absolute risk of coronary heart disease (CHD), stroke and total mortality compared to older postmenopausal participant (Rossouw *et al.*, 2007; Rapp *et al.*, 2003). Finally, the Kronos Longevity Research Institute (KLRI) conducted a 4 year double blind placebo-based study that evaluated the effects of early ET for peri-menopausal women on cardiovascular disease, cognition and mood. In October of 2012, The

Kronos Early Estrogen Prevention Study (KEEPS) published positive results with regard to mood and CHD, supporting post-study WHI results inferring that ET is effective when began early on in the menopausal transition. Cumulatively, the data from the WHI and KEEPS studies identify a very important period of time for which ET can be beneficial, otherwise known as the 'timing hypothesis'.

One issue that these data cannot address is the factor of aging. It remains unclear if advanced age and/or the length of E_2 deprivation is causing dichotomous effects of HT. In either case, the wealth of clinical data suggest a 'switch' in the molecular mechanisms by which estrogen signaling takes effect, but the basic science data on this subject are lacking. *This dissertation is aimed at understanding some of the molecular mechanisms regulating estrogen receptor beta (ERβ) signaling in an aged, estrogen-deprived neuroenvironment to further our knowledge on the effects of HT in the growing population of women living without ovarian hormones.*

CHAPTER II

ESTROGEN SIGNALING AND THE AGING BRAIN: CONTEXT-DEPENDENT CONSIDERATIONS FOR POSTMENOPAUSAL HORMONE THERAPY
(MOTT, NN ET AL., ISRN ENDOCRINOLOGY, JUL 7;2013:814690)

Literature Review

According to the CDC (2008), the average lifespan for women in the US was ~81 years of age. While the average lifespan has been steadily increasing over the past century (~48 years in 1900), the average age at which reproductive senescence, menopause, occurs has remained relatively constant at approximately 51 years of age (Bengtsson *et al.*, 1979; Singh *et al.*, 2002). Including the prepubescent years, this leaves women living about half of their lives without high levels of circulating ovarian hormones. The two primary ovarian hormones are E_2) and progesterone, both of which are required for female reproduction. Many positive anecdotal experiences are reported during times in the reproductive cycle when E_2 is high, sparking further investigation into the role of E_2 in various non-reproductive processes, including those pertaining to cognition and mood. The vast majority of basic science studies have described positive effects of E_2 on cognitive processes at a molecular level, and importantly, older postmenopausal females, exhibit significant deficits when performing tasks that require proper cognitive function including use of working memory, attentional processing, and executive function

(Verhaeghen and Cerella, 2002; Wroolie *et al.*; Sherwin, 1994a; Sherwin, 1996; Sherwin, 1994b; Phillips and Sherwin, 1992). The natural aging process is coincident with menopause, which confounds studies attempting to differentiate between the molecular mechanisms specific to menopause versus aging. Therefore, studies examining the physiological and molecular functions of estrogen receptors during periods of estrogen deprivation with respect to natural aging are requisite to understanding how reintroducing estrogens in aged postmenopausal women will affect neurological processes. In spite of the wealth of studies investigating the effects of HT on relevant health concerns, there are still very few conclusive arguments for or against HT to ameliorate neurological issues. Moreover, it is very likely that the actions of estrogens regulate opposing processes depending upon brain region and genetic composition of neurons involved, creating complex issues regarding the lack of specificity of E_2 treatment. Nevertheless, some insight into general functions of E_2 in the brain can be gleaned from existing data that demonstrate 1) there is a critical window of time surrounding menopause for which HT can be beneficial, suggesting aging is an important factor, 2) progestins are not likely to be beneficial for cognitive and affective neurological issues, and 3) the type of estrogen used may be crucial. Given these important conclusions this review will focus on the molecular mechanisms of E_2 signaling, with specific attention to the role of estrogen receptor β (ERβ) in the brain, and how variables that might contribute to these signaling patterns can be altered by age.

The menopausal transition: E_2 *decline and health concerns*

Menopause is defined by the Mayo clinic as "the permanent end of menstruation and fertility, occurring 12 months after your last menstrual period." Menopause is marked by a reduced oocyte number attributable to progressive atresia of ovarian follicles, and declining circulating levels of E_2 and progestins. The peri-menopausal transition is typically 4-8 years, during which, most women experience symptoms including, hot flushes, night sweats, mood swings, sleep disturbances, vaginal dryness and atrophy, urinary incontinence, most of which are alleviated by hormone (E_2) replacement therapy (HT/ET). Until recently, a great deal of evidence suggested that estrogens have positive effects on cognition, neuroprotection, memory, anxiety, depression, bone and cardiovascular health (Lindsay *et al.*, 1976; Rossouw *et al.*, 2007; Zhang *et al.*; Sherwin, 1994a; Krezel *et al.*, 2001; Ostlund *et al.*, 2003).

The paramount studies to present negative consequences of HT were the Women's Health Initiative (WHI), and ancillary studies including the Women's Health Initiative Study on Cognitive Aging (WHISCA) and the Women's Health Initiative Memory Study (WHIMS). Data from these studies showed that a combination therapy of conjugated equine estrogen/medroxyprogesterone acetate (CEE/MPA) increased risk for mild cognitive impairment and decreased global cognitive functioning, but CEE alone did not have any significant effect on cognitive functioning (Shumaker *et al.*, 2003; Shumaker *et al.*, 2004; Rapp *et al.*, 2003). Post-

study analyses have revealed many confounding factors in the WHI studies ranging from the choice of a reference group (previous HT users) to the age of participants and the choice of ET used (CEE) (Henderson *et al.*, 2005; Garbe and Suissa, 2004; Wroolie *et al.*), as well as the use of MPA, which has been shown to have adverse effects on memory after one dose in adulthood (Braden *et al.*). While the WHI studies showed negative or neutral effects of ET, many other basic science and observational studies have shown just the opposite. The Kronos Early Estrogen Prevention Study (KEEPS) recently announced findings that suggested E_2 therapy had a positive effect on mood and memory. Participants receiving CEE showed significant improvement in symptoms of depression, anxiety and a trend toward reduced feelings of anger/hostility. Importantly, CEE treatment or Premarin® (Wyeth-Ayerst, Philadephia, PA) is a mixture of several estrogenic compounds, but primarily estrone sulfate and ring B unsaturated estrogens such as equilin and equilinen, which can differentially activate ER isoforms as compared to E_2 alone (Bhavnani *et al.*, 2008) Participants receiving CEE self-reported a trend toward better recall of printed materials as compared to placebo, and women using transdermal E_2 tended to report fewer memory-related complaints. Another study performed a meta-analysis of 36 randomized HT clinical trials (RCT) focusing on cognition (Hogervorst and Bandelow). The length of treatment, type of memory, variety of hormone, and age of the participant were all variables that drastically altered the outcomes of each trial. Results from the meta-analysis indicated that

verbal memory was most often affected by HT, and younger women tended to have a better outcome in this category. There was also a trend toward worse outcomes on memory tests in patients treated with CEE treatment alone compared to those treated with biologically identical E_2. Moreover, treatment with estrogens alone (i.e. absent co-treatment with progestins) were overall associated with positive results on memory tests. In conclusion, data from these clinical trials have revealed the importance of using bioidentical hormones for HT and that downstream signaling processes for memory and mood can be affected by the choice of estrogen and/or combination of hormones used as therapeutics.

Estrogen receptor signaling

Estrogen signaling is mediated primarily through two receptors (ERα and ERβ). ERs are class I members of the nuclear hormone superfamily of receptors, deemed as a ligand inducible transcription factors (Mangelsdorf *et al.*, 1995). Classically, ERs were thought to be localized in the cytoplasm bound to intracellular chaperone proteins until induced by ligand to translocate to the nucleus, according to the two-step hypothesis coined by Elwood Jensen (Jensen *et al.*, 1968). Following ligand binding, ERs undergo a conformational change that allows for dimerization, translocation to the nucleus and DNA binding or association with other transcription factors to regulate gene transcription; however, we now know that ER signaling is not as dogmatic as previously thought.

For example, ERs are involved in other 'non-genomic' molecular functions including RNA processing, second-messenger signaling cascades and rapid dendritic spine formation in neurons. Of particular importance in the brain, the discovery of rapid signaling processes implicates E_2 as a neuromodulator, however local synthesis of E_2 has been the subject of fervent debate. While it is likely there is *de novo* synthesis of E_2 within the parenchyma, due to technical challenges, the exact levels and changes with age and circulating hormones have yet to be identified (Naftolin *et al.*, 1996; Roselli *et al.*, 1998). It is also difficult to determine how local E_2 may affect ER action. Most reports suggest an implicit role for local E_2 at the synapse and membrane (Balthazart and Ball, 2006), but whether nuclear/genomic activities of ERs are affected has yet to be established. Recent data from our laboratory demonstrate that E_2 can alter miRNA expression (Pak *et al.*), and others have shown that ERα can associate with miRNA processing enzymes such as Drosha (Yamagata *et al.*, 2009). Data from our laboratory (unpublished observations) and others have shown that ERs are involved in alternative splicing processes, and one study has demonstrated direct interaction of phosphorylated ERα with splicing factor (SF)3a p120 that potentiates alternative splicing through EGF/ E_2 crosstalk (Masuhiro *et al.*, 2005). These relatively novel ER functions may be explained by examining well-studied components of classic NR signaling such as the structural properties of the receptors.

Structural contributions to ER activity

Class I nuclear receptors (NRs) including ERα and ERβ have a characteristic structure comprised of five functional domains labeled A-E, and a sixth domain (F) unique to ERs (Fig. 1). The A/B domain contains an activator function-1 (AF-1) like domain that allows for associations with coregulatory proteins and other transcription factors. Notably, the A/B domain is the least conserved domain between ERα and ERβ (17% homology), and may be responsible for the observed ligand-independent actions of ERβ (Tremblay *et al.*, 1999a). The C domain, is a DNA binding domain that allows the receptor to bind a specific DNA sequence called an Estrogen Response Element (ERE) to regulate transcription of genes containing this sequence within their promoter region. Two zinc fingers forming a helix-loop-helix structure allow for appropriate spacing (3 nucleotides) between an inverted hexameric palindromic repeat that is described as the canonical ERE. The exact nucleotide sequence of hormone response elements can vary and in part, dictate the affinity a NR has to regulate a particular gene (Meijsing *et al.*, 2009). The D domain is a hinge-like region that allows the receptor to undergo a conformational change once activated and also contains a nuclear localization sequence. The best-studied region of ERs is the E domain, also referred to as the ligand binding domain (LBD). Characterization using x-ray crystallography has shown that the LBD consists of 12 ordered alpha helices that are essential for conferring ligand specificity (Bourguet *et al.*, 2000). The orientation of helix 12 is critical to the conformation NRs adopt once

bound to a particular type of ligand, and ultimately influence the ability of the receptor to bind other proteins and activate gene transcription. Helix 12 contains the core residues of the activator function 2 (AF-2) domain, a short amphipathic conserved alpha helix that interacts with coregulatory proteins through an LxxLL motif. Adjacent to the AF-2/E domain is the less characterized F domain that is unique to ERs. ERα has a larger F domain than ERβ, and the two receptors only share about 18% homology within this region. ERα dimerization and interactions with coregulators are altered when the F domain is deleted or modified, demonstrating that the F domain is a relevant structure for ERα transcriptional regulation, but a clear role for this domain for ERβ has yet to be determined (Koide *et al.*, 2007; Skafar and Koide, 2006). Importantly, naturally occurring human ERβ splice variants have altered E and F domains, which can affect hormone responsiveness in tissues that express these variants.

While the overall sequence homology between ERα and ERβ is greater than 60%, the specific gene targets of each receptor appear to be vastly different. For example, a variety of cancer cell models have identified an anti-apoptotic, proliferative role for ERα, whereas ERβ tends to promote apoptosis and regulate anti-proliferative genes (Chang *et al.*, 2006; Zhu *et al.*, 2004; Petersen *et al.*, 1998; Helguero *et al.*, 2005). It is well known that ERα and ERβ are readily able to form heterodimers when expressed in the same cell, adding another layer of complexity to the regulation of estrogen responsive genes. ERα and ERβ both bind EREs, but

the affinity for one receptor or the other can depend highly on the specificity of the DNA sequence being regulated and the ligands present (Kulakosky *et al.*, 2002; Grober *et al.*; Vivar *et al.*). Therefore, it is important to consider the overlap in ERα and ERβ preferred response elements when both receptors are expressed in the same system.

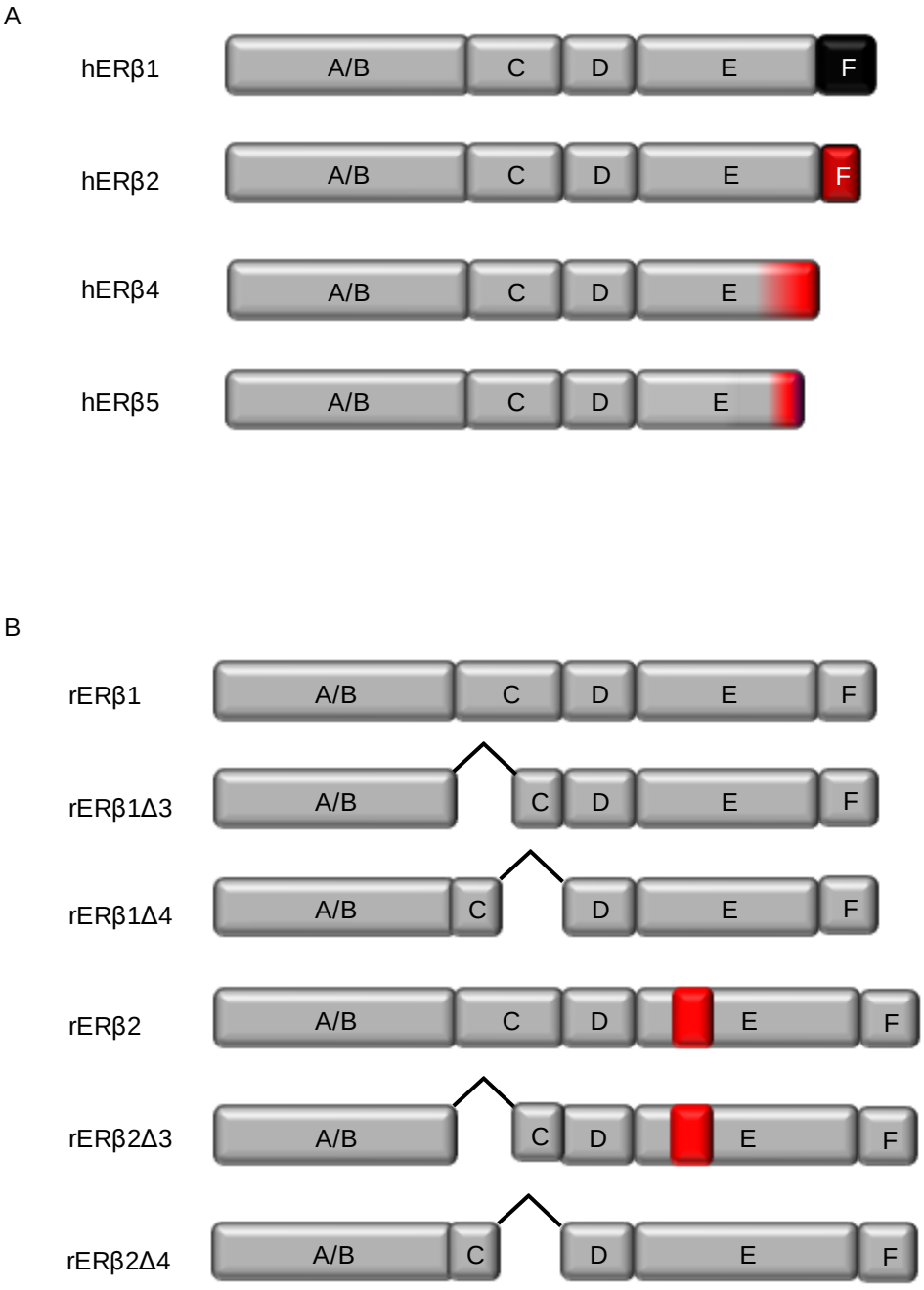


Figure 1. Representative image of domains within human and rat ERβ splice variants. Human ERβ splice variants (A) contain truncations and changes in amino acid sequence in the C-terminus E and F domains. Rat ERβ splice variants (B) contain an 18 amino acid insert in the LBD/E domain and/or exon 3/4 exclusions in the DNA binding domain.

Expression of ERs in the brain: A complex story

The principal determinant of E_2 action is the expression of ERα, ERβ, their alternatively spliced variants, or some combination of each, which is cell-type specific even within distinct brain nuclei. ER expression has been studied extensively, yet there are few definitive statements that can be made about the regulation of ERβ expression. It can be noted that ER expression profiles can vary throughout the life span, in particular when there are dramatic changes in circulating hormone levels, such as puberty and menopause (Fig. 2). Not only can ER expression vary dependent upon sex, age and E_2 treatment, but these factors can also direct subcellular localization, which ultimately dictates ER functions. Accordingly, contextual studies that map the exact cellular expression patterns of each receptor and their splice variants are a critical first step in creating a comprehensive examination of E_2 -regulated processes in any system.

The female vertebrate reproductive organs tend to be dominated by the expression of ERα, whereas ERβ is expressed largely in non-reproductive tissues. ERβ was first cloned from prostate tissue (Kuiper *et al.*, 1996), and has since been shown to have the highest levels of expression in the central nervous system and cardiovascular tissue, as well as lung, kidney, colorectal tissue, mammary tissue and the immune system (Kuiper *et al.*, 1997). Consequently, some of the most prominent phenotypic problems observed in mice lacking a functional ESR2 gene (βERKO mice) are neurological deficits. By contrast, ERα knockout mice have no

gross brain-related phenotypes, but exhibit decreased E_2-mediated neuroprotection following an ischemic event (Dubal *et al.*, 2001). Overall, the phenotypes observed in ERα- and ERβ-null mouse models suggest that ERβ is potentially more important for mediating non-reproductive E_2-governed processes than ERα.

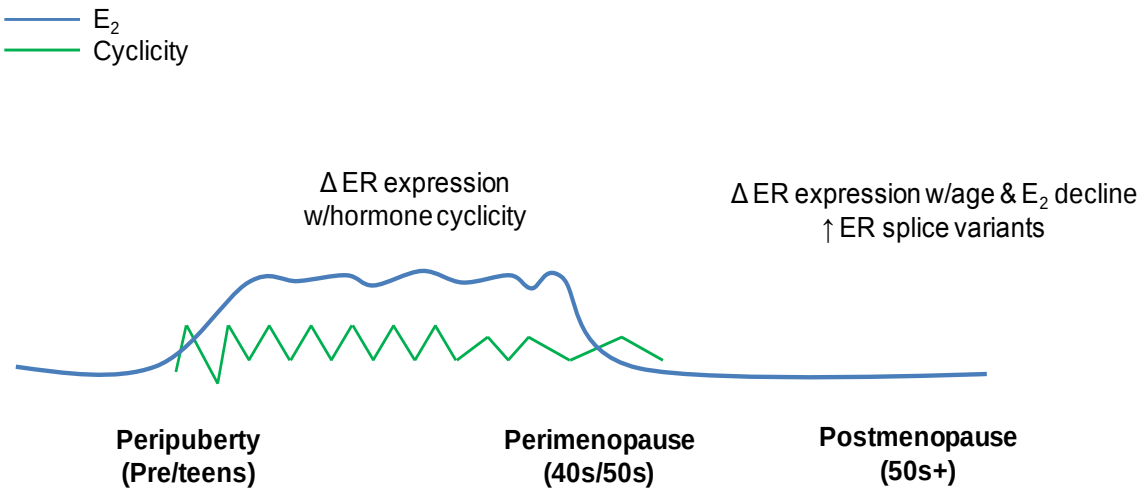


Figure 2. Timeline showing factors affecting ER gene expression throughout the female life span. Brain ER gene expression patterns are altered with age, sex and exposure to circulating hormone. Circulating hormones fluctuate with age, most dramatically at the time of puberty and menopause thereby contributing to changes in ER gene expression. Additionally, alternative splicing increases with age, thus potentially diversifying the ER gene expression profile.

ERα and ERβ are coexpressed in some regions of the hypothalamus, such as the medial amygdala (MeA), bed nucleus of the stria terminalis (BNST) and the periaqueductal grey area. However, ERα is predominant in hypothalamic nuclei that control reproduction, sexual behavior and appetite (e.g., arcuate (ARC), medial preoptic (MPoA), ventromedial(VM)) but ERβ is the predominant isoform in the non-reproductive associated nuclei (e.g., paraventricular (PVN), supraoptic (SON) and suprachiasmatic (SCN)) as well as the hippocampus, dorsal raphe nuclei, cortex and cerebellum (Shughrue *et al.*, 1998; Shughrue *et al.*, 1997). In the hippocampus, mRNA and protein for both ERs have been detected and are well established as mediating both genomic and non-genomic processes (Milner *et al.*, 2001; Milner *et al.*, 2008; Milner *et al.*, 2005). Nuclear and extranuclear ERβ mRNA and immunoreactivity (IR) have been detected in principal cells as well as in many other nuclei of cells within the ventral CA2/3 (Milner *et al.*, 2001; Shughrue *et al.*, 1997). Although not as prevalent as ERβ, ERα has also been detected in the hippocampus, primarily within GABAergic interneurons (Milner *et al.*, 2001; Milner *et al.*, 2005).

ER expression is also often found to be sexually dimorphic. As one would expect, many regions of the hypothalamus exhibit a great deal of sexual dimorphism due in part to differences in sexual behavior and regulation of gonadotrophic hormones, but regions such as the BNST also display some sex-related differences in ER expression. For example, ERα in the BNST can be induced in somatostatin positive neurons of male, but not female, rats (Herbison and Theodosis, 1993). ERs

have also been shown to be sexually dimorphic in the developing rodent hippocampus, but not in adults (Kalita *et al.*, 2005; Ivanova and Beyer, 2000). However one report identified ERβ mRNA in the adult female, but not male, rhesus macaque basal ganglia and hippocampus (Pau *et al.*, 1998). Importantly, a lack sexually dimorphic regional ER expression does not preclude differential responses to estrogens, as other effector molecules can alter estrogen-responsive processes.

Expression of ERs can vary not only with chromosomal sex, but also in response to the hormonal milieu. For instance, it is well accepted that ERα expression is autoregulated by E_2, primarily through proteosomal degradation, (Wijayaratne and McDonnell, 2001) but also perhaps on a transcriptional level by E_2-bound ERβ (Bartella *et al.*). The ERβ gene (ESR2) promoter region has not been extensively characterized, but it has been shown to contain E_2 responsive *cis* sequence binding sites for Oct-1 and Sp-1, which interact with ERs via *trans* factors suggesting a molecular mechanism for E_2- mediated autoregulation of its receptor. There is also an *Alu* repeat sequence that may contain an ERE that could act as an ER-dependent enhancer (Li *et al.*, 2000). Conversely, *in vitro* and *in vivo* studies investigating the effects of E_2 on ERβ expression have yielded inconsistent conclusions depending upon cell type, animal species and age. For instance, in the T47D human breast cancer cell line E_2 upregulated ERβ (Vladusic *et al.*, 2000). However, ERβ expression was decreased by E_2 in mammary glands of lactating mice that co-express ERα (Hatsumi and Yamamuro, 2006). ERβ was also decreased in

the PVN of rats subjected to OVX + E_2 (Patisaul *et al.*, 1999). Thus, it appears that E_2 may regulate ERα and ERβ, however this effect is highly dependent upon cell-type, and possibly the co-expression of other ERs.

In addition to sex and E_2, aging also appears to dictate ER expression. Overall, decreased nuclear E_2 binding has been reported in the hypothalamus and anterior pituitary of aged female rats compared to young, but the change in E_2 binding was not necessarily attributed to a decrease in total ER expression (Brown *et al.*, 1990; Rubin *et al.*, 1986), suggesting a shift in the ratio of ERs and/or subcellular localization. While overall nuclear E_2 binding within the hypothalamus may decrease with age, changes to ER expression patterns with age remain contentious. In general it appears that age alone does not eliminate ERα expression in the brain, but regional specificity and E_2 availability may be important factors (Funabashi *et al.*, 2000; Wilson *et al.*, 2002) and an increase in ESR promoter methylation has been correlated with age in other systems (Post *et al.*, 1999; Issa *et al.*, 1994). One study reported varied middle age-specific reduction in hypothalamic ER with E_2 treatment (Funabashi and Kimura, 1994), yet another study showed that E_2 decreased hypothalamic ER expression significantly in all ages tested (3, 11, and 20 month)(Miller *et al.*, 1994). Specific to ERα, work by Chakraborty and colleagues determined immunoreactive cell numbers did not always change following OVX and E_2 replacement, rather their study revealed that with advanced age (24-26 months compared to 3-4 and 10-12 months) the number

of ERα positive cells was increased or stayed the same in different hypothalamic nuclei (Chakraborty *et al.*, 2003a). Moreover, in the hippocampus, ERα was decreased after long term estrogen deprivation (LTED, 10 weeks), regardless of E_2 replacement following LTED, but E_2 deprivation had no effect on ERβ (Zhang *et al.*). The same report demonstrated decreased levels of ERβ in very old rats (24 month females compared to 3 month diestrus females). In general, most reports suggest that ERβ expression with age is either decreased or neutral, but like ERα may be highly region-specific. A decrease in cortical ERβ expression with age is supported by evidence showing a corresponding increase in CpG methylation of the ESR2 promoter in middle aged (9-12 month) rats (Westberry *et al.*). Other reports describe decreases in ERβ protein and message in some areas but not in others (Wilson *et al.*, 2002; Chakraborty *et al.*, 2003b). Taken together, there are a number of reports attempting to identify the parameters that control ER expression such as age, sex and response to E_2, however with such vast deviations in expression with cell type there is still much to be learned about expression of these receptors, especially in brain regions controlling non-reproductive behaviors.

ERβ alternative splice variants

Based upon the highly variable reports that differ in sex and age of animals as well exposure to hormone it may be possible that these studies are unknowingly detecting changes in splice variant expression, which could change E_2 responsiveness as well as downstream gene regulation. Not only can ERs

heterodimerize to regulate gene transcription, but there are a number of alternatively spliced variants of each receptor that are endogenously expressed and potentially contribute to the diverse tissue specific actions of E_2. Alternative splicing of ERs alters inherent signaling properties of the receptor including ligand and DNA binding affinity, nuclear localization and dimerization, depending on where the alternative splice site is encoded. A number of ER splice variant transcripts and other proteins have been identified in demented human brains, breast and prostate, and in some reports, an increase in alternative splicing is correlated with pathology (Poola *et al.*, 2000; Ishunina and Swaab, 2009; Ishunina and Swaab, 2008; Ishunina *et al.*, 2000; Ishunina *et al.*, 2007). Also interesting, age alone may increase alternative splicing of some gene products (Tollervey *et al.*). The identified ERβ human splice variants are truncated at the C-terminus of the receptor (Figure 1A), however experimental evidence suggests that the C-terminus of the receptor is not required for ERβ-mediated transcription, especially with regard to the identified human splice variants (Mott and Pak). Unlike the human splice variants, rodent ERβ splice variants identified to date been shown to have either an exon inclusion in the ligand binding domain, creating (rERβ2), or an exon deletion in the DNA binding domain rERβ1Δ3, rERβ1Δ4 or both rERβ2Δ3 and rERβ2Δ4 (Figure 1B) (Petersen *et al.*, 1998; Inoue *et al.*, 1996; Skipper *et al.*, 1993). Exon inclusion (rERβ2 variants) has been shown to produce a protein that binds E_2 with a 35-fold decrease in affinity. In contrast, ERs with exon 3 and 4 deletions are unable to bind

DNA, but can still mediate transcription through protein:protein interactions with other transcription factors such as AP-1, and bind E_2 as well as rERβ1 (Petersen *et al.*, 1998; Price *et al.*, 2000). Importantly, the transcriptional functions of rERβ1 are significantly altered when co-expressed with other splice variants, likely due to a weaker interaction with coactivator proteins (Chu and Fuller, 1997; Lu *et al.*, 1998). Despite lower E_2 binding and/or lack of DNA binding, the rodent and human splice variants retain a constitutive ligand-independent transcriptional function, at both basic and complex promoters (Pak *et al.*, 2006; Pak *et al.*, 2007; Mott and Pak), suggesting that these splice variants have an important endogenous biological function. Indeed, unliganded or apo-ERβ1 has been reported to bind to and regulate a subset of genes distinct from those regulated by ERβ1 when bound to E_2 (Vivar *et al.*). Conversely, the human splice variants do not bind ligand with great affinity (Leung *et al.*, 2006), and might therefore only regulate the class of genes that unliganded ERβ target.

The downstream target genes of ERβ splice variants might be an important consideration at the time of menopause, as ER expression profiles and alternative splicing tend to change with age (Tollervey *et al.*). One recent report demonstrated an increase in ERβ2 expression in the hippocampus of 9-month old, middle aged rats following short-term (6 days) E_2 deprivation that was significantly decreased compared to the sham group after E_2 administration (Wang *et al.*). Importantly, E_2 replacement no longer affected ERβ2 expression in the hippocampus after LTED

(180 days). That study also reported a decrease in hippocampal neurogenesis and increased floating behavior in a forced swim test, thereby functionally correlating increased ERβ2 with mood regulation and potentially cognition. Thus, the expression and functions of ERβ splice variants are absolutely critical to understand the effects of estrogen particularly at times of sustained E_2 deprivation with regard to cognition and affect. While ERβ2 expression has been assessed in the young male rat brain (Chung *et al.*, 2007), and other variants have been described in some brain regions (Price *et al.*, 2000; Price *et al.*, 2001), there is a general lack of data on most ERβ splice variants, especially in aged female brains.

Some of the splice variants identified to date have been characterized as dominant negative receptors, serving to inhibit activation of the full length receptor (Wang and Miksicek, 1991), however most identified variants do not bind ligand with the same affinity and have the potential to differentially regulate target genes. While several splice variants for ERβ have been identified in many model systems including mouse (Kuppers and Beyer, 1999), rat (Shughrue *et al.*, 1998; Shughrue *et al.*, 1997) and monkey (Gundlah *et al.*, 2000), there is a general lack of comparative studies on expression and functionality of human ERβ variants, especially in neuronal systems. Further, changing expression levels of one or more alternatively spliced variants during a period of E_2 deprivation may drastically change general receptivity and downstream functions of E_2.

Novel protein:protein interactions for E_2-mediated nuclear processes

Protein:protein interactions are an essential relay in the regulation of dynamic cellular processes. Immediately following translation, ERs typically associate with a chaperone protein to ensure proper folding, protect from degradation and assist the ER in becoming poised to accept ligand. Once bound to ligand, ERs can dimerize and act as transcription factors to mediate gene regulation or associate with membrane proteins to initiate a signaling cascade. When acting as transcription factors, ERs associate with a number of coregulatory proteins that assist in activating or repressing E_2 -regulated genes. Coregulatory interactions are more characterized for ERα than ERβ, and importantly, less clear is how ERβ mediates ligand-independent transcription. In addition to the well-established ER interaction partners, many novel interacting proteins have not yet been characterized and could be critical for nuclear processes not limited to gene transcription.

HSPs and Chaperone proteins

According to the classical two-step hypothesis inactive steroid hormone responsive nuclear receptors are constantly accompanied and protected from degradation by a number of chaperone proteins, typically members of the heat shock protein (HSP) family. This receptor:chaperone complex has been studied extensively, and while the idea of a protective role for chaperones stands, this complex performs other functions. For instance, HSP:ER complexes can serve to

pre-activate a hormone receptor by forcing a conformational change in ER such that it is able to bind its cognate hormone. The initial HSP complex consists of the ER, HSP70, HSP70-interacting protein (HiP) as well as other accessory and scaffolding proteins (Morishima *et al.*, 2000). HSP90 is recruited to the complex, and HSP70 dissociates, creating the mature HSP:ER complex (Dittmar and Pratt, 1997). HSP90 induces a conformational change in the nuclear receptor and the ER is released from the complex, ready to dimerize and bind DNA or other transcription factors to regulate gene transcription. However, some studies suggest that HSPs could have a broader and more critical role than originally thought. For example, in Drosophila HSPs are required for DNA binding, and in some instances may regulate NR action (Kang *et al.*, 1999). Interestingly, aging alters HSP70 in a cell-type specific manner, and E_2 increases HSP70 levels in female hypothalamus (Olazabal *et al.*, 1992). Therefore, changes in chaperone levels with age or E2, could potentially alter the activational state of ERs.

Transcriptional proteins and ERs

The process of transcribing DNA into RNA is a systematic process that involves multi-protein complexes binding to DNA, modifying histone marks and initiating RNA synthesis. ERα, but not ERβ, has been shown to directly interact with TFIIB, IIE, IIF and TIID proteins that initiate transcription (Sabbah *et al.*, 1998; Wu *et al.*, 1999). However, experimental evidence from co-immunoprecipitation studies has demonstrated interactions between ERβ coregulatory proteins as well as other

transcription factors. Coregulatory proteins are transcriptional accessory proteins that enhance or repress transcription of target genes. In general, coactivators enhance, whereas corepressors block gene transcription. However, recent data suggest that seemingly non-transcriptional proteins may have context-dependent coregulatory functions. Importantly, certain coregulators can also be governed by age and E_2 (Ghosh and Thakur, 2008; Frasor *et al.*, 2003; Frasor *et al.*, 2005), thus recent discoveries imply that ER-mediated gene regulation is not as well understood as previously thought.

The best studied and well-established group of coregulatory proteins that selectively associate with NRs is the steroid receptor coactivator (SRC/p160) family. The SRC family is composed of three members, SRC-1, SRC-2 and SRC-3, all of which contain canonical LxxLL motifs known as the nuclear receptor (NR) box. This motif interacts with AF-2 domains in ERβ, as well as other NR family members such as glucocorticoid receptor (GR), progesterone receptor (PR), thyroid hormone receptor (TR) and ERα (McKenna and O'Malley, 2002). SRC members have intrinsic histone acetyltransferase activity (HAT, DNA activating) and interact with CREB binding protein (CBP) (Yao *et al.*, 1996). CBP/p300 proteins are also coactivators that have intrinsic HAT activity and can recruit ASC-2 and other known coregulatory proteins (Hanstein *et al.*, 1996). Confirmed coregulatory interaction partners for several NRs that do not belong to the SRC family include estrogen receptor association protein (ERAP 140) (Halachmi *et al.*, 1994), nuclear corepressor (NCoR)

(Horlein *et al.*, 1995), silencing-mediator of retinoic acid and thyroid hormone receptor (SMRT) (Chen and Evans, 1995) and many others. As is the case with our understanding of ERβ interactions with basic transcriptional machinery, studies investigating ERβ:coregulator interactions are sparse which may be due to uniquely challenging issues associated with ERβ, such as a lack of high fidelity biochemical tools, complicated structural properties, and or pleotropic physiological actions that are specific to ERβ.

In 2010, Bert O'Malley and colleagues directed a high throughput study (not including ERβ) aimed at compiling a database for the endogenous coregulator pool "nuclear receptor complexome" (Malovannaya *et al.*). In this study, a number of novel protein interactions were identified, and studies such as these are identifying proteins as 'coregulators' that had been previously thought to serve completely different functions. One group of relatively novel coregulatory proteins are the E3 ubiquitin-protein ligases such as E6-associated proteins (E6-AP) (Nawaz *et al.*, 1999) . While these proteins were thought to serve primarily as ubiquitin conjugating enzymes, they have recently been highlighted as transcriptional enhancers of NR-mediated activity independent of ligase function. Similarly, a group of E3-ligases that conjugate small ubuquitin like modifier (SUMO) proteins to a target protein called PIAS are also now considered NR coregulators and utilize a typical LxxLL motif. In one study, a decrease in ER expression following LTED or with advanced age coincided with an increase in ER association with an E3-

ubiquitin ligase, CHIP (Zhang *et al.*). Together, these newly described roles for HSPs and E3 ligases raise novel questions about estrogen signaling, such as when is an E3-ligase:ER complex targeted for transcriptional regulation versus degradation? Also, when are HSPs merely performing a chaperone/protective function versus directing transcriptional processes? Future efforts aimed at elucidating the complexity of age-related changes in receptor structure and recruitment of coregulatory proteins could provide important insight into these seemingly paradoxical findings.

Nuclear actin: setting the stage

Coregulatory interactions may be poised upon a bed of nuclear actin, which has recently been identified as a dynamic molecular stage for which many nuclear processes are performed such as transcription, chromatin remodeling, mRNA processing and nuclear import/export. The general events that initiate transcription are well established; however the process by which all of the molecular components are temporally layered into a complex is still unclear. Nuclear actin is essential in forming the pre-initiation complex on a promoter, elongation and RNP organization, as well as remodeling of chromatin (Zheng *et al.*, 2009; Hofmann *et al.*, 2004; Tokunaga *et al.*, 2006), and as mentioned previously, ERs are also key factors in these processes. In one study, ERα and β-actin were co-immunoprecipitated on the E_2 responsive pS2/*TFF*1 promoter, indicating that ER and nuclear actin may work in concert to regulate transcriptional processes under control of estrogens (Metivier *et*

al., 2003). An actin binding protein gelsolin, caps actin filament ends and also has been shown to be a NR coactivator (Shao *et al.*; Nishimura *et al.*, 2003). Gelsolin may assist in actin polymerization, allowing transcriptional machinery to be brought in proximity of target genes, however it remains unclear how gelsolin enhances AR/ER transcriptional activity.

Actin is also commonly associated with ubiquitous multifunctional RNA binding proteins such as heterologous nuclear riboproteins (HnRNPs), which also associate with ERs (Nalvarte *et al.*). HnRNPs associate within the matrix of nuclear actin, accompany transcripts out of the nucleus, participate in alternative splicing and can modulate transcription (Miau *et al.*, 1998). Phosphorylated HnRNP K has been shown to mediate translation of specific mRNAs (Ostareck-Lederer *et al.*, 2002), and HnRNP H is involved in splicing and mRNA polyadenylation (Bagga *et al.*, 1998; Markovtsov *et al.*, 2000). In the past, the association of NRs with HnRNPs was thought to be non-specific due to the ubiquitous nature of these proteins, but recent studies are no longer ruling out an important interaction between NRs and HnRNPs that may assist in transcription and/or splicing (Jung *et al.*, 2005; Hong *et al.*, 2002). Some data demonstrate a dynamic interaction between ERα and HnRNPs and furthermore, that E_2 might regulate expression of members of the HnRNP family (Shao *et al.*, 2012). As noted previously, age-related increases in splicing could lead to aberrant signaling, not only for E_2-mediated processes, but for cellular processes in general.

Nuclear ER interaction partners have historically been a distinct class of nuclear receptor coregulators that seemed to solely assist ERs in gene transcription; however the number of interaction partners for ERs is increasing. Further investigation into ERβ-associated proteins is required, as far as NRs are concerned; data specific to ERβ are inadequate to make broad conclusions. Moreover, posttranslational modifications to coregulatory proteins, ERs or changes in their expression patterns due to age or sustained estrogen deprivation could all contribute to an altered microenvironment, setting the stage for atypical estrogen signaling upon therapeutic reinstatement of hormones (Fig. 3).

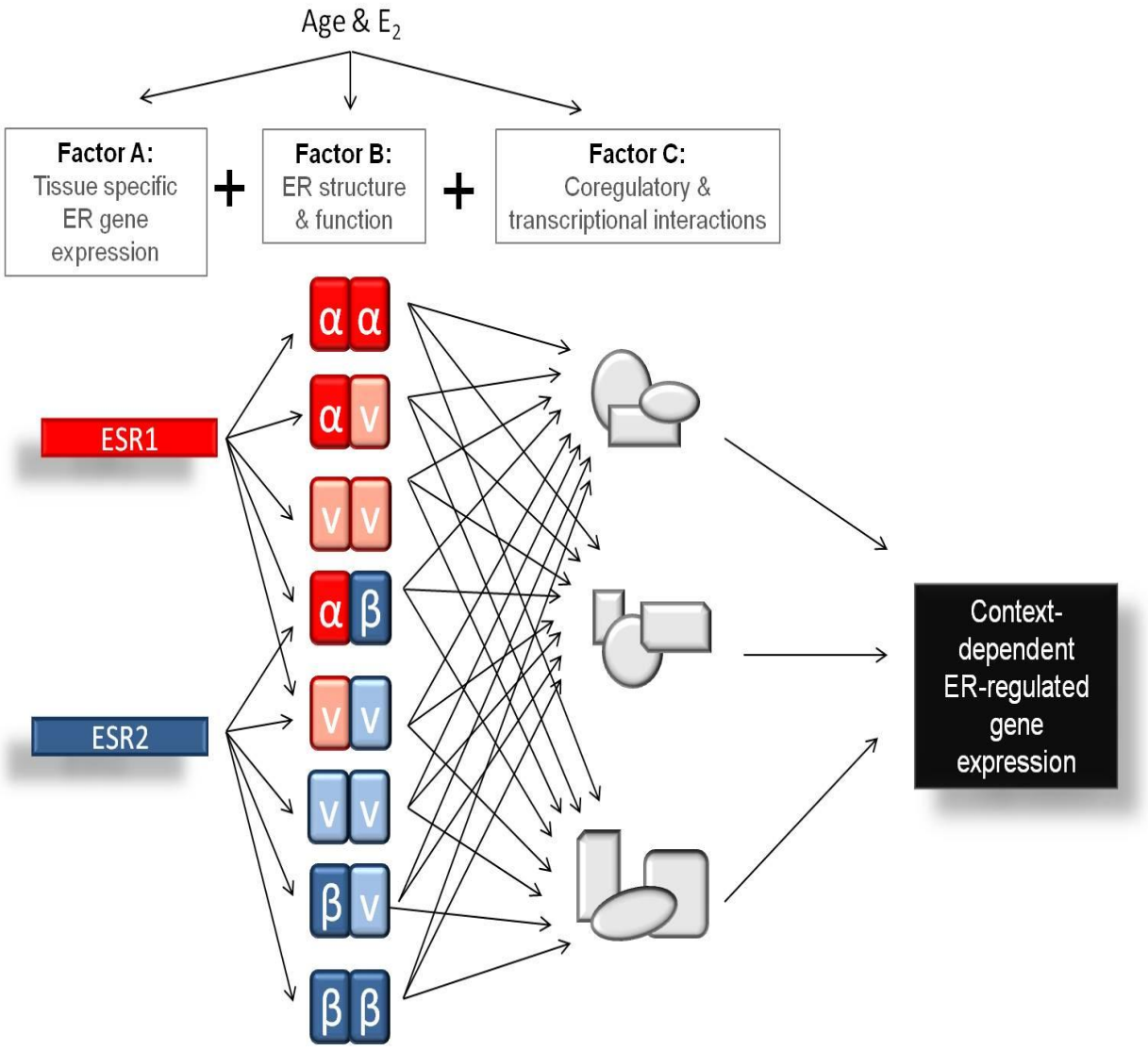


Figure 3. Age and hormonal milieu exponentially increase the potential diversity of estrogen receptor signaling leading to context dependent gene regulation. Age and E_2 influence ER gene expression, alternative splicing, coregulatory protein expression and interaction, which ultimately direct ER-target gene transcription.

Post-translational modifications of ERβ

Apart from hormone binding and protein induced structural changes, fine tuning of ER activation and can be achieved through a variety post-translational modifications (PTMs) to the receptors including phosphorylation, ubiquitination, sumoylation, acetlyation, methylation, palmitoylation and so on. Among PTMs, phosphorylation of ERβ is the most thoroughly studied, yet many putative sites have not been empirically tested and the exact molecular consequences of this modification have not been fully elucidated. Even less is known about other modifications to ERβ, and the influence of menopause and aging on ERβ. Most of the pioneering work ERβ PTMs comes from A. Tremblay's group and, presents a strong argument that relatively small modifications can completely change the functionality of the receptor within a given cellular context, which could present a mechanism for alterations in ERβ function in the midst of estrogen deprivation.

Phosphorylation is the best studied modification to ERs and is known to alter ERα transcriptional functions by modulating ligand/DNA binding, protein:protein interactions, and receptor stability. However, posttranslational modifications of ERβ are severely understudied. To date phosphorylation sites on the rodent ERβ are only putative homologous sites derived from mouse and human ERβ, and only one site on hERβ1 has been empirically examined. Murine ERβ serine^{106} mediates ligand-independent transcriptional activity initiated through signaling of stromal cell-derived factor 1 (SDF-1) (Sauve *et al.*, 2009) or EGF. Also, phosphorylation of

both serine^{106} and serine^{124} induce ligand-independent recruitment of SRC-1, ubiquitination and degradation (Tremblay *et al.*, 1998; Picard *et al.*, 2008; Tremblay *et al.*, 1999a; Tremblay and Giguere, 2001); however once again, yet to be determined is the signal following phosphorylation that determines whether the receptor will be degraded or sent to regulate gene transcription. Most identified ERβ phosphorylation sites are located in the N-terminus or hinge region of the receptor, however point mutations to the C-terminus of ERα and ERβ can induce a constitutive transcriptional active state (Tremblay *et al.*, 1998). There is even less known about phosphorylation of alternatively spliced variants of ERβ. The lack of data in this area underscores the importance of investigating phosphorylation of not only full-length ERβ, but also expressed splice variants, and the role of kinases with age and E_2 availability.

Data from our lab and others have recently identified another modification to ERβ, conjugation of a small ubiquitin-like modifier (SUMO-1) (Picard *et al.*). SUMOylation regulates activities including nuclear translocation and protein:protein interactions. Nuclear steroid hormone receptors, including ERβ (Tirard *et al.*, 2007; Sentis *et al.*, 2005; Poukka *et al.*, 2000; Picard *et al.*; Le Drean *et al.*, 2002; Duma *et al.*, 2006; Daniel *et al.*, 2007) are all acceptors of SUMOylation in presence and absence of hormones. For example, in the absence of progestins SUMO-1 conjugation to the progesterone receptor (PR) abolished ligand-independent transcription of target genes (Daniel and Lange, 2009). The family of small

ubiquitin-like modifiers (SUMO) is a group of proteins that can be attached covalently to a lysine residue through a series of ligase reactions catalyzed first by a SUMO activating enzyme, E1, then a SUMO-specific conjugating enzyme, E_2 (i.e. Ubc9) and finally a SUMO-ligating enzyme, E3 (i.e., PIAS). SUMOylation is best known for modifying a protein to alter protein:protein interactions in a rapid and dynamic fashion. For transcription factors, SUMOylation is often indicative of transcriptional repression, however it can enhance the transcriptional activities of some factors, such as ERα (Sentis *et al.*, 2005). Interestingly, SUMO and SUMO-related proteins appear to be decreased in the brain with age, which could have serious implications for ER-mediated gene regulation.

While there is some evidence regarding hormone-induced PTMs, there are very few studies that examine changes in PTMS with age especially regarding ERβ, which has only been shown to be substrate for phosphorylation, ubiquitin, sumo and palmityol groups in very limited contexts (Pedram *et al.*, 2007). Further, the undertaking of integrating hormone binding, PTMs, protein:protein interaction and downstream functional activities is astounding, but must be addressed to fully understand ER signaling.

Estrogens and cognition

Most empirical and observational data give merit to the idea that estrogens have a positive effect on cognitive processes, increased spine densities (Woolley and McEwen, 1992; Woolley *et al.*, 1996), enhanced synaptic plasticity (Woolley, 1998;

Srivastava *et al.*, 2008; Ogiue-Ikeda *et al.*, 2008) and improved memory (Sandstrom and Williams, 2004; Hogervorst *et al.*, 2000), however the receptor(s) and mechanisms that regulate these processes remain unclear. There are a myriad of behavioral studies suggesting that E_2 enhances prefrontal cortex (PFC) and hippocampal-dependent tasks. For example, long term E_2 deprivation diminished aged female rhesus macaques' performance in a delayed response task, a PFC dependent task (Bailey *et al.*). E_2 also enhanced object recognition under a number of different paradigms (Walf *et al.*, 2006; Luine *et al.*, 2003; Fan *et al.*), and there are also multiple lines of evidence supporting E_2-mediated neuroprotection which may be important for cognition, especially after stroke (Dubal and Wise, 2001; Yang *et al.*, 2000; Simpkins *et al.*, 1997; Shi *et al.*, 1998) .

Pharmacological targeting of the receptors with ER selective ligands has been a standard method for investigating the behavioral, physiological and cellular actions of E_2 mediated distinctly through ERα and/or ERβ, however valuable insight has also come from the ERβ-null (βERKO) mice. βERKO mice have significantly fewer neurons in the cortex, hypothalamus, amygdala and ventral tegmental area compared to WT. They also exhibit neuronal shrinkage and hyperproliferation of glia by 3 months of age, as well as have high levels of apoE and apoE-dependent deposition of amyloid plaques throughout the CNS by 12 months of age (Zhang *et al.*, 2004). These mice also demonstrate spatial learning deficits in the Morris water maze (Rissman *et al.*, 2002) and a decrease in hippocampal- and amygdala-

dependent memory in a fear conditioning paradigm that is accompanied by decreased synaptic plasticity in hippocampal slice preparations (Day *et al.*, 2005). The critical role of ERβ in higher level brain functions has been deduced from these studies and others, warranting a full investigation of the wide-spread molecular actions of E_2 known contribute to cellular processes on at least two levels: at the synapse and on the genome.

Long term potentiation (LTP) is an important component of learning and memory. It represents an increase in synaptic transmission and plasticity that underlies cognitive behaviors, and is readily altered by E_2 in many circumstances. In fact, application of an aromatase inhibitor eliminates CA1 LTP generated by theta burst stimulation in intact female, but not male or OVX rats, posing a potentially serious concern for women using aromatase inhibitors for therapeutic treatment of breast cancer (Vierk *et al.*). E_2 can also enhance or suppress long term depression (LTD), reducing synaptic transmission, which may be dependent upon the specific receptors involved. In aged male CA1 cells, E_2 decreased LTD (Vouimba *et al.*, 2000), however E_2 enhanced LTP in the cerebellum where ERβ is the predominately expressed cognate receptor (Andreescu *et al.*, 2007). However to date, there is little data on the mechanisms by which ERβ regulates these processes.

Estrogens and mood regulation

A range of behavioral experiments indicate that E_2 modulation of stress, mood and affect is a complex story, with considerable conflicting data that may, as

in other processes, be explained in part by distinct roles for ERα and ERβ. Anecdotally, many women report mood fluctuations as corresponding to changes in circulating estrogen levels, such as what occurs during the menstrual cycle, peri-puberty, postpartum, and peri/post-menopause. Incidence of anxiety and depression are observed at peri-menopause and when hormone levels are fluctuating (Gonda *et al.*, 2008; Freeman, 2003). However, E_2 can also exhibit anxiogenic properties, and often anxiety and depression present in a comorbid fashion, especially in women (Lund *et al.*, 2005; Breslau *et al.*, 1995). Interestingly, after the age of 55, bouts of depression and anxiety appear to decrease in women (Bebbington *et al.*, 1998). As previously mentioned, peri-menopausal women receiving CEE in the KEEPs study reported an improvement in mood, and the primary actions of CEE tend to be mediated through ERβ (Bhavnani *et al.*, 2008). A plethora of behavioral studies have mounted in response to observational reports, and at first glance it appears that ERβ has an anxiolytic and antidepressive role, however there is still an immense void to be filled with respect to biochemical and molecular mechanisms of ERβ and affective disorders. Elucidating the precise molecular mechanisms that require ERβ in plasticity and neurotransmitter processing in brain regions regulating these behaviors will help clarify the role of E_2 in stress and mood related processes.

Contemporary hypotheses concerning the onset of affective disorders revolve around perturbations to the central processing of environmental stress.

The hypothalamic-pituitary-adrenal (HPA) axis is the 3-tiered hierarchical biological system that mediates physical or psychological response to stressors. The primary steroid regulating the HPA axis is cortisol/corticosterone (humans/rats, CORT), a glucocorticoid receptor (GR) ligand that is produced from the adrenals to exert negative feedback upon the HPA system to effectively modulate response to stressors. The central hypothalamic HPA structure, the PVN produces two neuropeptides, corticotropin releasing hormone (CRH) and arginine vasopressin (AVP), to activate the HPA axis. CRH and AVP synergistically stimulate release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which acts on the adrenal cortex to produce CORT. CORT binds GR and negatively regulates CRH and AVP expression and release through classical negative feedback mechanisms (Aguilera *et al.*, 1983; Papadimitriou and Priftis, 2009). ERβ is the main ER expressed in the PVN (Suzuki and Handa, 2004; Miller *et al.*, 2004; Lund *et al.*, 2005; Isgor *et al.*, 2003), and regulation of AVP is an interesting example of how ER action can vary. AVP expression fluctuates during the menstrual cycle and is usually highest when E_2 is low. In fact, oral contraceptives appear to decrease AVP expression, and E_2 is thought to inhibit AVP in the human SON (Forsling *et al.*, 2003). In the rodent system ERβ and its splice variants activate the rodent AVP promoter independent of ligand (Pak *et al.*, 2007), however the human promoter is repressed by ERβ and splice variants. This discrepancy between the human and rat was mediated by an AP-1 response element on the human AVP promoter that is not

present in the rat. Importantly, ERβ acted similarly in the two systems when the AP-1 sequence was deleted from the human promoter, underscoring the striking alterations small changes in DNA sequence can invoke in E_2 signaling pathways and the importance of understanding the experimental context from which such conclusions are based (Mott and Pak). On the contrary, rat and human CRH expression was increased in response to E_2 in rodent, monkey, and human hypothalamus, but inhibited in the placenta (Lalmansingh and Uht, 2008; Roy *et al.*, 1999; Ni *et al.*, 2002; Vamvakopoulos and Chrousos, 1993).

In addition to AVP and CRH, glutamatergic and GABAergic projects from regions like the BNST, AMY, PFC and hippocampus all express ERβ (Shughrue *et al.*, 1998; Shughrue *et al.*, 1997) and are likely targets for E_2 to exert effects on the HPA axis. Moreover, decreased ERβ mRNA in postmortem locus coeruleus has been found to correlate with suicide (Ostlund *et al.*, 2003) and even more recently, ERβ-mediated hippocampal nitric oxide levels have been implicated in affective behaviors in females, but not males (Hu *et al.*). Neurotransmitter release from these regions influences mood, affect and stress responses, and E_2 increases the rate of monoamine oxidase degradation and serotonin transport which enhances serotonin at the synapse; E_2 also increases serotonin receptor expression (Summer and Fink, 1995; Smith *et al.*, 2004). Dopamine and serotonin (Imwalle *et al.*, 2005)are diminished in the BNST, POA, and hippocampus and caudate putamen (dopamine) of βERKO mice (Imwalle *et al.*, 2005) further implicating an important role for ERβ

in the regulation of emotion and mood. βERKO mice also display serious morphological and functional abnormalities in the brain that correlate to increased depression and anxiety (Tomihara *et al.*, 2009; Walf *et al.*, 2009; Walf *et al.*, 2008a; Walf *et al.*, 2008b; Krezel *et al.*, 2001). In addition to βERKO studies, administration of ERβ selective agonists (diarlyproprionitrol, DPN) decrease both stress markers and anxiety-related behaviors in rats (Lund *et al.*, 2005). In fact, there have been several studies implicating ERβ and its variants in affective behaviors, but the molecular mechanisms remain poorly understood.

Summary

Estrogen receptor-mediated signaling in the brain regulates neurological processes many of which translate to cognitive and affective behavioral outputs. When estrogen is declining and becomes replete, as in menopause, a number of neurophysiological changes occur, producing some unwanted changes. The most common and logical remedy is replacement of bioidentical hormone, E_2, however this treatment can be problematic dependent upon the length of time a woman has been in a postmenopausal, estrogen-deprived state. This suggests that there is a molecular switch in estrogen-mediated signaling that may allow for drastic change in ER signaling, not to mention the interaction of E_2 signaling components and the natural aging process. These changes are likely to include alterations to receptor profiles including expression of alternatively spliced variants that respond

differently to E_2, changes in the cellular microenvironment that can alter the protein:protein associations which ultimately leads to changes in ER-mediated gene transcription, and synaptic transmission. ERβ in particular is widely expressed and implicated positively in the regulation of memory and mood fluctuations, two of the most commonly reported neurological issues in postmenopausal women. It is important to understand the actions of ERβ in the areas regulating these processes to identify what, when, how and for whom hormone therapy may be a useful treatment to rectify cognitive and affective issues.

Hypothesis and Aims

During menopause, aging and deprivation of 17β-estradiol (E_2) induce changes in gene transcription by influencing the actions of estrogen receptors alpha and beta (ERα and ERβ). Through molecular interactions with effector proteins ERβ modulates processes (i.e., transcription of target genes such as AVP) that lead to changes in stress response, mood, and memory that can be manifested throughout menopause. Little is known about how ERβ functions in an aged microenvironment devoid of E_2, but alternatively spliced variants that do not respond to E_2, but are constitutively active, have been shown to increase under these circumstances. Therefore, I hypothesized that both molecular interactions and inherent factors in the splice variants of estrogen receptor beta (ERβ) contribute to changes in ERβ

function as a result of the aging process and in the absence or reinstatement of E_2.

Thus, I have developed the following two aims to test my hypothesis:

Aim 1: Identify the contribution of C-terminal truncations of human (h)ERβ splice variants to ligand-independent regulation of promoter activity of the full-length receptors in neuronal cells.

There are several identified alternative ERβ_ splice variants in mouse, rat and human tissues, however the sequence homology between variants in different species is not well conserved. Our laboratory and others have confirmed the ligand-independent transcriptional actions of rat ERβ1 and several of the rat alternative splice variants, however, to date there have been no reports of human ERβ1 or any alternative splice variants thereof exhibiting ligand-independent activity on target genes. The mechanisms by which constitutive, ligand-independent transcriptional activity occurs is postulated to by influenced by MAPK signaling, and there are consensus sequences for p38 phosphorylation present on the human receptors. There is also a lack of data with regard to brain region specific expression of ERβ1 splice variants. I therefore sought to answer the following questions:

1) Are hERβ splice variants differentially expressed in the brain regions of aged patients that control affective processes such as the amygdala?
2) Do all of the identified hERβ splice variants retain DNA binding activity?

3) Can hERβ splice variants modulate promoter activity of mediated by basic cis-acting elements such as an ERE or AP-1 site independent of ligand or in response to agonists/antagonists?
4) Do hERβ splice variants modulate the hAVP promoter in the same fashion as the rodent ERβ splice variants, and do they respond to ligand?
5) What elements on the hAVP promoter contribute to ERβ splice variant gene repression?
6) Could p38 play a role in mechanism of hERβ splice variant-mediated ligand-independent activity?

Overall, data from this aim identified that several splice variant transcripts are differentially expressed in the amygdala, and within the same patient, splice variant expression can vary depending upon the region of the amygdala. I also used an electrophoretic shift assay to determine that each of the splice variants bind a consensus ERE *in vitro.* Then, by employing the use of luciferase-fused promoter constructs, I was able to determine that each splice variant transactivates and represses ERE- and AP-1 mediated promoter constructs respectively. Each hERβ splice variant can also repress the hAVP promoter, independent of the presence of E_2, agonist or antagonist. I also found that hERβ splice variant-mediated activity on the hAVP promoter was elicited through an AP-1 site. Finally, I showed that p38

inhibition can block hERβ splice variant mediated AP-1 and hAVP repression in neuronal cells and that estradiol can potentiate AP-1 activity when p38 is inhibited.

Aim 2: Determine the accompaniment of molecular proteins associated with hERβ in young and aged animals upon a brief E_2 withdrawal and replacement.

Transcriptional regulation by ERβ requires a cohort of regulatory proteins. Protein associations with ERβ depend upon the status of the receptor regarding ligand binding and post-translational modifications that could alter the charge or structure of the receptor. Ligand-independent transcriptional activity mediated by ERβ is not well understood, nor are the protein:protein interactions required to achieve this function. Thus, with this aim I attempted to answer the following questions:

1) What proteins comprise the cohort of non-DNA bound nuclear proteins that associate with ERβ?

2) Is there a specific subset of nuclear proteins that differentially interact with ERβ following E_2 replacement and does age change which proteins prefer to associate with ERβ upon?

3) Does age alone alter the interactions between ERβ and its interaction partners in the absence of E_2?

3) Do the expression levels of identified interaction partners change with age and E_2, contributing to changes in their interaction with ERβ?

From this aim I was able to identify a subset of non-DNA bound ERβ associated proteins. Using quantitative 2-Dimensional-Differential Electrophoresis (2D-DIGE), I was able to determine that a subset of ERβ-associated proteins were differentially associated with ERβ dependent upon age and E_2. This list includes heat shock protein 70 (HSP70), annexins I and V (ANXAI and ANXAV), heteronuclear riboprotein H (HnRNP H), gelsolin (GELS), α-enolase (ENO1), valosin containing protein (VCP), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (For a complete list of proteins identified see Tables 1 and 2). Overall, data from this aim suggests that age and E_2 can significantly alter protein associations with ERβ and that some inherent changes in ERβ, rather than expression of the interaction partners is a major factor in the changes of these interactions with age and E_2 reinstatement after a brief period of hormone deprivation.

CHAPTER III

C-TERMINAL-INDEPENDENT STRUCTURAL REQUIREMENTS FOR HUMAN ESTROGEN RECEPTOR BETA (ERβ) TRANSCRIPTIONAL REGULATION IN NEURONAL CELLS

(MOTT NN. ET AL., J. NEUROENDOCRINOLOGY, 2012 OCT:24 (10):1311-21)

Introduction

Estrogen receptors (ERs) are critical regulators of many processes involved in functions of the central nervous system, including homeostasis, reproduction, memory, anxiety and synaptic plasticity ((Lund *et al.*, 2005; Geary *et al.*, 2001; McEwen *et al.*, 1975; Ogawa *et al.*, 1998; Sherwin, 1994a; Krezel *et al.*, 2001; Weiser *et al.*, 2008). The actions of estrogens are mediated primarily by high affinity ERα and ERβ, both of which belong to the nuclear receptor superfamily. Similar to the case in rodents, the human full-length ERβ (hERβ1) is the most recently identified ER. At least three alternatively spliced variants of hERβ are present in the human brain, although the exact expression patterns and precise actions of these receptor splice variants remain largely unknown (Leung *et al.*, 2006; Moore *et al.*, 1998). However, the naturally occurring variations in ERβ structure have the capacity to provide important clues about the functional significance of the receptor domains, ultimately giving insight into the mechanisms regulating receptor action in various tissue-specific microenvironments. The physiological importance of these variants has been recently highlighted in a study showing the elevated expression of a

dominant negative rat ERβ2, which is structurally distinct from human ERβ2, diminished the effectiveness of hormone therapy following ovariectomy in rats (Wang *et al.*). On a molecular level, previous studies from our laboratory have demonstrated that the rat ERβ splice variants constitutively activated a variety of minimal and complex promoters in neuronal cells, and this activation was not dependent on the presence of ligands [17β-estradiol (E_2), growth factors, etc.]; however, no such phenomenon has been reported for the human ERβ splice variants. Importantly, the rodent ERβ splice variants are substantively different in structure from the human specific splice variants raising the question of relevance and translatability from the rodent studies to human health.

The structural differences in human ERβ splice variants suggest the intriguing possibility that these receptors could have specific functions that are not dependent on the presence of ligands, or that they are resistant to normal ligand effects, which could have detrimental consequences for therapeutic hormone treatment strategies if these variants are highly expressed during menopause or in disease states. The human ERβ variants identified to date contain variable length deletions and substitutions in exon 8 (e.g. hERβ1, hERβ2, hERβ4 and hERβ5) (Fig. 4A), resulting in serially truncated receptor proteins at the C-terminus (Moore *et al.*, 1998). The C-terminus of ERβ houses the ligand binding domain, a domain that is absolutely required for ligand-induced actions of the receptor. Notably, the hERβ splice variants lack varying portions of the E and F domains, which alter their innate

functional properties and ability to bind ligand. Specifically, truncations to this region render hERβ2 unable to bind E_2, whereas hERβ4 and hERβ5 have a very low affinity for E_2, such that binding would occur only in conditions with supraphysiological levels of E_2 (Leung *et al.*, 2006).

Amino acids encoded in the E domain (ligand binding domain; LBD) form a secondary structure consisting of 12 α-helices. These helices are considered essential for ligand binding and associations with coregulatory proteins. Indeed, helices 3, 5, and 12 are arranged in a pattern that forms a 'hydrophobic pocket' called the activation function-2 (AF-2) region. Upon ligand binding, the LBD undergoes a conformational change that results in a positional shift of helix 12, which alters the opening of the pocket; however, only hERβ1 has the necessary coding region for the normal configuration of this helix. Helix 12 of hERβ2 is encoded such that it is positioned in antagonism to ligand (Fig. 4B) and hERβs 4 and 5 do not contain this sequence (Leung *et al.*, 2006). Upon ligand binding, this region can interact with the common nuclear box consensus LxxLL motifs that are contained in nuclear receptor coregulatory proteins such as steroid coactivator-1 (SRC-1) (11–13). In addition to ligand binding, the E / F domains of ERα contain the interface for receptor dimerization and possibly other regions important for the binding of coregulatory proteins (Peters and Khan, 1999).

Unique from all other steroid hormone receptors, only ERs contain an F domain that, for ERβ, consists of approximately 30 amino acids at the extreme end

of the C-terminus. Little is known about the functional significance of the F domain on ERβ, although recent studies suggest that the F domain may be important for transcriptional activation of ERα. For example, mutations to the F domain of ERβ enhanced dimerization, possibly by unmasking the dimerization interface of the E domain (Yang *et al.*, 2008). Importantly, one study showed that specific amino acid sequences in this region alter the transcriptional responsiveness of ERα in the presence of a typical ER agonist such as E_2 or an antagonist, such as tamoxifen or fulvestrant (ICI 182 780) (Koide *et al.*, 2007). This change in transcriptional responsiveness may be related to the ability of the F domain to associate with coregulators. For example, alterations to the F domain of ERα also lead to increased associations with coactivators, and ultimately enhanced transcriptional activity in yeast (Yang *et al.*, 2008). The present study provides the first examination of transcriptional actions mediated by human ERβ splice variants in neuronal cells. Specifically, I hypothesized that the E and F domains were important for conferring ligand-dependent (rather than ligand-independent or constitutive) activity when regulating minimal promoters with an estrogen response element (ERE) or activator protein-1 (AP-1) enhancer site, and that these effects may be a result of endogenous kinase activity. Taken together, our results demonstrate that the previously observed constitutive activity of ERβ in neuronal cells is conserved between rodents and humans. Moreover, the constitutive activity is regulated independent of the C-termini truncations deleting the AF-2 region and F domain of

the receptor and alternative splice variants. These data highlight the possibility that the ERβ splice variants play an important functional role in the brain especially when E_2 becomes replete, as is the case at menopause.

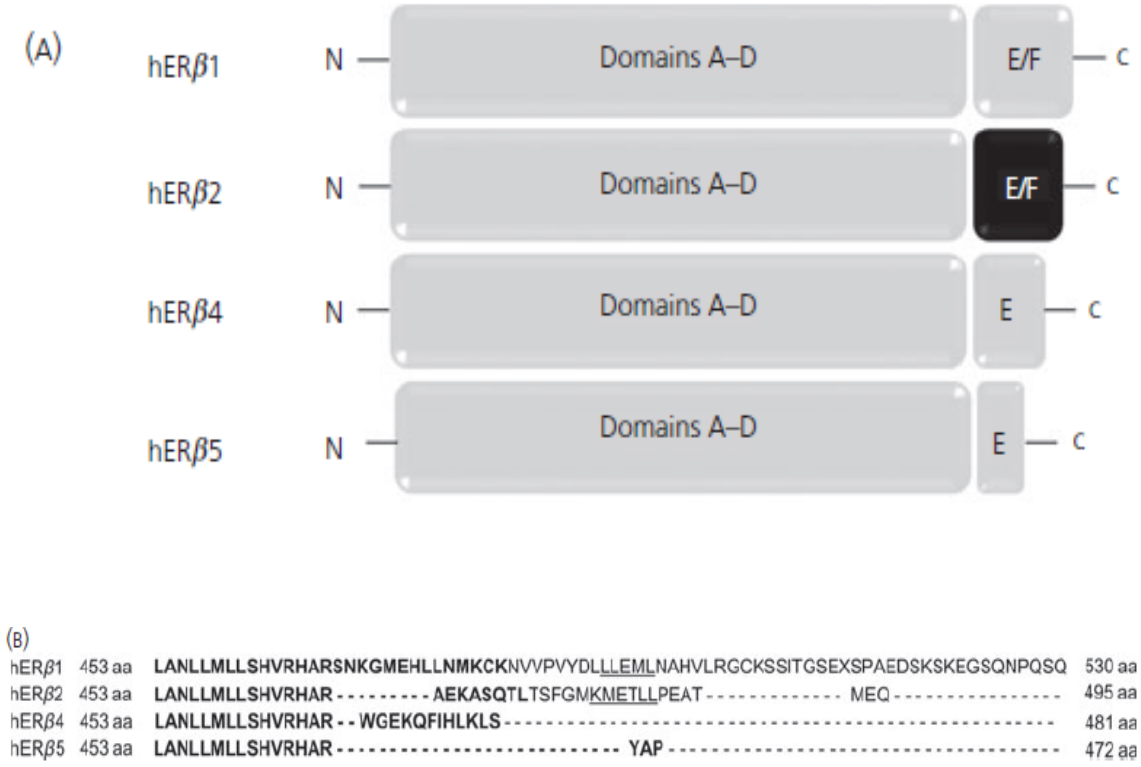


Figure 4. Schematic representation of specific human estrogen receptor (hERβ) splice variants. (A) Identified receptors are encoded by identical A-D domains. Alternative splice sites in the E domain produce splice variants with altered C-termini E and F domains. (B) Amino acid sequence of C-termini of hERβ splice variants. Bold letters represent amino acids comprising helix 11. Underlined amino acids are representative of helix 12 in ERβ1 and its corresponding sequence alignment with hERβ2.

Results

hERβ splice variants are expressed in human amygdala

Expression of the human ERβ splice variants have not been well explored, particularly in the brain. Existing reports show low levels of transcripts from whole brain homogenate, which does not give an accurate picture of heterogeneity in the brain, not only amongst different brain regions, but within subnuclei of those regions. To determine whether hERβ splice variants were expressed in specific brain regions relevant to affective issues and cognition, 3 human amygdala were obtained through the Netherlands brain bank (Female, non-demented: ages 77, 84 and 85). At least three distinct regions of the each were obtained from 2mm microdissected punches. Specific primers for the hERβ 2 and 5 were designed small unique regions of the C-termini of each receptor (See Methods for primer sequences). hERβ2 was shown to be expressed in 2 of the three patient samples obtained, but differentially so within each distinctly punched region (Fig. 5). hERβ4 appeared to be ubiquitously expressed in all of the subjects and within each region, however, the hERβ4 primers were also specific for hERβ1 and therefore require subtractive methods to determine the actual levels of expression for each sample. hERβ5, similar to hERβ2 was differentially expressed in each patient and between regions in the amygdala of each patient.

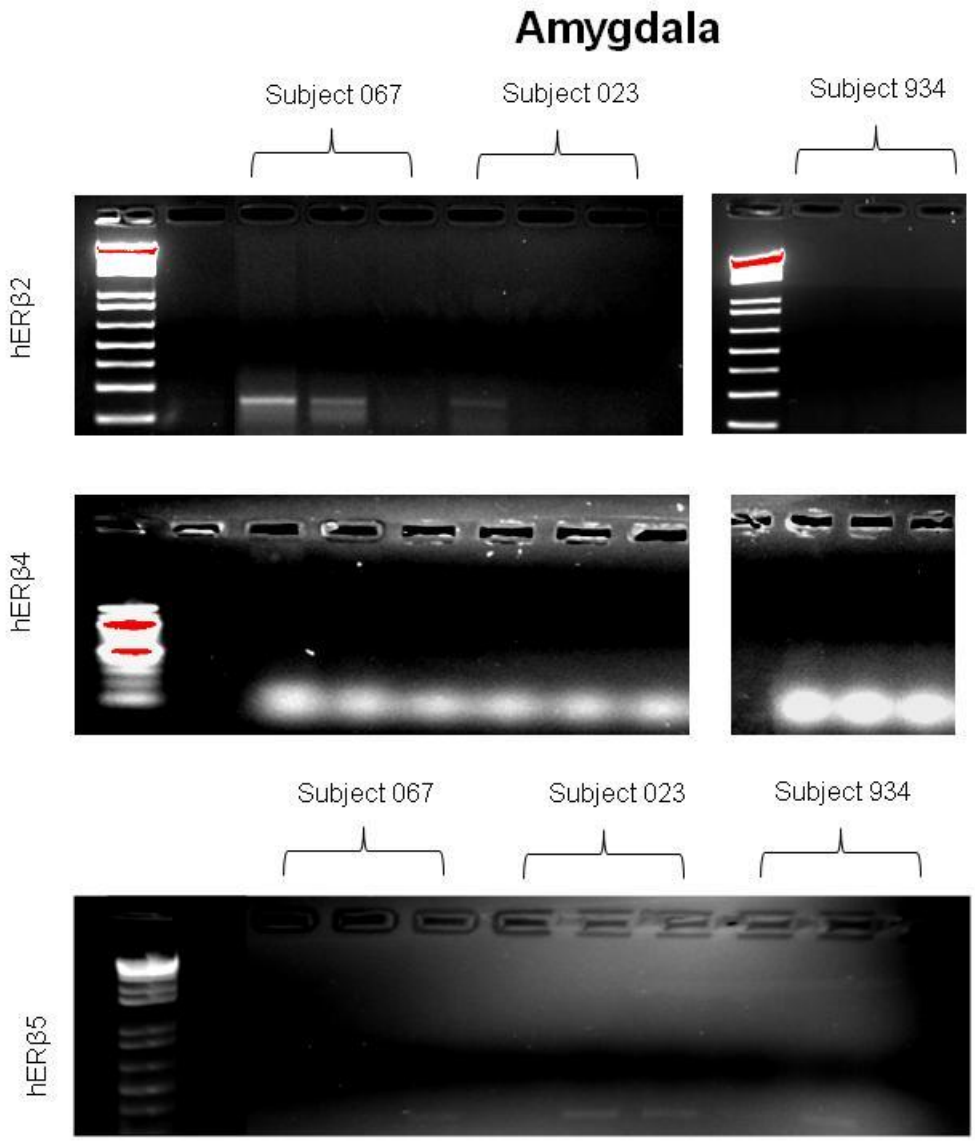
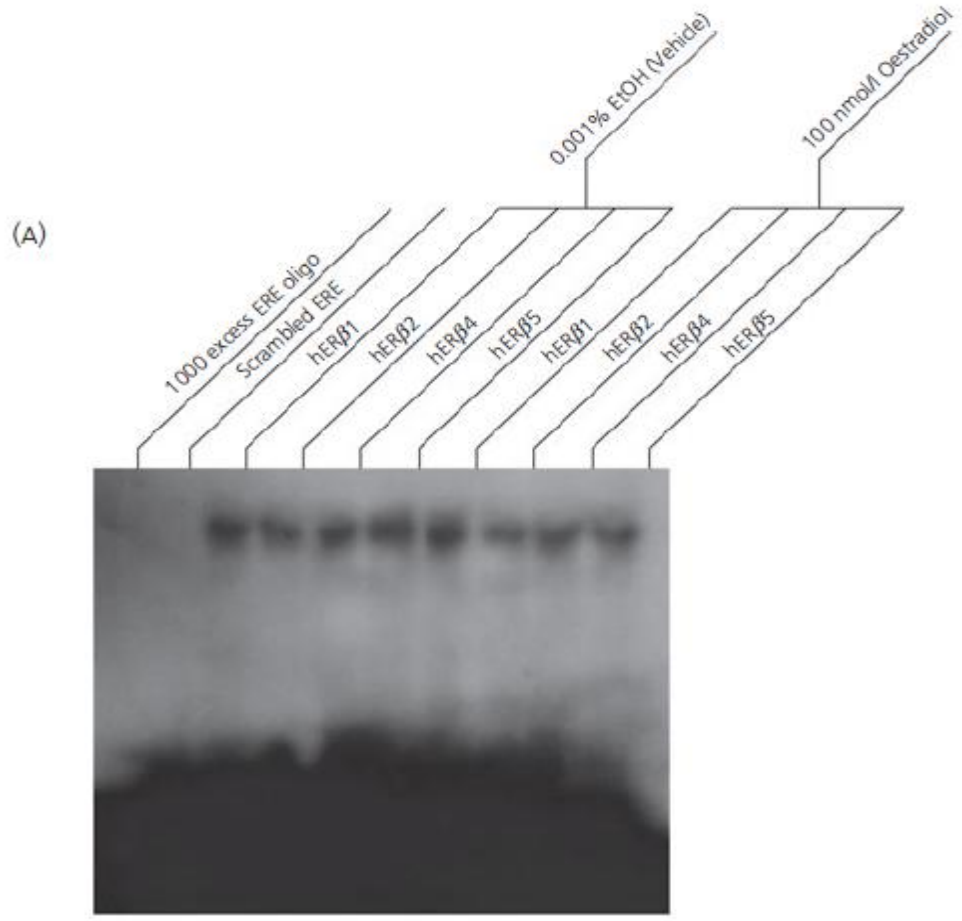


Figure 5. Expression of hERβ splice variants in human brain tissue. Unique primers targeted against the C-terminus of each receptor were designed to amplify respective mRNA transcripts from human amygdala. Samples were derived from non-demented human female brains (Ages Subject 067: 77, Subject 023: 85, Subject 934:84)

hERβ splice variants bind a consensus ERE in the absence of ligand

The classical model of ER action requires that the receptor first bind to a ligand or undergo an activational event before it is capable of binding to DNA at an ERE. This model contrasts with previous data from our laboratory and others showing that rodent ERβ1 and ERβ2, and human ERβ1, bind an ERE consensus sequence in the absence of ligand (Pak *et al.*, 2005). Notwithstanding these previous binding

studies, it was unknown whether human-specific ERβ2, ERβ4 or ERβ5 had the ability to bind a consensus ERE in the absence of ligand. Therefore, EMSAs were performed to determine whether hERβ1, hERβ2, hERβ4 and hERβ5 could bind a consensus ERE sequence in the absence of E_2 and also to determine whether the presence of E_2 altered DNA binding. The results obtained showed that all human ERβ splice variants caused a strong shift of ERE-P32 oligos, demonstrating the ability of all human-specific splice variants to bind an ERE in both the presence and absence of E_2 (Fig. 6A). Similar to the rodent splice variants reported previously, there were no significant differences between vehicle and E_2 treated lysates that contained hERβ1, hERβ2, hERβ4 or hERβ5 (Fig. 6B).



(B)

Treatment	Vehicle				100 nmol/l Oestradiol			
Receptor	hERβ1	hERβ2	hERβ4	hERβ5	hERβ1	hERβ2	hERβ4	hERβ5
Relative OD (± Std)	216260 (± 7316)	211077 (± 26)	211083 (± 5)	211079 (± 24)	211093 (± 3)	211067 (± 18)	204405 (± 9414)	211080 (± 30)

Figure 6. Ligand-independent DNA binding activity of human estrogen receptor (hER)β1, hERβ2, hERβ4 and hERβ5. (A) In-vitro translated hERβ splice variant proteins were incubated with 0.01% EtOH (Vehicle, lanes 3–6) or 100 nM 17β-estradiol (E_2) (lanes 7–10) for 18 h at 4 ^{0}C before incubation with 0.2 pM 32PATP labeled vitellogenin consensus estrogen response element (ERE) oligonucleotide. The binding product was resolved on a 6% DNA retardation gel for 35 min at 200 V. Gels were subsequently dried and exposed to autoradiography. To determine DNA binding specificity, unlabeled oligonucleotide was added in 1000-fold excess of 32P-ERE (lane 1). A scrambled ERE sequence was used as a negative control (lane 2). (B) Densitometric quantification of gel bands. Data are shown as the mean SD.

These results suggest that the presence of E_2 does not enhance, nor is it required, for hERβ1, hERβ2, hERβ4 or hERβ5 to bind an ERE, which is consistent with their reported inability to bind E_2 with high affinity. A scrambled sequence ERE oligo and competition with 1000-fold excess unlabelled ERE effectively demonstrated specific DNA binding to an ERE for hERβ1 (Fig. 6A, lanes 1, 2) and each of the splice variants (data not shown). In control experiments, none of the splice variants caused a shift in EMSAs using a SP-1- P32 oligo, indicating the selectivity of these splice variants for the ERE oligo (data not shown).

Apo-hERβ splice variants activate ERE -and AP-1-mediated promoter activity

Liganded ER-mediated activation or repression of promoter activity is accomplished through direct DNA binding at an ERE site when the receptor is in the cis-acting conformation, or in the trans-acting conformation through protein:protein tethering at alternative regulatory transcription sites, such as an AP-1 site. To test the transcriptional activation of ERE- or AP-1- mediated promoters by human-specific ERβ splice variants, I co-transfected hippocampal-derived HT-22 neuronal cells with a 2xERE-tk-luciferase reporter construct or an AP-1-tk-luciferase reporter construct, and varying concentrations of expression vectors containing full-length hERβ1,hERβ2, hERβ4 or hERβ5 (Figs. 7 and 8). All hERβ splice variant expression vectors significantly increased basal ERE-tk-luc activity in the absence of ligand (Fig. 7). Furthermore, I found that there were no significant differences between the

increasing concentrations of expression vectors, with the exception of hERβ2, because the lowest dose did not cause a statistically significant increase in promoter activity at an ERE.

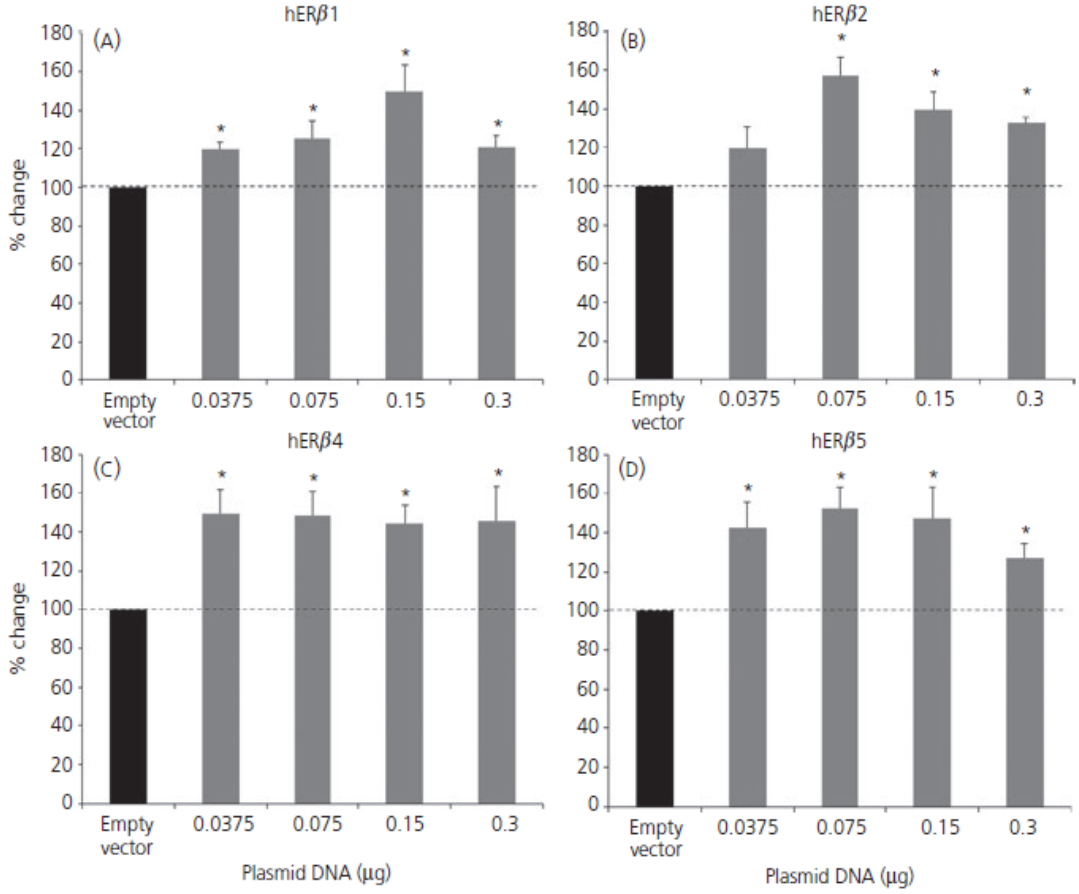


Figure 7. Apo-human estrogen receptor (hERβ) splice variants on estrogen response element (ERE)-mediated promoter activity. HT-22 cells were transiently transfected with 0.15ug ERE-luciferase reporter construct and increasing amounts of plasmid expression vectors containing hERβ1 (A), hERβ2 (B), hERβ4 (C) or hERβ5 (D). Data represent the percentage change in relative light units compared to empty vector controls. *P < 0.05: statistically significant differences from empty vector control.

Notably, with the exception of hERβ2, all of the other hERβ splice variants increased ERE-tk-luciferase activity with concentrations as low as 0.0375 μg/well and maintained consistent activation at all doses, demonstrating the ability of these ERβ splice variants to constitutively activate ERE-mediated promoter activity. ER-mediated regulation of promoters through an AP-1 site requires protein associations with members of the Jun and Fos family of proteins. Therefore, AP-1-mediated promoter regulation can be highly complex and variable depending upon the cellular context. For example, rodent ERβ has been shown to have both activational and repressive actions on AP-1-mediated promoter activity (26, 27). In these experiments, I examined transcriptional activity of the human-specific ERβ splice variants on AP-1-mediated promoter activity in hippocampal HT-22 neuronal cells. Our results showed that hERβ1, hERβ2, hERβ4 and hERβ5 significantly repressed basal AP-1 mediated promoter activity (Fig. 8) in a constitutive manner. Unlike our observations using the ERE-tk-luc reporter construct, there were significant differences between the highest and two lowest concentrations of ERβ1 plasmid and the highest and lowest plasmid concentrations of ERβ5 (Fig. 8).

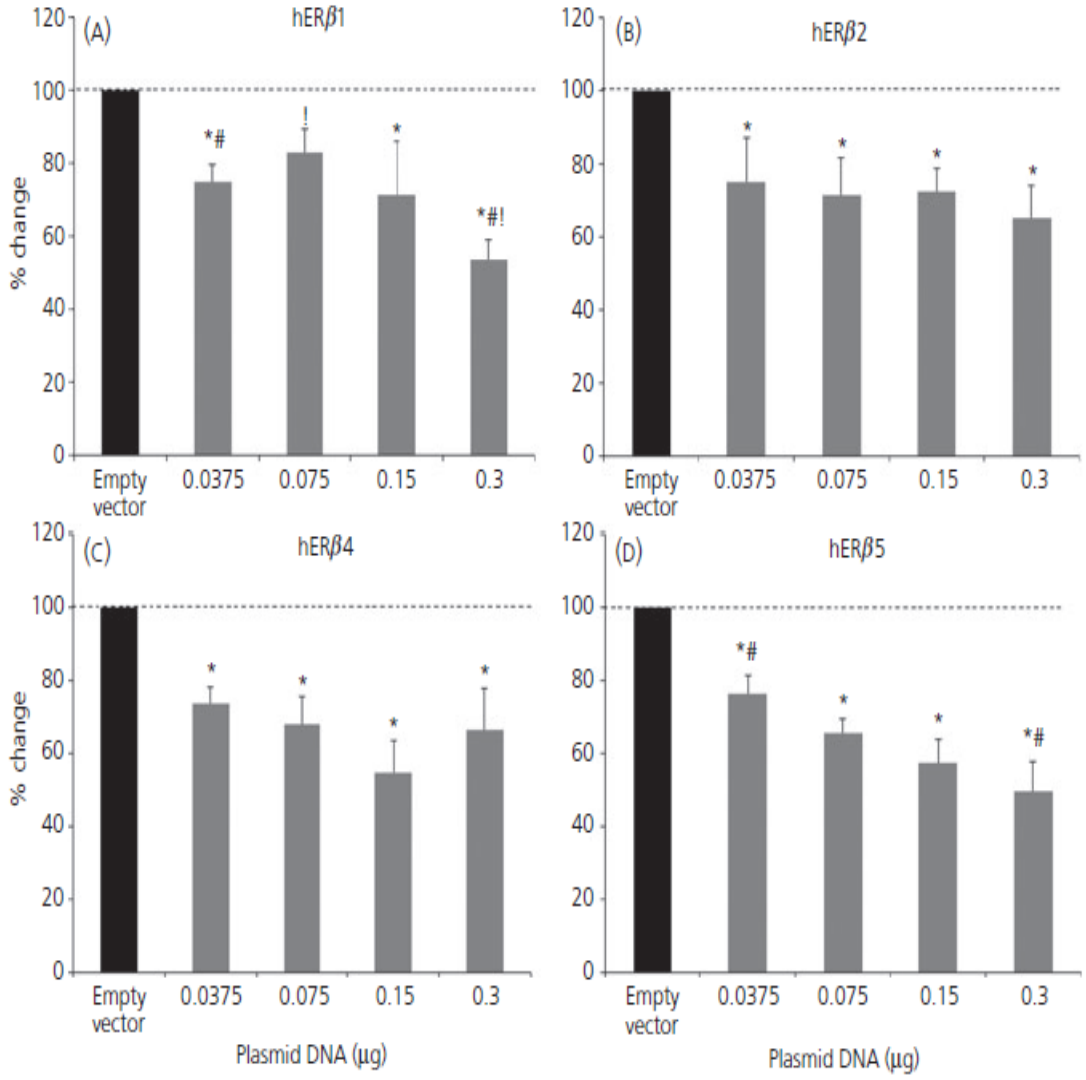


Figure 8. Apo-human estrogen receptor (hER)β splice variants on activator protein-1 (AP-1)-mediated promoter activity. HT-22 cells were transiently transfected with 0.15µg of AP-1-luciferase reporter construct and increasing amounts of expression vectors containing hERβ1(A), hERβ2 (B), hERβ4 (C) and hERβ5 (D). Data represent the percentage change in relative light units compared to empty vector controls. * Denotes statistically significant differences from empty vector control ($P < 0.05$). # Denotes statistical significance between groups.

ERβ agonists and antagonists do not alter hERβ splice variant constitutive regulation of ERE- and AP-1-mediated promoters

Human-specific ERβ2, ERβ4 and ERβ5 have very limited binding affinity for E_2, despite the fact that ERs are classified as ligand activated nuclear receptors. Therefore, to determine whether the inherent structural differences in the C-terminus of the human specific ERβ splice variants alter the ability of agonists or antagonists to activate ERE- or AP-1-mediated promoter activity, I assessed ERE- and AP-1-tk-luciferase activity in the presence of E_2 and 3β-diol (an ERβ-selective agonist), or the ER antagonist ICI182 780. Consistent with experiments shown in Figures 7 and 8, the presence of hERβ splice variants alone caused a statistically significant constitutive increase in ERE-mediated promoter activity (Fig. 9A, black bars). Conversely, a significant constitutive hERβ mediated repression was observed for all of the splice variants on AP-1-mediated promoter activity (Fig. 9B, black bars). As expected, E_2 further potentiated the constitutive hERβ1-mediated effects at an ERE, but not AP-1 site, whereas the ER antagonist, ICI 182 780 abolished the constitutive hERβ1-mediated response at both an ERE and AP-1 site (Fig.9). The presence agonist or antagonist, did not alter the constitutive effects of hERβ2, hERβ4 or hERβ5 on ERE- or AP-1-mediated promoter activity (Fig. 9). Moreover, the selective ERβ agonist 3β-diol did not further potentiate the constitutive effects of hERβs on ERE- and AP-1 mediated promoter activity (Fig. 9).

These results suggest that the presence of ligand does not alter the constitutive activity of the human ERβ splice variants at these promoter elements in neurons.

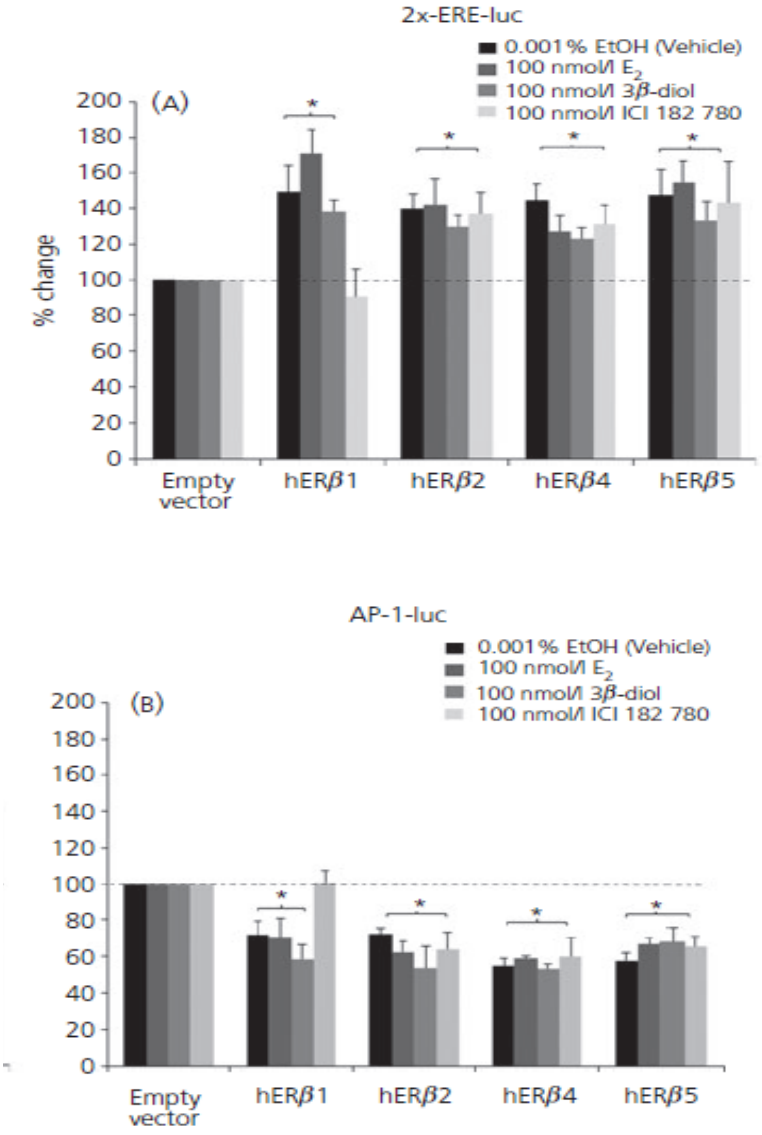


Figure 9. Effects of 17β-estradiol (E_2), 5α-androstane-3β, 17β-diol (3β-diol) and ICI 182 780 on human estrogen receptor (hER)β splice variant-mediated estrogen response element (ERE) and activator protein-1 (AP-1) promoter activity. HT-22 cells were transiently transfected with 0.15μg of (A) 2x-ERE- or (B) AP-1-luciferase reporter constructs and 0.15μg of expression vectors containing hERβ1, hERβ2, hERβ4 or hERβ5. Twenty-four hours post transfection, cells were treated with vehicle (0.001% EtOH), 100nM of E_2, 3β-diol or ICI 182 780 for 12 h. Data represent the percentage change in relative light units compared to empty vector controls. *P < 0.05: statistically significant differences from empty vector, vehicle-treated control.

hERβ-mediated repression of hAVP promoter is dependent upon AP-1 and p38 activity

Arginine vasopressin (AVP) regulates a number of neurological processes, and it is well established that E_2 and ERs alters vasopressinergic systems (De Vries *et al.*, 1994; Han and De Vries, 2003; Brot *et al.*, 1993; Ebner *et al.*, 1999). Previous data from our laboratory and others, demonstrated that rodent ERβ induced a robust ligand-independent increase of the rodent AVP promoter (Shapiro *et al.*, 2000; Pak *et al.*, 2007). Furthermore, we determined that the region on the rodent AVP promoter important for rodent ERβ1-mediated constitutive activation was a non-ERE or AP-1 site between -1.3/-740 kb upstream of the transcription start site. We co-transfected the hAVP-luciferase reporter construct with the individual hERβ splice variants into human neuroblastoma-derived SK-N-SH cells and measured luciferase activity. All human-specific ERβ splice variants significantly repressed hAVP promoter activity in a constitutive manner, which, for hERβ1-mediated repression, was blocked by ICI 182, 780 (Fig. 10). All other splice variants/ligand combinations did not alter the constitutive repression of hAVP promoter activity.

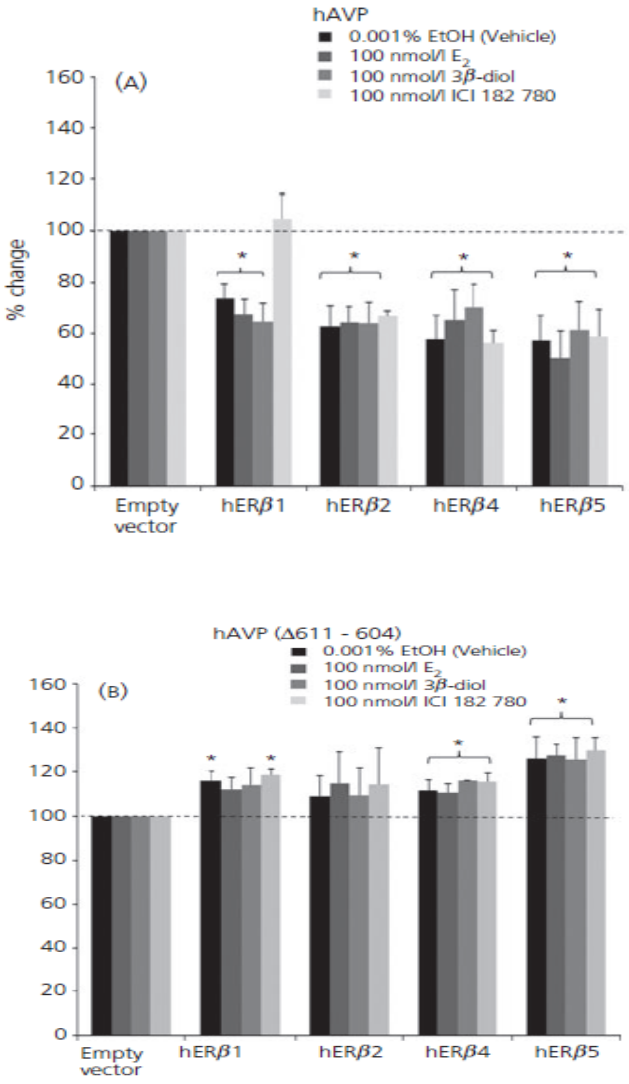


Figure 10. Effects of 17β-estradiol (E_2), 5α-androstane-3β, 17β-diol (3β-diol) and ICI 182 780 on human estrogen receptor (hERβ) splice variant-mediated arginine vasopressin (AVP) promoter activity before and after deletion of an activator protein-1 (AP-1) site. SK-N-SH cells were transiently transfected with 0.15µg of (A) human AVP (hAVP)-luciferase or (B) hAVP Δ 611 – 604-luciferase reporter constructs and 0.15µg of expression vectors containing hERβ1, hERβ2, hERβ4 or hERβ5. Twenty-four hours post transfection, cells were treated with vehicle (0.001% EtOH), 100 nM E_2, 3β-diol or ICI 182 780 for 12 h. Data represent the percentage change in relative light units compared to empty vector, vehicle-treated controls. *P < 0.05: statistically significant differences from control.

The results from the complex hAVP promoter were strikingly similar to the results obtained using the AP-1 minimal promoter construct shown in Fig. 10B. Therefore, I hypothesized that the primary element regulating this portion of the hAVP promoter activity would be an AP-1 site. Indeed, examination of the hAVP promoter sequence showed the presence of an imperfect AP-1 site located 611 bp upstream from the transcription start site. To examine whether the constitutive repression in hAVP promoter activity was a result of this imperfect AP-1 site, I used site-directed mutagenesis to create a mutant promoter construct lacking the imperfect AP-1 site (hAVPΔAP-1) and then subjected the hAVPΔAP-1 to the same reporter gene analysis described in Fig. 10A. Site-directed mutagenesis of the imperfect AP-1 site eliminated the constitutive repression of transcriptional activity that was detected for the full-length hAVP promoter in the presence of hERβ1, hERβ2, hERβ4 or hERβ5, and also allowed for significant activation of the AVP promoter by hERβ1, hERβ4 and hERβ5 (Fig. 10B). The presence of agonists, E_2 or 3β-diol had no affect hAVPΔAP-1 promoter activity mediated by any of the hERβ splice variants compared to vehicle-treated controls. However, unlike our previous results obtained using the minimal promoters, the antagonist ICI182 780 was unable to reduce the constitutive activation of hERβ1-mediated hAVPΔAP-1 activity back to baseline levels.

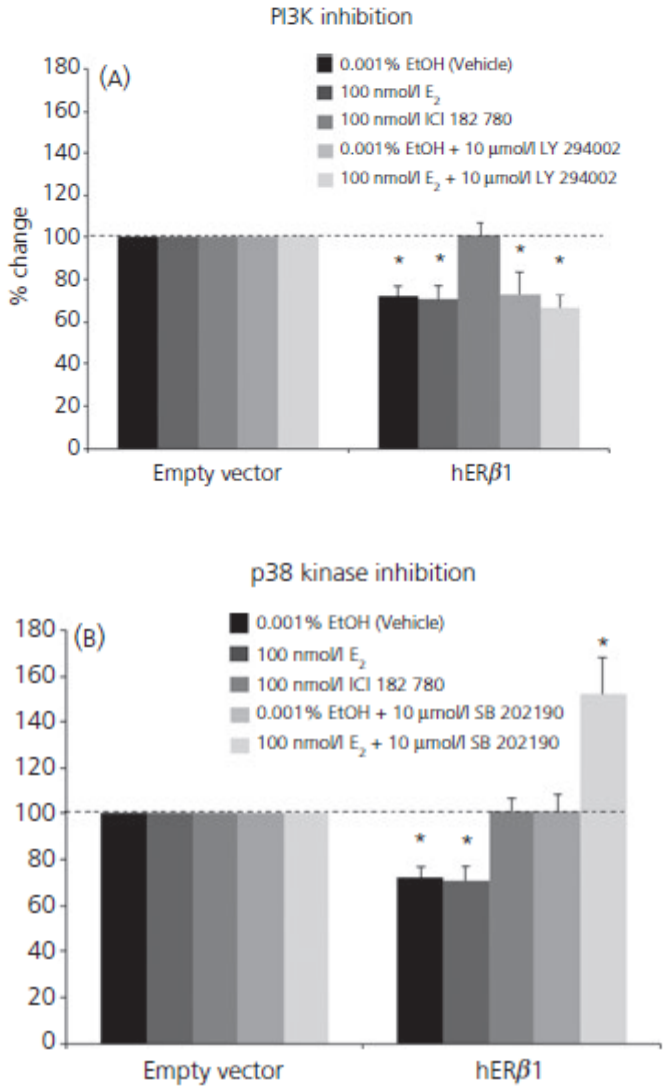


Figure 11. Role of phosphoinositide 3-kinase (PI3K) and p38 kinase inhibition on human estrogen receptor (hER) β1-mediated repression of activator protein-1 (AP-1) promoter activity. HT-22 cells were transiently transfected with 0.15µg of AP-1-luciferase reporter construct and 0.15µg of an expression vector containing hERβ1. Twenty-four hours post transfection, cells were treated with vehicle (0.001% EtOH), 100 nM 17β-estradiol (E_2), or (A) 10 lM LY294002, or (B) 10 lM SB202190 and 100 nM ICI 182 780 for 12 h. Data represent the percentage change in relative light units compared to empty vector, vehicle-treated controls. *P < 0.05: statistically significant differences from control.

The mechanisms by which hERβ acts as a constitutive transcription factor remain unclear, however phosphorylation of the receptor has been implicated as a potential signal to activate constitutive function. Specifically, p38, members of the MAP kinase family, have been identified as potential regulators of N-terminal phosphorylation of hERβ (Picard *et al.*, 2008). We tested the effects of endogenous kinase activity on constitutive actions of hERβ1 using specific kinase inhibitors. A blockade of p38 activity using the kinase inhibitor, SB202190, restored hERβ1-mediated AP-1 repression to baseline levels similar to treatment with ICI 182 780 (Fig. 11B). Notably, concomittant administration of the p38 kinase inhibitor and E_2 not only restored baseline promoter activity, but also enhanced AP-1-mediated promoter activity (Fig.11B).

Next, I tested whether inhibition of p38 kinase activity would block the constitutive repression observed by all of the hERβ splice variants on hAVP promoter activity. Our results showed that inhibition of p38 kinase blocked the repression of hAVP activity mediated by both hERβ1 and hERβ2, but not by hERβ4 or hERβ5 (Fig. 12B). Similar to the results observed with p38 kinase inhibition at the AP-1 site, when the kinase inhibitor was administered concomitant with E_2, the promoter activity was significantly increased above baseline (Fig. 12B). I also tested whether inhibition of another signaling pathway, AKT / PI3K, would abolish hERβ-mediated constitutive activity. Notably, PI3K has not been previously implicated in mediating hERβ transcriptional activation. Using the PI3K inhibitor LY294002, I

found no effect of PI3K inhibition on hERβ-mediated activity of either promoter (Figs 11A and 12A), suggesting that this pathway is not involved in the constitutive activation of hERβ.

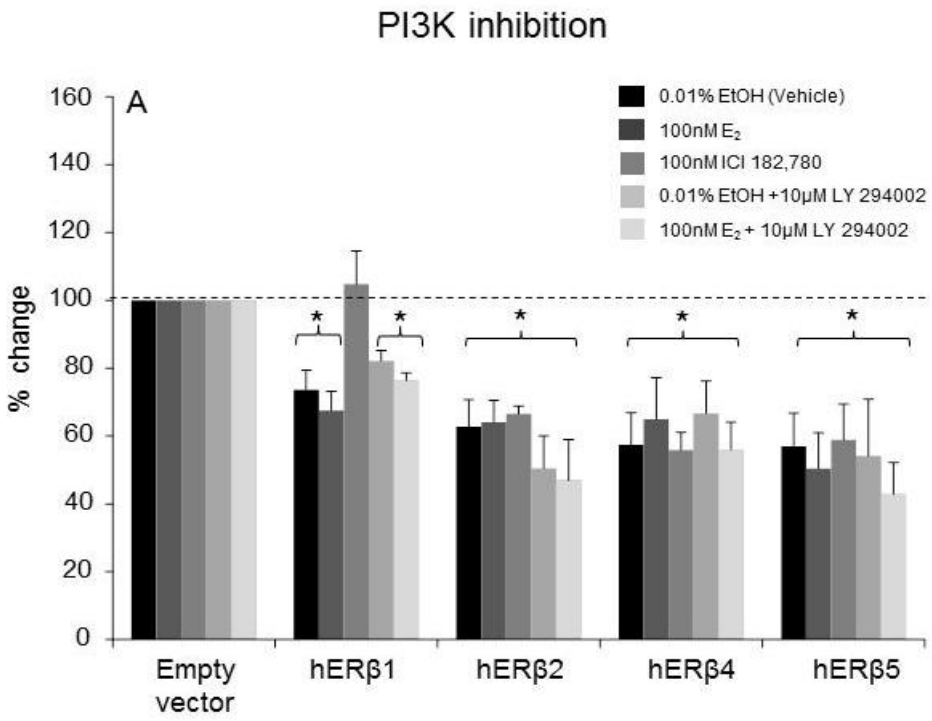


Figure 12A. Phosphoinositide 3-kinase (PI3K) inhibition on human estrogen receptor beta (hERβ) splice variant-mediated repression of human AVP (hAVP) promoter activity. SK-N-SH cells were transiently transfected with 0.15μg of hAVP-luciferase reporter construct and 0.15μg of an expression vector containing hERβ1, hERβ2, hERβ4 or hERβ5. Twenty-four hours post transfection, cells were treated with vehicle (0.001% EtOH) or 100 nM 17β-estradiol (E_2), (A) 10μM LY 294002 or (B) 10μM SB202190 and 100 nM ICI 182 780 for 12 h. Data represent the percentage change in relative light units compared to empty vector, vehicle-treated controls. *P < 0.05 denotes statistically significant differences from control.

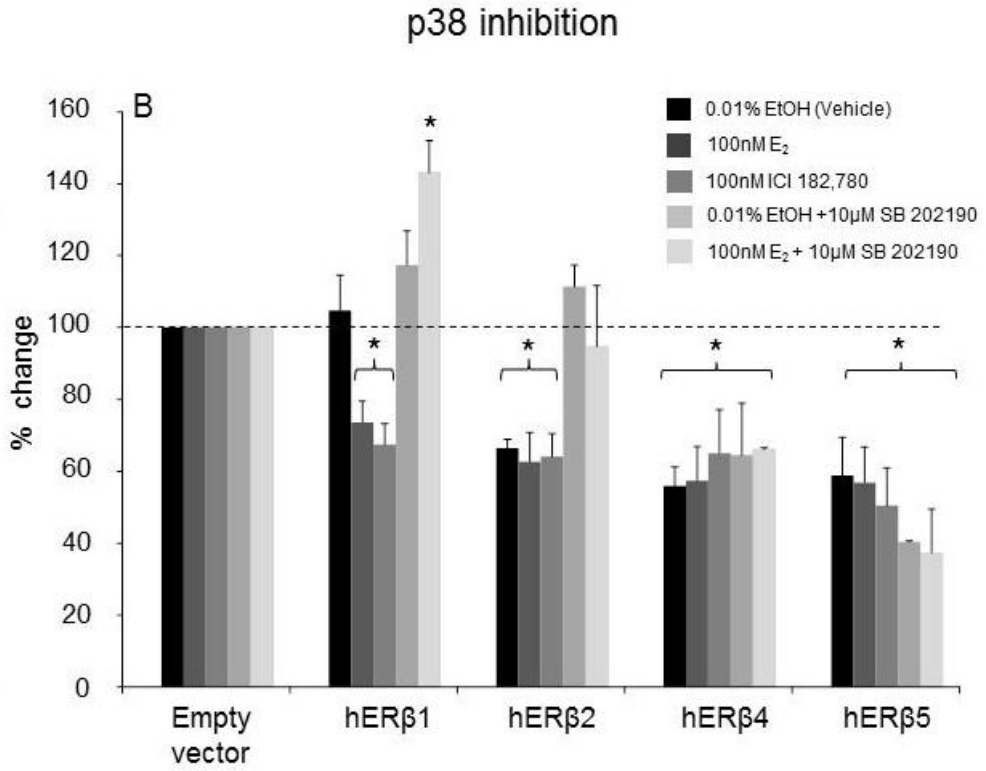


Figure 12B. p38 kinase inhibition on human estrogen receptor beta (hERβ) splice variant-mediated repression of human AVP (hAVP) promoter activity. SK-N-SH cells were transiently transfected with 0.15μg of hAVP-luciferase reporter construct and 0.15μg of an expression vector containing hERβ1, hERβ2, hERβ4 or hERβ5. Twenty-four hours post transfection, cells were treated with vehicle (0.001% EtOH) or 100 nM 17β-estradiol (E_2), (A) 10μM LY 294002 or (B) 10μM SB202190 and 100 nM ICI 182 780 for 12 h. Data represent the percentage change in relative light units compared to empty vector, vehicle-treated controls. *$P < 0.05$ denotes statistically significant differences from control.

Discussion

The key novel findings obtained in the present study demonstrate that human-specific ERβ splice variants are not only expressed in human amygdale but exhibit marked constitutive activity in neuronal cells at both minimal and complex promoters, which can be blocked by inhibition of endogenous p38 kinase activity, raising the possibility that these splice variant receptors are important mediators of centrally-regulated processes in the presence and absence of cognate ligands. Our studies demonstrate that the human-specific ERβ splice variants are largely unresponsive to ligand and induce modest, yet significant constitutive increases in ERE-mediated promoter activity, and robust decreases in AP-1-mediated promoter activity. Although the changes in ERE-mediated promoter activity were modest, these fine-tuned changes could have important biological consequences. Taken together, these data contribute to our overall understanding of ER splice variants in neuronal cells.

I have presented strong evidence that the human-specific ERβ splice variants bind DNA at a canonical ERE sequence, and modulate transcription in a ligand-independent manner at both ERE- and AP-1- mediated minimal promoters. Treatment with E_2 and 3β-diol did not significantly enhance the constitutive effects of hERβ1 on ERE- or AP-1-mediated promoter activity; however, the antagonist ICI 182 780 abolished all ERβ1-mediated constitutive activity. Previous ligand binding analyses have shown that both rodent and human ERβ splice variants have

a decreased ability to bind ligands compared to full-length hERβ1 (Peng *et al.*, 2003; Leung *et al.*, 2006). Indeed, ligands, whether agonist or antagonist, had no effect on ERE- or AP-1-mediated promoter activity via the splice variants hERβ2, hERβ4 and hERβ5, which is consistent with their reported inability to bind ligand (Leung *et al.*, 2006). Interestingly, the results shown in Figures 7 and 8 demonstrate a trend toward a dose-responsive effect of receptor concentration on promoter activity, which may be significant in brain regions where varying proportions of particular splice variants are expressed. The results from the present study suggest that further investigation of these splice variants is warranted, particularly concerning coexpression of the splice variants. These data indicate that hERβ splice variants are transcriptional active, and therefore could be physiologically relevant in neuronal cells despite fluctuations in steroid hormone levels.

The human ERβ splice variants in the present study are truncated at the C-terminus, which is useful for studying one of the two functional activation regions (AF-1 compared to AF-2) because there is a natural disruption in the AF-2 region of the splice variants. Our laboratory and others have shown that both human and rodent ERβ display constitutive activation (in the complete absence of growth factors or other nontraditional ligands) in many different promoter contexts and cell systems (Tremblay *et al.*, 1999a; Tremblay *et al.*, 1998; Tremblay *et al.*, 1999b; Tremblay and Giguere, 2001; Pak *et al.*, 2006; Pak *et al.*, 2007; Pak *et al.*, 2009; Pak *et al.*, 2005). These studies describe ligand-independent activation events (e.g.

phosphorylation and coactivator recruitment to murine and human ERβ) occurring primarily at the N-terminal AF-1 domain, whereas ligand-dependent activation occurs at the C-terminal AF-2 domain. I also propose that the N-termini of hERβs may function in concert with other activational events originating from domains A–D that depend upon the cellular context to facilitate a basal level of transcription. In congruence with published reports of other ERs, I hypothesized that p38-mediated phosphorylation of N-terminal serines (S87 and recently S105) could be an activating signal for the constitutive action of hERβs. With this in mind, p38 and PI3K activity was inhibited prior to measuring the effects on hERβ-mediated repression of AP-1 and hAVP promoter activity. Consistent with the literature p38, and not PI3K, inhibition blocked the activity of hERβ1 and, representing a novel finding, p38 inhibition blocked the dominant negative actions of hERβ2 on a complex promoter. However, p38 inhibition did not block the repressive actions of hERβ4 and 5. It is possible that p38 may phosphorylate a portion of the missing C-termini of hERβ4 and 5; however, there are no p38 consensus sequences present on this portion of the receptor to support this prediction. A more likely explanation for the inability of p38 kinase inhibition to restore promoter activity would be that a misfolding of hERβ4 and 5 occurs as a result of these variants lacking of a large region encoding the E and F domains. It is possible that this region is important for allowing a favorable conformation that results in the ability for the receptor to be constitutively active at certain promoters. Also, the presence of E_2 enhanced the

hERβ1-mediated activation of both AP-1 and hAVP promoters when administered with SB 202190. This may reflect an alternate mechanism for transcriptional activation of hERβ1 when estrogens are present. Although I report that p38 inhibition blocks the repressive effects of hERβ1 and 2, I cannot determine from these studies whether this effect is the result of the direct phosphorylation of the receptors. Overall, the conclusions drawn from the present study exclude only the possibility that the C-terminus (part of the E and all of the F) domains are required for constitutive activity of the receptor in neuronal cells, and that p38 kinase inhibition blocks the repressive actions of hERβ1 and 2. It stands to reason that there are multiple mechanisms working in concert to support the constitutive activity of ERβ, including posttranslational receptor modifications, cell-type and response element specific associations with coregulators, and even allosteric modulation through DNA : receptor interactions.

The complexity of AVP gene regulation by ERs is most apparent in physiological studies where the actions of ERs have been shown to be both anxiogenic and anxiolytic in vivo (Lund *et al.*, 2005). The findings of the present studies revealed that, unlike the rodent ERβ splice variants, which increased AVP promoter activity in the absence of ligand, the human-specific ERβ splice variants uniformly decreased human AVP promoter activity. The human and rodent AVP promoters are highly homologous in the 1000-bp region proximal to the transcription start site, with the exception of an imperfect AP-1 site located 611 bp

upstream of the hAVP transcriptional start site, which is absent in the rodent promoter. Deletion of this site resulted in significant activation of promoter activity mediated by hERβ1, hERβ4 and hERβ5, mimicking our previous findings in the rodent system. Conversely, hERβ2 displayed a trend towards activation of this promoter, although the differences were not significant from control. Antagonism with ICI 182 780 did not block hERβ1- mediated ligand-independent activation of hAVP when the AP-1 site was removed. This lack of antagonism suggests that ligand independent activation of the hAVPΔAP-1 promoter may be quite different from traditional ERE-mediated promoter activation, where ICI 182 780 blocks activation by hERβ1. These data imply that unique regulatory elements may exist in the proximal rodent and human AVP promoters that could conserve some aspects of hERβ-mediated AVP promoter activity in the absence of AP-1-mediated activity. Indeed, the region responsible for ligand-independent activation by ERβ in both the rodent and human promoters did not contain classical ERE or AP-1 sequences.

Overall, the results of the present study provide insight into the structural requirements for ERβ-mediated constitutive activity, which appear to be independent of the C-terminal domains. I also confirmed the lack of splice variant-mediated constitutive effects on various promoters. Taken together, these data contribute significantly to the growing body of information detailing the molecular mechanisms of estrogens receptor signaling. Importantly, these studies

demonstrate the novel potential for human ERβ to mediate transcriptional activation of a variety of genes in the absence of ligand in the brain.

CHAPTER IV

AGE ALTERS THE DYNAMICS OF ERβ PROTEIN:PROTEIN ITNERACTIONS IN THE VENTRAL HIPPOCAMPUS DEPENDENT UPON 17β-ESTRADIOL

(MOTT NN ET AL., MOL CELL PROTEOMICS.2014 JAN 5)

Introduction

The neuroprotective and beneficial effects of estrogens in the brain have been reported for decades, yet recent evidence from clinical trials suggested that the benefits of estrogens in postmenopausal women might not outweigh the risks. Specifically, the risk of cardiovascular disease and invasive breast cancer was significantly increased in postmenopausal women given hormone therapy (HT) as part of the largest clinical trial performed to date (Women's Health Initiative (WHI). These results sharply contradicted substantial evidence from numerous studies in animal models, prompting a re-evaluation of the data from the WHI studies. Later it was determined that factors contributing to the observed detrimental effects of HT in the WHI study included advanced age, the types of synthetic estrogens and progestins used in the study, and perhaps most importantly, the number of years post-menopause prior to the initiation of HT. However, more than 10 years after these studies concluded there is little to no mechanistic explanation for how aging contributes to a change in estrogen signaling. One possibility is that there is an age related change in the way the brain receives and responds to estrogens; however

the mechanisms by which this could occur are unclear. I hypothesized that there are intrinsic changes in the function of ERβ in the brain with advanced age, and ERβ in particular, has been shown to be a critical regulator of many neurobiological functions. An important component of ERβ signaling is that it requires associations with a cohort of intracellular regulatory protein partners. Therefore, one likely possibility is that the protein:protein interactions required for ERβ signaling are altered with age and estrogen bioavailability.

Traditional functions of ERs depend heavily upon dynamic interactions with transcription factors and coregulatory proteins that influence ER-mediated gene expression. Previous studies have shown that ERβ can associate with traditional coregulators in the brain such as steroid coactivator-1 (SRC-1) and estrogen receptor associated protein (ERAP) 140 (Greco *et al.*, 2001; Paramanik and Thakur, 2010; Shao *et al.*, 2002) , and that these associations are modified by multiple factors including age. One study demonstrated a decreased association between ERAP 140 and ERβ in the aged hippocampus, despite an overall increase in ERAP 140 expression (Paramanik and Thakur, 2010). These results raise the interesting possibility that age causes intrinsic changes in the functional properties of ERβ, which alters its ability to interact with other proteins irrespective of protein availability. Also gelsolin (GELS) and GAPDH, proteins that have often been considered artifacts when observed in nuclei, are actually proteins shown to activate nuclear receptors such as androgen receptor (AR) (Nishimura *et al.*, 2003;

Harada *et al.*, 2007). Similarly, an actin binding protein in the same family as GELS, Flightless I, has also been deemed a nuclear receptor coactivator for ER-mediated gene expression (Lee *et al.*, 2004).

The neuroprotective effects of estrogens are particularly important for post-menopausal women in brain regions such as the hippocampus; a brain area that is functionally subdivided into ventral and dorsal regions. The ventral hippocampus, forged by connections to the hypothalamus and amygdala, modulates affective processes such as responses to stress and emotion, whereas the dorsal hipopcampus is important for mediating cognitive functions, especially memory (Fan *et al.*; Fanselow and Dong, 2010; Hampson *et al.*, 1999). Importantly, estrogens regulate both cognitive and emotional processes, and ERβ may be of particular importance in postmenopausal women for mediating the effects of fluctuating E_2. As the predominant estrogen receptor in the hippocampus, ERβ is largely responsible for a number of neurobiological functions ranging from gene transcription to synaptic transmission. Indeed, transcriptional regulation is the conventional mechanism of action for ERβ-mediated processes; however evidence suggests that ERs have a much broader physiological role and can interact with proteins that regulate alternative splicing and mRNA processing, as well as a number of cytoplasmic signaling events (Nalvarte *et al.*; Masuhiro *et al.*, 2005; Ambrosino *et al.*). Therefore, the actions of estrogens and their receptors may extend to more cellular processes than previously recognized.

Our aim in these studies was to quantitatively assess age-related changes in cellular proteins that associate with ERβ in the hippocampus using young (3 mo. old) and aged (18 mo. old) female rats. I also quantified how E_2 affected these protein:protein interactions at each age in order to better understand a potential mechanism for the differential effects of HT that have been observed in postmenopausal women. I specifically focused on proteins that associated with ERβ in the absence of DNA binding as an indicator of how age and/or E_2 affects non-conventional ERβ signaling pathways. Our approach was novel in that all experiments were performed *in vivo* in an out bred strain of rats using highly sensitive 2D-difference gel electrophoresis (2D-DIGE) coupled with liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) to both quantify and identify novel ERβ:protein interactions. Our results demonstrated that E_2 altered the association of ERβ with number of previously unidentified coregulatory proteins depending on age. Some of these novel proteins included actin binding proteins, mRNA alternative splicing proteins, and multifunctional metabolic proteins. Together, the work presented here sheds light on two important and very novel findings that further our understanding of the molecular and physiological functions of ERβ in the brain by 1) identifying novel ERβ:protein interactions that could delineate previously unknown roles for ERβ, and 2) by demonstrating how age and E_2 alters these protein interactions *in vivo*.

Results

Global quantification of ERβ protein associations as a function of age and E_2

To determine whether protein:protein interactions with ERβ are altered by age and E2 *in vivo*, female Fisher344 rats (3 mo (N = 40)- and 18-mo (N= 39)) were bilaterally ovariectomized (OVX) and allowed to recover post-OVX for 7 days. After 7 days post-OVX the animals received once/day subcutaneous injections of 2.5ug/kg E_2 or safflower oil (vehicle) for 3 consecutive days. Animals were sacrificed by rapid decapitation 24 hours after the last injection and trunk blood and brains were collected further analysis (Fig. 13). Circulating E_2 was measured by using an enzyme-linked immunoassay system (EIA, Cayman Chemical). The limit of detection for the assay was 6.6 pg/ml. Levels of plasma E_2, for young treated animals were determined to be 53.67 (SEM+/- 7.24) pg/ml and 50.56 (SEM+/- 8.78) pg/ml in aged animals, within the physiological range for post-menopausal patients receiving hormone replacement therapy (17-75pg/ml) (Schmidt *et al.*, 1994), but E2 levels in vehicle treated animals were not detectable (Fig. 14).

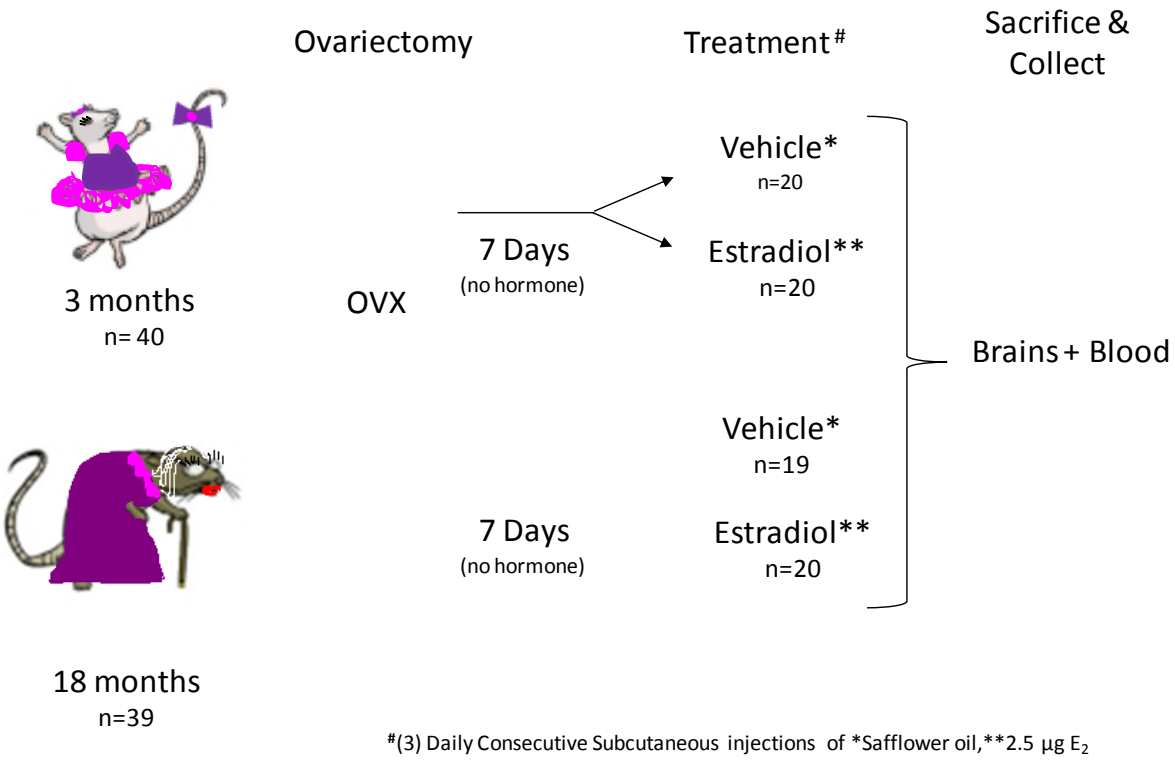


Figure 13. Hormone treatment paradigm. Female Fisher 344 rats (3-mo (N = 40) and 18-mo (N= 39)) were bilaterally ovariectomized (OVX). After 7 days post-OVX the animals received once/day subcutaneous injections of 2.5ug/kg 17β-estradiol or safflower oil (vehicle) for 3 consecutive days. Animals were sacrificed by rapid decapitation 24 hours after the last injection and trunk blood and brains were collected further analysis.

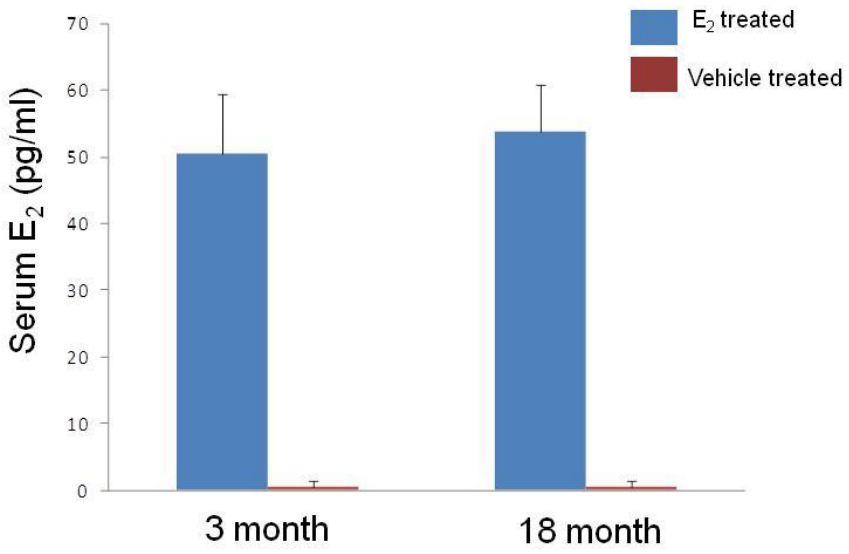


Figure 14. Serum E_2 levels from young and aged animals following treatment paradigm. Circulating 17β-estradiol was measured by using an enzyme-linked immunoassay system (EIA, Cayman Chemical). Trunk blood was collected in tubes coated with 20-50 units of porcine heparin (Sigma) per ml of blood collected. Blood was then centrifuged at 4000 x g for 7 minutes and plasma was removed subjected to immunoassay per manufacturer's instructions. The limit of detection for the assay was 6.6 pg/ml.

To examine the molecular weights of the proteins co-immunoprecipitated with ERβ, surface-enhanced laser desorption ionization time-of-flight (SELDI-tof) mass analysis was performed rather than a 1 dimensional molecular weight gel to make best use of scarce samples obtained *in vivo.* In general it was determined that 1-D analysis of the samples for molecular weight using a gel or mass spectrometry alone was insufficient to examine the array of proteins in the samples (Fig. 15).

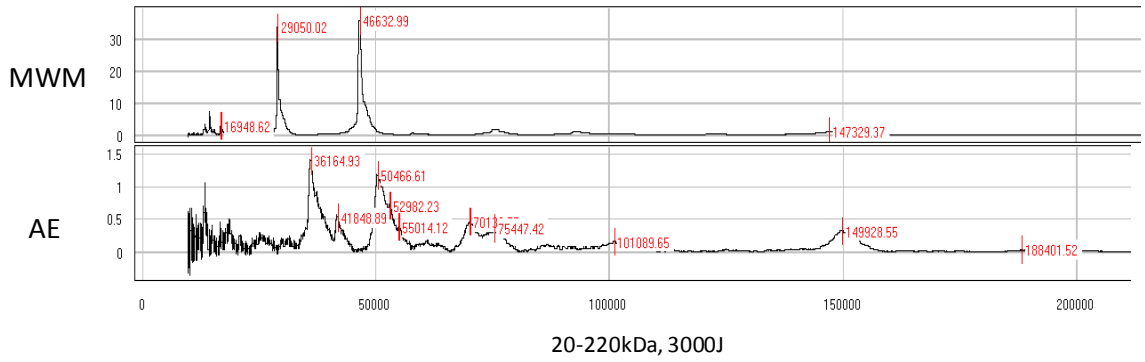


Figure 15. Representative SELDI-tof mass analysis of proteins co-immunoprecipitated with ERβ in the ventral hippocampus. Following co-immunoprecipitation of ERβ and associated proteins, samples (aged E_2-treated, AE shown) were subjected to SELDI-tof mass analysis using an assortment of standard peptides as a molecular weight marker (MWM).

Global quantification of ERβ protein associations as a function of age and E_2

To determine changes in protein 'spots' co-immunoprecipitated with ERβ from the ventral hippocampus, proteins were subjected to 2D-DIGE and the protein spot patterns were analyzed using DeCyder software (GE Healthcare) described in more detail in Figure 16. Briefly, Cy5 and pooled internal standards from each experimental group were labeled with Cy3. Cy3 and Cy5 labeled proteins were resolved first on a 3-11NL Immobiline isoelectric focusing gel (GE Healthcare) and then resolved for molecular weight on a 12% SDS-PAGE gel. Gel plugs representing spots of interest were excised from preparative gels and subjected to LC-ESI-MS/MS for peptide identification (Fig. 16).

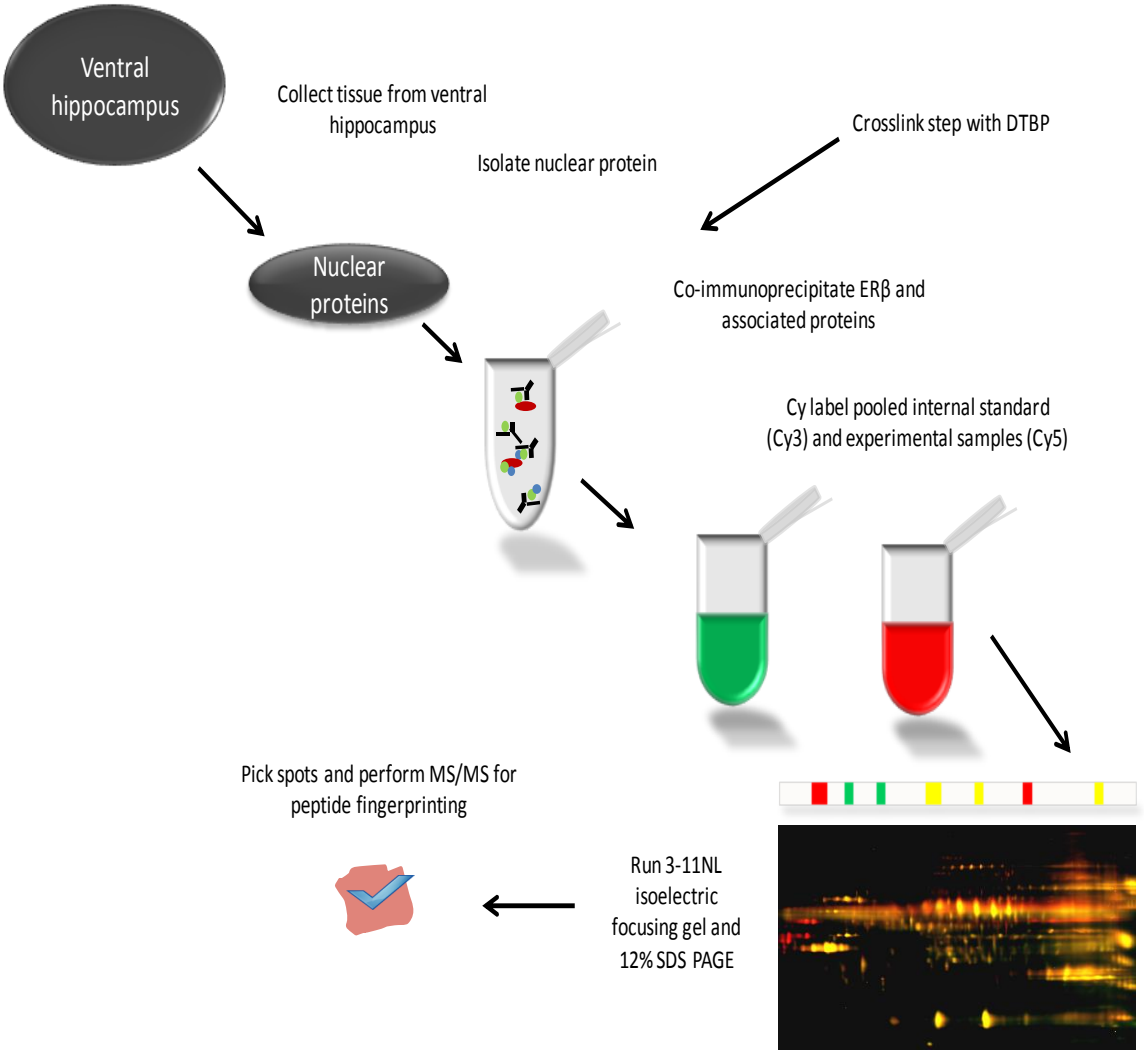


Figure 16. Proteomic experimental design. The ventral hippocampus was microdissected and snap frozen (-80^{0}C). Nuclear proteins were isolated and subjected to co-immunoprecipitation of ERβ (or crosslinked prior with DTBP). After preparing samples for 2D-electrophoresis, experimental samples were labeled with Cy Dyes (standard Cy3, experimental Cy5) and subjected to isoelectric focusing and subsequent separation of proteins based on molecular weight. Finally, spots of interest were excised from a preparative gel and subjected to LC-ESI-MS/MS and peptide fingerprinting for identification.

Overall, there were a total of 19 protein spots that were significantly altered with E_2 between all young and aged replicates examined (19/741), equivalent to 2.56% of total protein spots examined in this paradigm (Fig. 17). I was able to identify 17 of the 19 proteins interacting with ERβ that were significantly altered by E_2, dependent upon age (Fig. 18). Notably, E_2 treatment significantly altered ERβ association with all 19 protein spots in the young animals, yet only 5 of the 19 spots were changed in aged animals. Quantification of individual gels demonstrated that E_2 treatment significantly increased the log standard abundance of 7 protein spots, and decreased the log standard abundance of 12 protein spots in young animals (young vehicle: (YV) to young E2: (YE), Table 1, Fig. 17A). By stark contrast, E_2 treatment increased only 3 spots in aged animals and decreased just 2 spots (aged vehicle: (AV) to aged E2: (AE), Table 1, Fig.17B). Interestingly, E_2 treatment in aged animals failed to significantly alter the log standard abundance of the majority of proteins that were co-immunoprecipitated with ERβ.

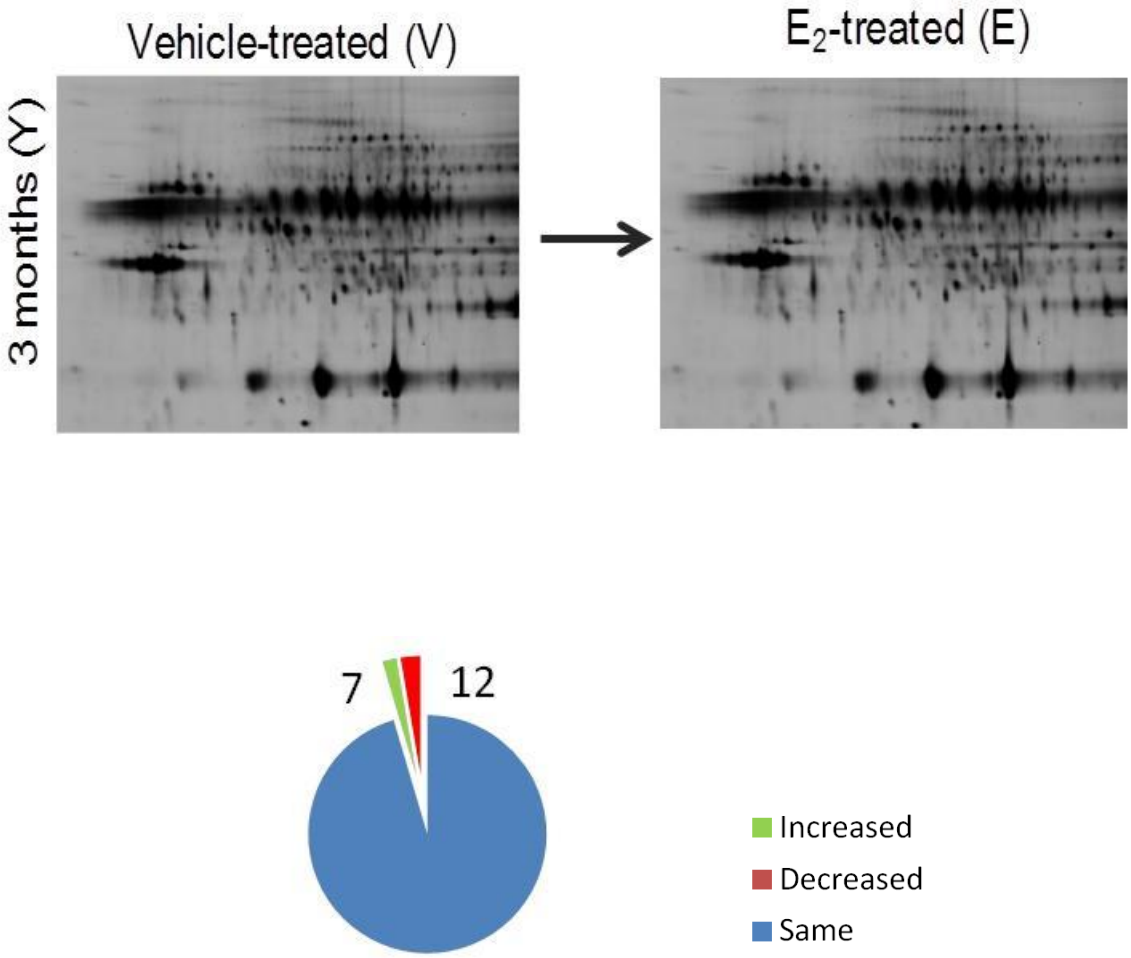


Figure 17A. Representative analytical gel images of 3 month old vehicle and E_2 treated samples and overall number of protein spots altered by E2 treatment. Pie chart represents the total number of protein spots that were significantly changed from vehicle to estradiol groups. Total number of protein spots calculated from all confirmed and matched spots in BVA module. Increased/decreased spots correspond to protein spots that displayed a significantly increased/decreased standard abundance compared to vehicle treatment, indicating and increase in association with ERβ. (n=3, 1-way ANOVA $p<0.05$)

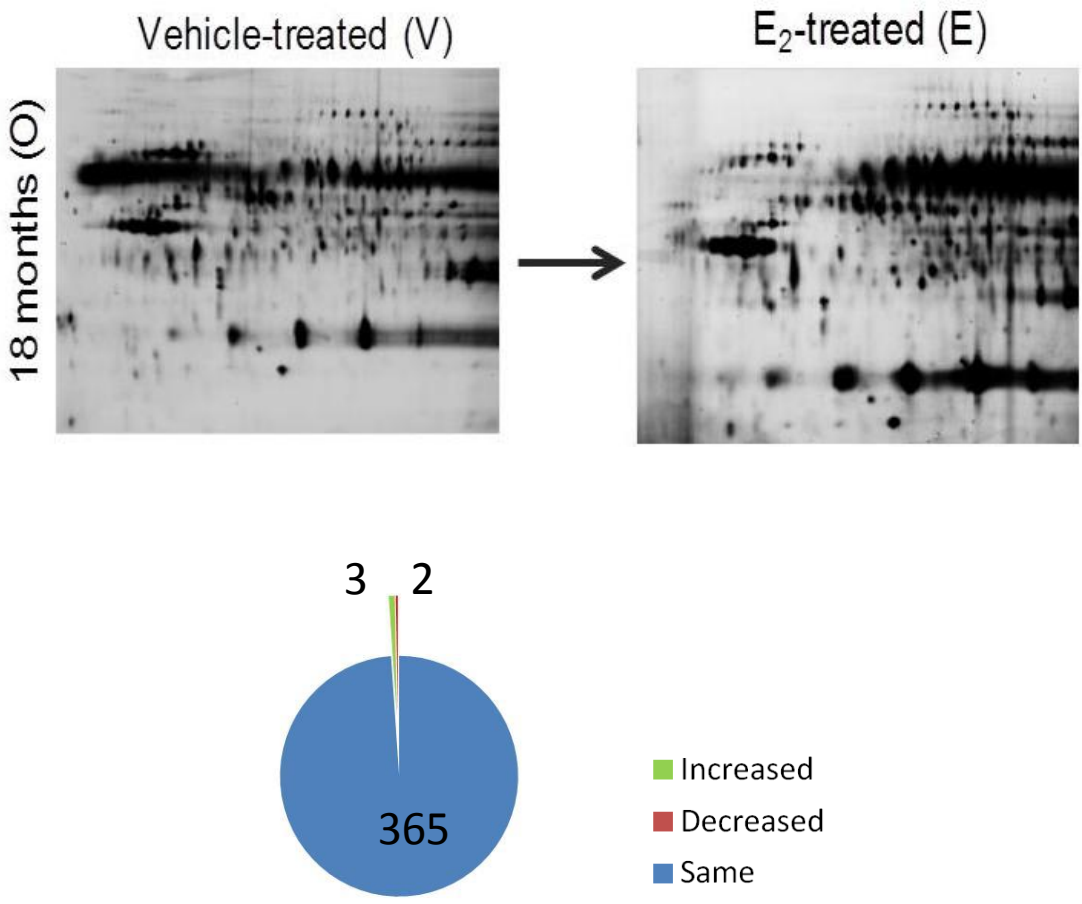


Figure 17B. Representative analytical gel images of 18 month old vehicle and E_2 treated samples and overall number of protein spots altered by E_2 treatment. Pie chart represents the total number of protein spots that were significantly changed from vehicle to estradiol groups. Total number of protein spots calculated from all confirmed and matched spots in BVA module. Increased (green)/decreased (red) spots correspond to protein spots that displayed a significantly increased/decreased standard abundance compared to vehicle treatment, indicating and increase in association with ERβ. (n=3, 1-way ANOVA $p<0.05$)

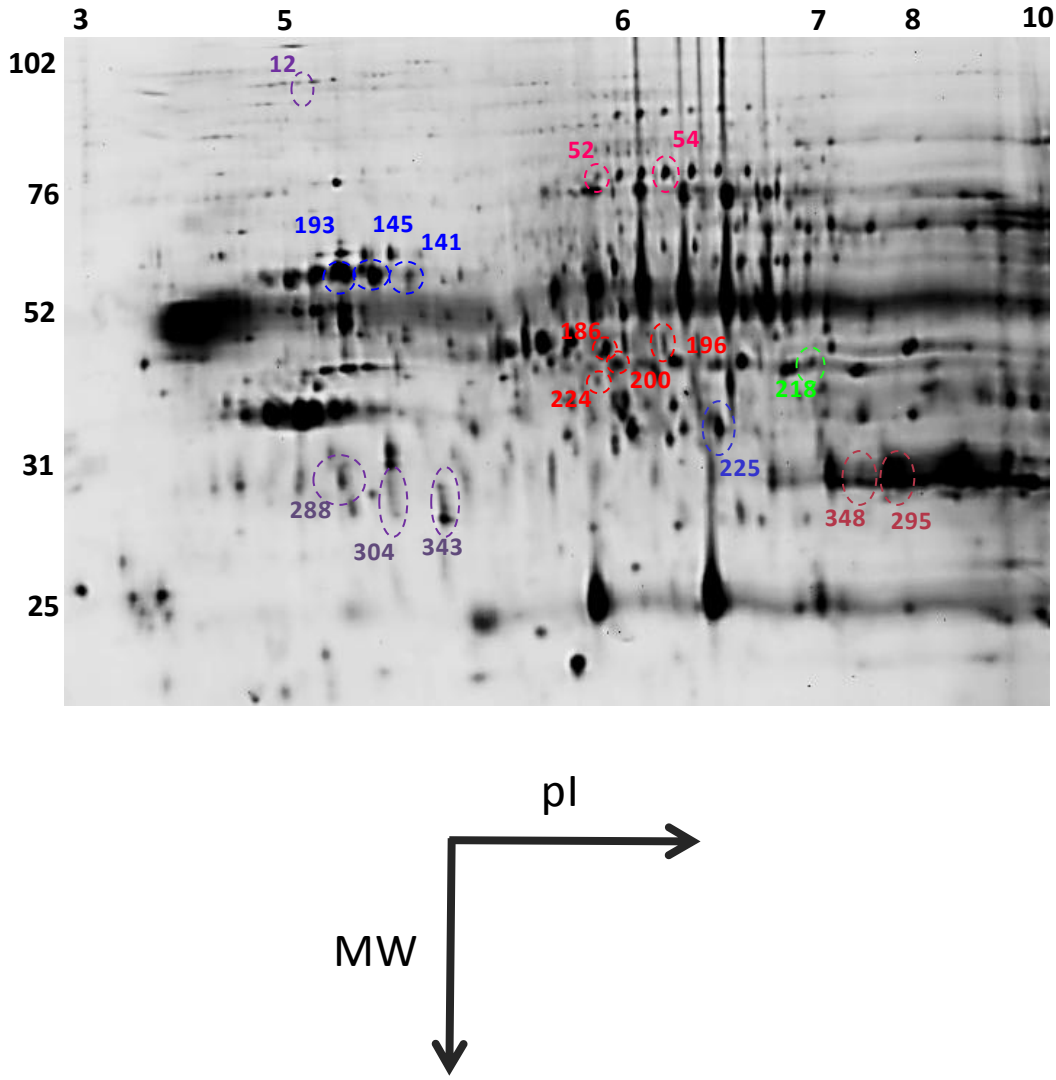


Figure 18. Representative 2D-gel image for identified proteins co-immunoprecipitated with ERβ in the ventral hippocampus that were altered by age or E_2 treatment. Representative 2D-image of Cy labeled proteins (indicated by BVA no.) co-immunoprecipitated with ERβ that were significantly altered by age or E_2 treatment with from rat ventral hippocampus (N=3, 1-way ANOVA, $p<0.05$).

Identified proteins altered by age and E_2												
Pick Spot No.	BVA Spot No.	Accession No.	Molecular weight (Kda)	Estimated Isoelectric Point	PEAKS score	% Coverage	ID	Log Standard Abundance				Function
								YV	YE	OV	OE	
22	288	gi|28373861	36	4.92	99	25.71	Annexin V (ANXAV) Spot A	0 -	0.05 ↑	-0.06 -	0 ↑	multifunctional
22	304	gi|28373862	36	4.92	99	25.71	Annexin V (ANXAV) Spot B	-0.26 -	-0.06 ↑	0.04 -	-0.04 ↓	multifunctional
22	343	gi|28373863	36	4.92	99	25.71	Annexin V (ANXAV) Spot C	-0.03 -	0.24 ↑	0.03 -	-0.03 -	multifunctional
15	195	gi|120538378	47	5.7	93.2	10.72	Heterogeneous nuclear ribonucleoprotein H (HnRNPH) Spot A	-0.03 -	0.23 ↑	-0.05 -	-0.01 -	RNA splicing
15	224	gi|120538378	47	5.7	93.2	10.72	Heterogeneous nuclear ribonucleoprotein H (HnRNPH) Spot B	-0.09 -	0.09 ↑	0.05 -	0.01 -	RNA splicing
15	186	gi|120538378	47	5.7	93.2	10.72	Heterogeneous nuclear ribonucleoprotein H (HnRNPH) Spot C	0.01 -	0.12 ↑	0.12 -	0.08 ↓	RNA splicing
15	200	gi|120538379	47	5.7	93.2	10.72	Heterogeneous nuclear ribonucleoprotein H Spot D	-0.15 -	0.03 ↑	-0.04 -	-0.01 -	RNA splicing
5	52	gi|149038929	80	5.75	49.4	6.43	Gelsolin (GELS) Spot A	-0.12 -	0.09 ↑	-0.03 -	-0.02 -	actin binding, coactivator
5	54	gi|149038929	80	5.75	49.4	6.43	Gelsolin (GELS) Spot B	0.01 -	0.14 ↑	-0.08 -	-0.04 -	actin binding, coactivator
21	225	gi|62662279	38	6.97	99.1	26.01	Annexin A1(ANXA1)	-0.02 -	0.08 ↑	0.04 -	0.02 -	coactivator
9	141	gi|116242506	74	5.97	93	14.58	Heat shock protein 70 (HSP70) Spot A	-0.08 -	-0.03 ↓	0 -	0.07 -	chaperone
9	145	gi|116242506	74	5.97	93	14.58	Heat shock protein 70 (HSP70) Spot B	0.06 -	-0.18 ↓	0.01 -	0.06 -	chaperone
9	193	gi|116242507	75	5.97	93	14.58	Heat shock protein 70 (HSP70) Spot C	0.13 -	0.07 ↓	-0.02 -	0.05 ↑	chaperone
16	218	gi|158186649	47	6.16	98.6	19.35	Enolase 1 alpha (ENO1)/Myc binding protein (MBP)	0.06 -	-0.01 ↓	-0.18 -	-0.07 ↑	multifunctional
2	12	gi17865351	89	5.1	88.6	13.4	Valosin-containing protein (VCP)	0.21 -	0.02 ↓	0.13 -	0.03 -	multifunctional
20	295	gi|62662278	36	8.14	68.2	6.57	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Spot A	0.1 -	-0.12 ↓	0.06 -	0.05 -	coactivator/ metabolism
20	348	gi|62662278	36	8.14	68.2	6.57	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Spot B	0.04 -	-0.16 ↓	-0.07 -	0.05 -	coactivator/ metabolism

Table 1. Identified proteins altered by age and E_2. ↑ ↓ indicate statistically significant changes in log standard abundance (1-way ANOVA, $p>0.05$)

Due to the nature of quantitative 2D-DIGE experiments using scarce samples from *in vivo* experiments, only 2 groups can be reliably compared in any given gel (i.e, YV vs. YE), therefore I performed a second set of experiments comparing vehicle-only treated samples in young and aged animals. This analysis was performed to establish whether aging alone, in the absence of E_2, altered the baseline of the identified protein interactions. From the 19 proteins significantly altered by E_2 treatment in young and/or aged animals, only 1 (BVA Spot #295 (GAPDH spot A), data not shown) was significantly changed by age alone. The log standard abundance of BVA spot #295 GAPDH Spot A was significantly decreased in aged vehicle treated animals compared to young, suggesting that the baseline interaction between ERβ and GAPDH may decrease with age, regardless of E_2 bioavailablity. As previously mentioned, only 2.56% of all the proteins that co-immunoprecipitated with ERβ in this paradigm were altered by age and E_2 treatment. Unaltered proteins fell into the same functional categories as those that were changed including chaperone proteins, structural proteins, coactivators, DNA/RNA binding proteins and multifunctional proteins (Table 2 Fig. 18B.).

Identified proteins unaltered by age and E_2							
Pick Spot No.	Accession No.	Estimated Isoelectric Point	Molecular weight (Da)	PEAKS score	% Coverage	Protein ID	Category
1	gi|209862801	5.45	106790	71.5	2.89	Alanyl-tRNA synthetase (AARS)	translation
3	gi|84781723	6.56	80461	60.8	1.84	TNF receptor-associated protein 1 (TRAP1)	chaperone
4	gi|28467005	4.93	84815	98.9	17.87	Heat shock protein 90 (HSP90A)	chaperone
4	gi|91234898	4.97	83282	98.9	21.13	Heat shock protein 84 (HSP90B)	chaperone
6	gi|6981504	8.29	83501	60.9	1.14	Spinocerebellar ataxia 1 (AT-1)	coactivator
7	gi|54400730	5.96	57458	90.8	5.23	T-complex 1 (TCP1)	chaperone
7	gi|149023097	5.88	53587	61.4	8.98	Glucose regulated protein 58 (GRP58)	chaperone
8	gi|25742763	5.97	72347	94.3	23.24	Glucose regulated protein 78 (GRP78)	chaperone
10	gi|12053837	5.78	47057	94.3	23.24	Glucose regulated protein 75 (GRP75)	chaperone
11	gi|38328248	4.93	50164	92.8	22.84	Alpha tubulin (TUBBA)	structural
11	gi|20799322	4.73	2050066	75.2	20.49	Neuron-specific class III beta tubulin	structural
12	gi|56383	5.91	60966	69.4	10.47	Heat shock protein (HSP60)	chaperone
12	gi|149028522	5.98	68350	60.9	1.47	Minichromosome maintenance deficient 7 (MCM7)	DNA helicase
13	gi|149039794	5.39	42801	78.1	11.4	Heterogeneous nuclear ribonucleoprotein K (HnRNP K)	RNA processing
14	gi|58865414	7.53	54161	99	13.32	Annexin A11 (ANXA11)	multifunction
17	gi|13592093	5.19	41279	63.2	4.08	Heat shock protein 70 interacting protein (HIP)	Chaperone
17	gi|488838	5	47220	97.7	12.76	Calcium-binding protein 1 (CaBP1)	multifunction
17	gi|1374715	5.18	51203	97.4	10.95	ATP synthase beta subunit	metabolism
17	gi|25742677	5.09	47408	65.5	8.61	Proteasome 26S	proteasome
18	gi|149053068	5.32	39540	94	7.76	Eukaryotic translation initiation factor 4A1 (EIF4A1)	RNA helicase
19	gi|119959830	5.29	31747	95	16.61	Beta actin (ACTA)	structural
19	gi|149043182	5.23	34264	93.8	7.53	Alpha actin (ACTB)	structural
19	gi|149036532	5.31	33670	97	12.58	Gamma actin (ACTG)	structural

Table 2. Identified proteins unaltered by age and E_2

Peptide fingerprinting and analysis of ERβ associated proteins.

In humans, E2 administration has dichotomous effects dependent upon age and/or menopausal status; however there is little biochemical evidence to explain this phenomenon. Our results showed that E_2 treatment differentially altered ERβ associated proteins in young, compared to aged animals, providing evidence for a putative mechanism for the age-dependent effects of E_2. I identified several clusters of protein spots based on their shared isoelectric point, molecular weight, and migration pattern (Fig. 19). Some spot clusters were pooled into a single sample for peptide fingerprinting using LC-ESI-MS/MS, and the proteins with the highest PEAKS score (>60), matching molecular weight and isoelectric range were identified as representing the entire cluster. These results were confirmed by subjecting replicate spots within a cluster for peptide identification. Finally, these clusters have been functionally grouped into those that had an overall increase and those that had an overall decrease in log standard abundance following E_2 treatment in young animals.

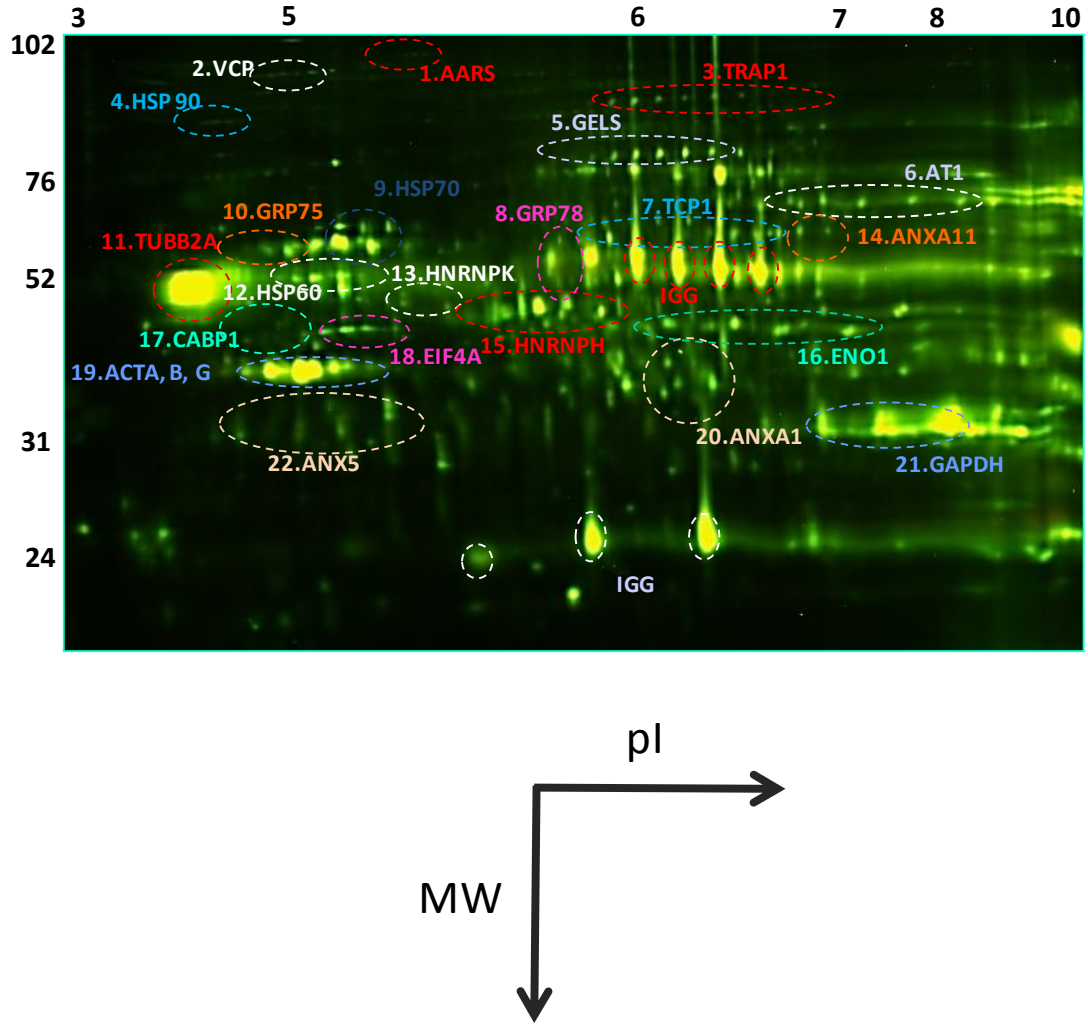


Figure 19: Representative 2D-gel image for identified protein clusters co-immunoprecipitated with ERβ in the ventral hippocampus. Representative 2D-image of Cy-labeled proteins co-immunoprecipitated with ERβ identified by tandem mass spectrometry (PEAKS score >60) from rat ventral hippocampus.

Spot clusters that increased in E2-treated young animals.

Annexin V (ANXAV) was identified as the most highly abundant protein in a cluster of 3 spots that changed following E_2 treatment. Further, E_2 treatment had quantitatively distinct effects on the log standard abundance of each spot within this cluster. For instance, each spot increased following E_2 treatment in young animals (Table 1: BVA spot #288 (spot A), 304 (spot B), and 343 (spot C); Fig. 20). However, E2 treatment in aged animals increased the log standard abundance of spot A, decreased spot B, and had no significant effect on spot C (Table 1, Fig. 20). Moreover, BVA Spot No. 288, identified in a cluster of spots as Annexin V, was the only protein spot that E_2 similarly affected (increased) in both young and old animals.

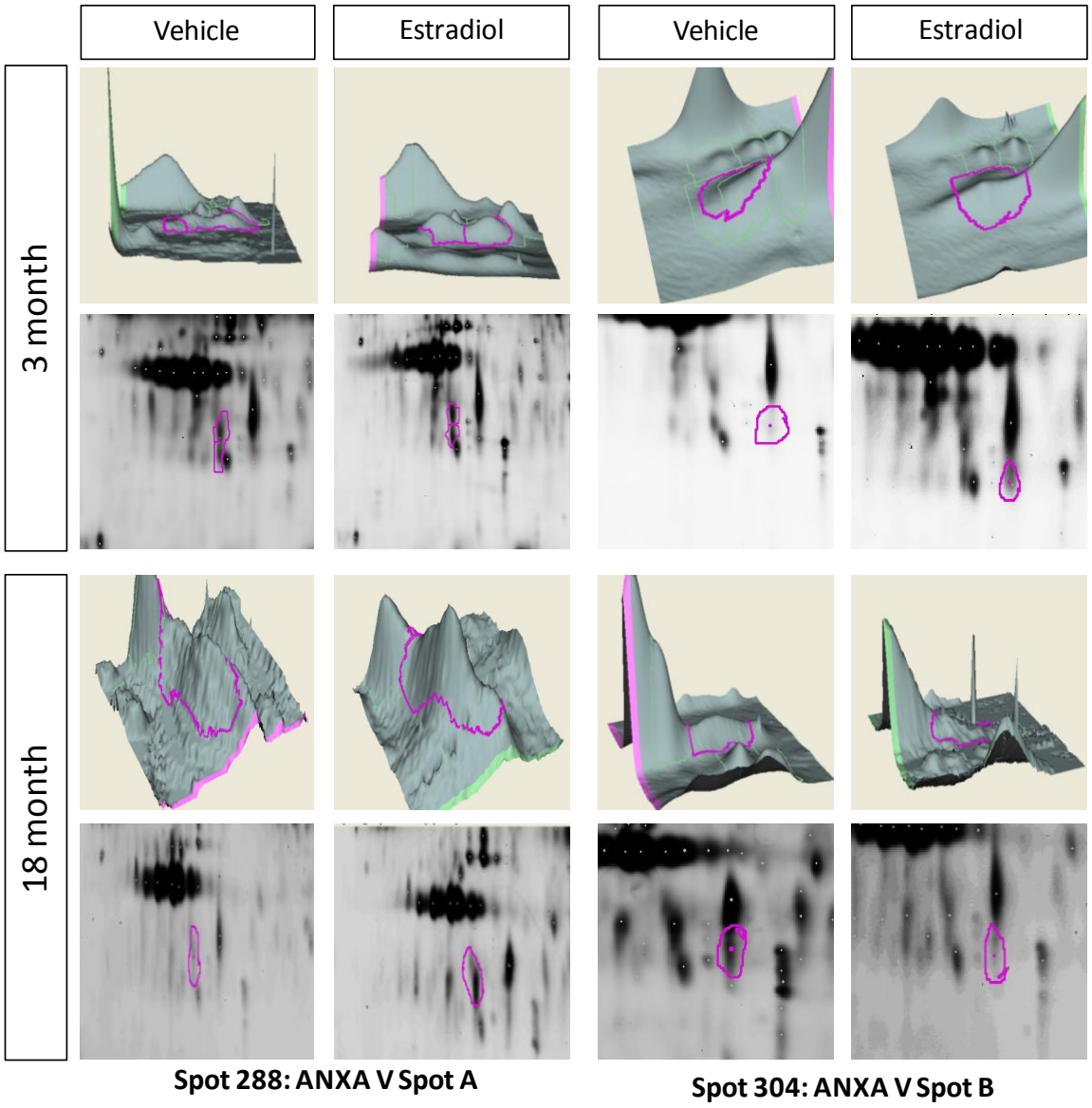


Figure 20A. DeCyder topographical, gel image analysis and average log standard abundance of annexin V (ANXAV) in response to E_2 in young and aged animals. For each panel from top left to right: 3 month: YV representative topography, YE representative topography, YV representative gel image, YE representative gel image; 18 month: AV representative topography, AE representative topography, AV representative gel image, AE representative gel image.

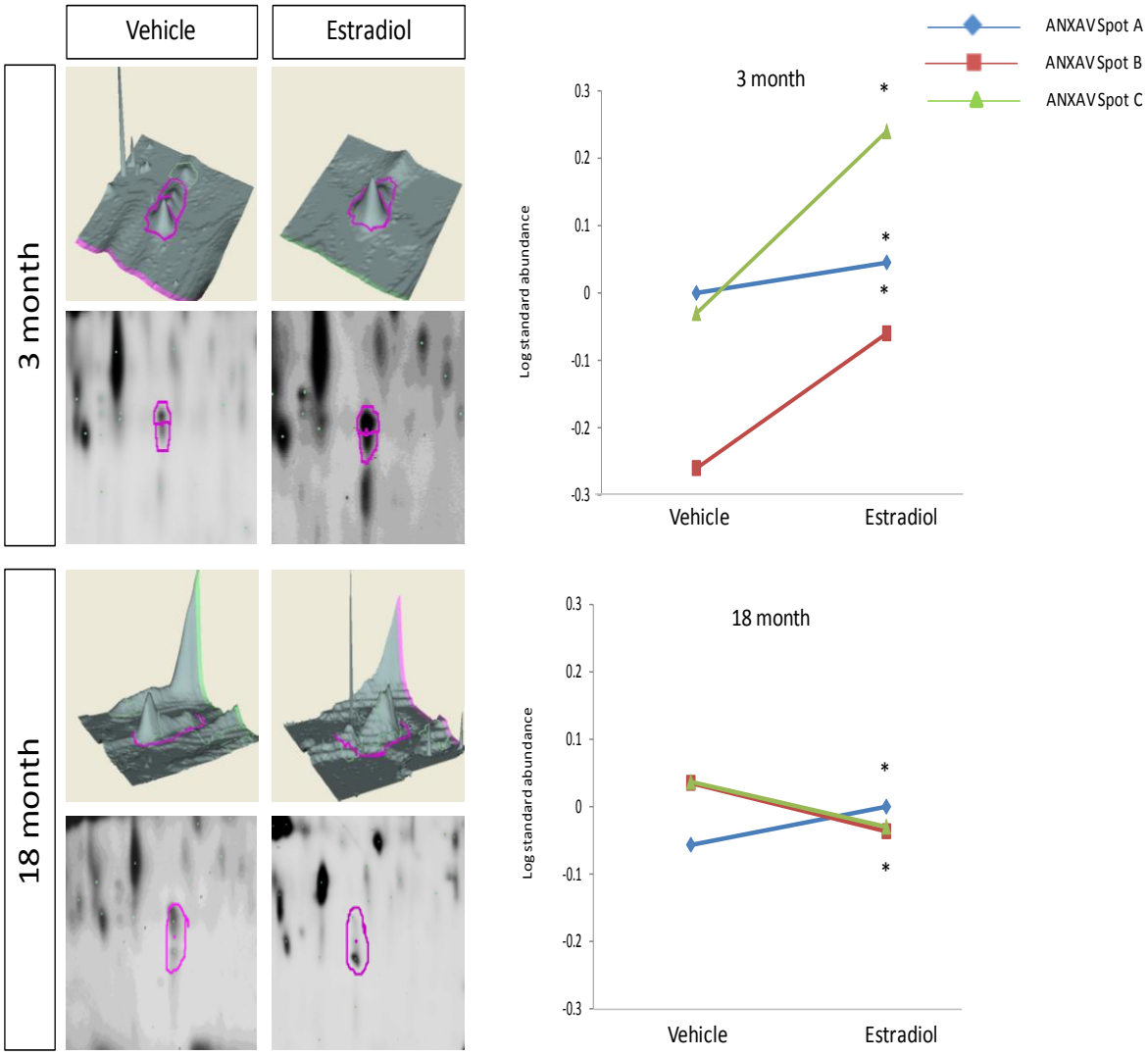


Figure 20B. DeCyder topographical, gel image analysis and average log standard abundance of annexin V (ANXAV) in response to E_2 in young and aged animals. For each panel from top left to right: 3 month: YV representative topography, YE representative topography, YV representative gel image, YE representative gel image; 18 month: AV representative topography, AE representative topography, AV representative gel image, AE representative gel image. Graphs represent log transformed average abundance normalized to internal standard and matched to master gel. (Top 3-mo., Bottom, 18-mo.) Average calculated from 3 independent experiments with a biological variance of 4 pooled animals/experiment. (N=3, BV=15) * denotes significance from vehicle $p<0.05$.

Similar to the ANXAV cluster, E_2 treatment significantly increased ERβ association with all 4 spots identified as HnRNP H in young animals (Table 1: BVA spot #195 (spot A), #224 (spot B), #186 (spot C), #200 (spot D); Fig. 21). By contrast, E2 treatment did not increase any of these spots in the aged animals, as spot C was significantly decreased and the other 3 were unaffected (Table 1, Fig. 21).

Young animals treated with E_2 had a significant increase in 2 spots identified as a cluster of gelsolin proteins (GELS, Table 1: BVA #52 (spot A), BVA #54 (spot B); Fig. 22), yet E_2 had no effect in aged animals. Notably, in this cluster GELS was the only predominant peptide match in the group of spots selected that corresponded to its approximate isoelectric point and size. Moreover, this spot cluster was split into three samples and GELS was the only protein identified and it was observed in all 3 samples (Spot 52, 54 and 56 (not significantly changed by E_2)), despite a PEAKS score lower than 60 (Pick Spot No. 5, PEAKS score of 49.5).

Similar to the results from GELS, log standard abundance of annexin 1 (ANXA1, Table 1: BVA #225, Fig. 23) was significantly increased by E_2 in young animals but was not significantly altered by E_2 in aged animals. Taken together, these data suggest that E_2 may enhance some ERβ:protein interactions in young animals but has an opposite or little effect on the same interaction in older animals

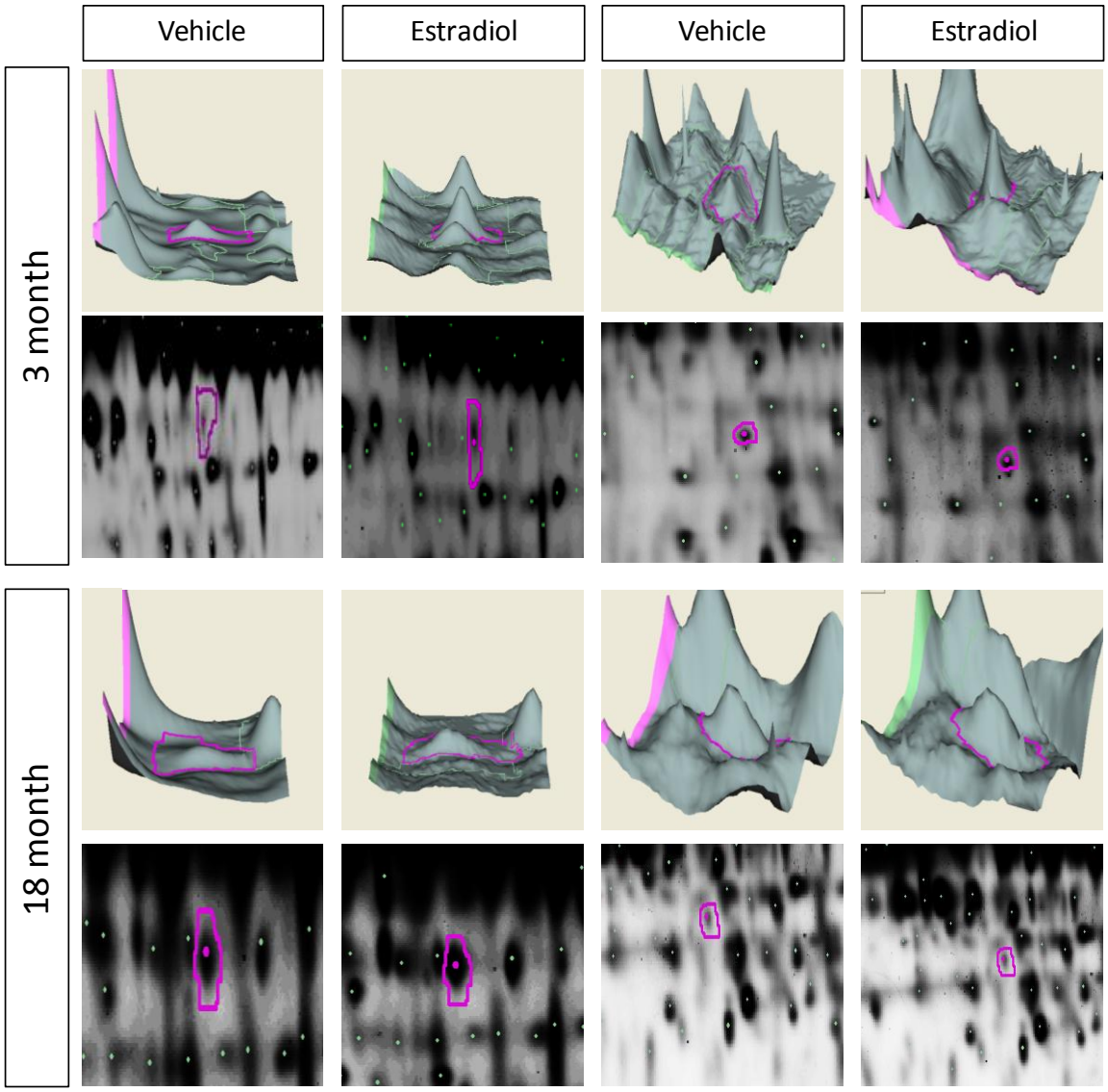


Figure 21A. DeCyder topographical, gel image analysis and average log standard abundance of heteronuclear riboprotein H (HnRNP H) in response to E_2 in young and aged animals. For each panel from top left to right: 3 month: YV representative topography, YE representative topography, YV representative gel image, YE representative gel image; 18 month: AV representative topography, AE representative topography, AV representative gel image, AE representative gel image.

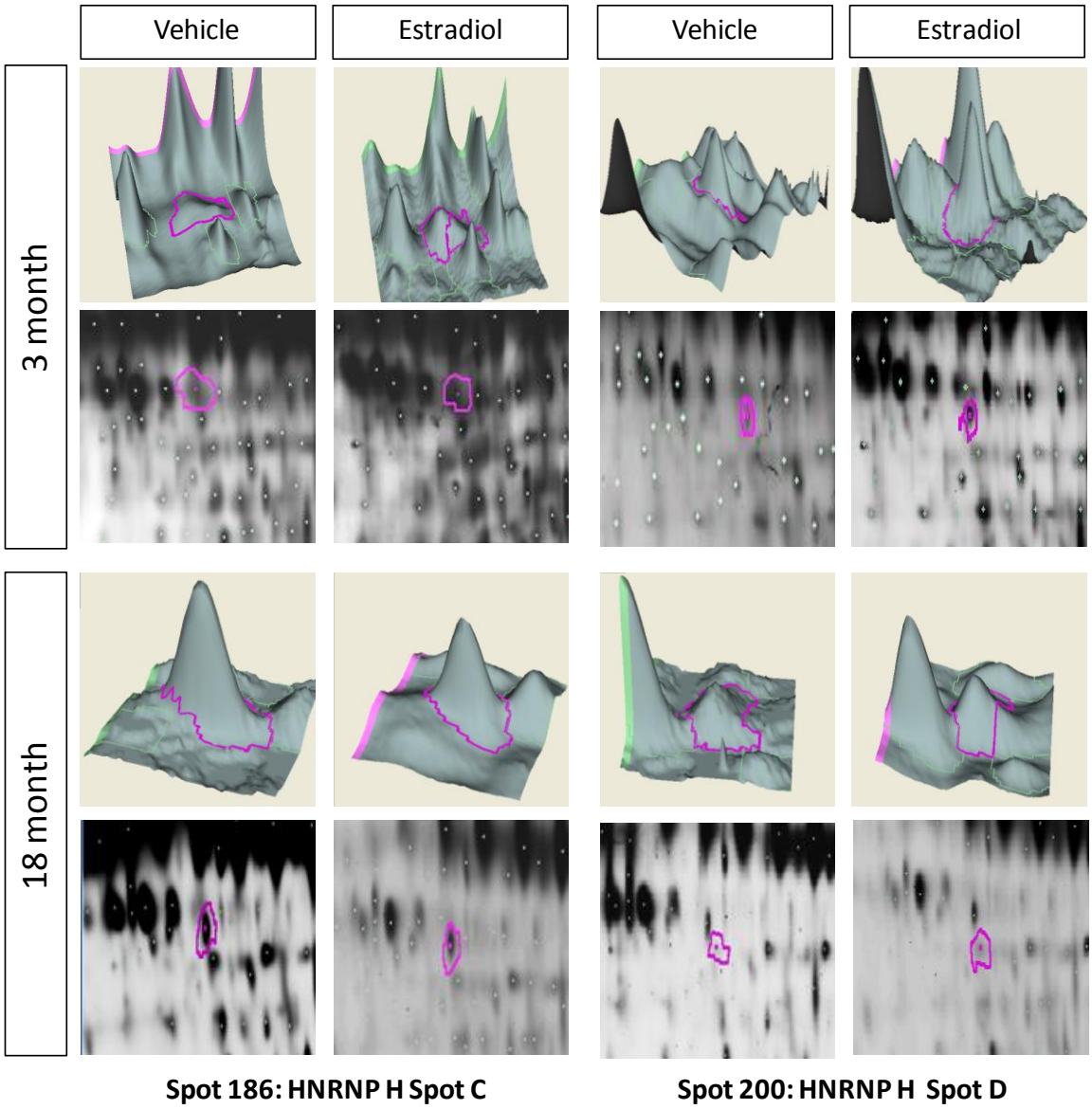


Figure 21B. DeCyder topographical, gel image analysis and average log standard abundance of heteronuclear riboprotein H (HnRNPH) in response to E_2 in young and aged animals. For each panel from top left to right: 3 month: YV representative topography, YE representative topography, YV representative gel image, YE representative gel image; 18 month: AV representative topography, AE representative topography, AV representative gel image, AE representative gel image.

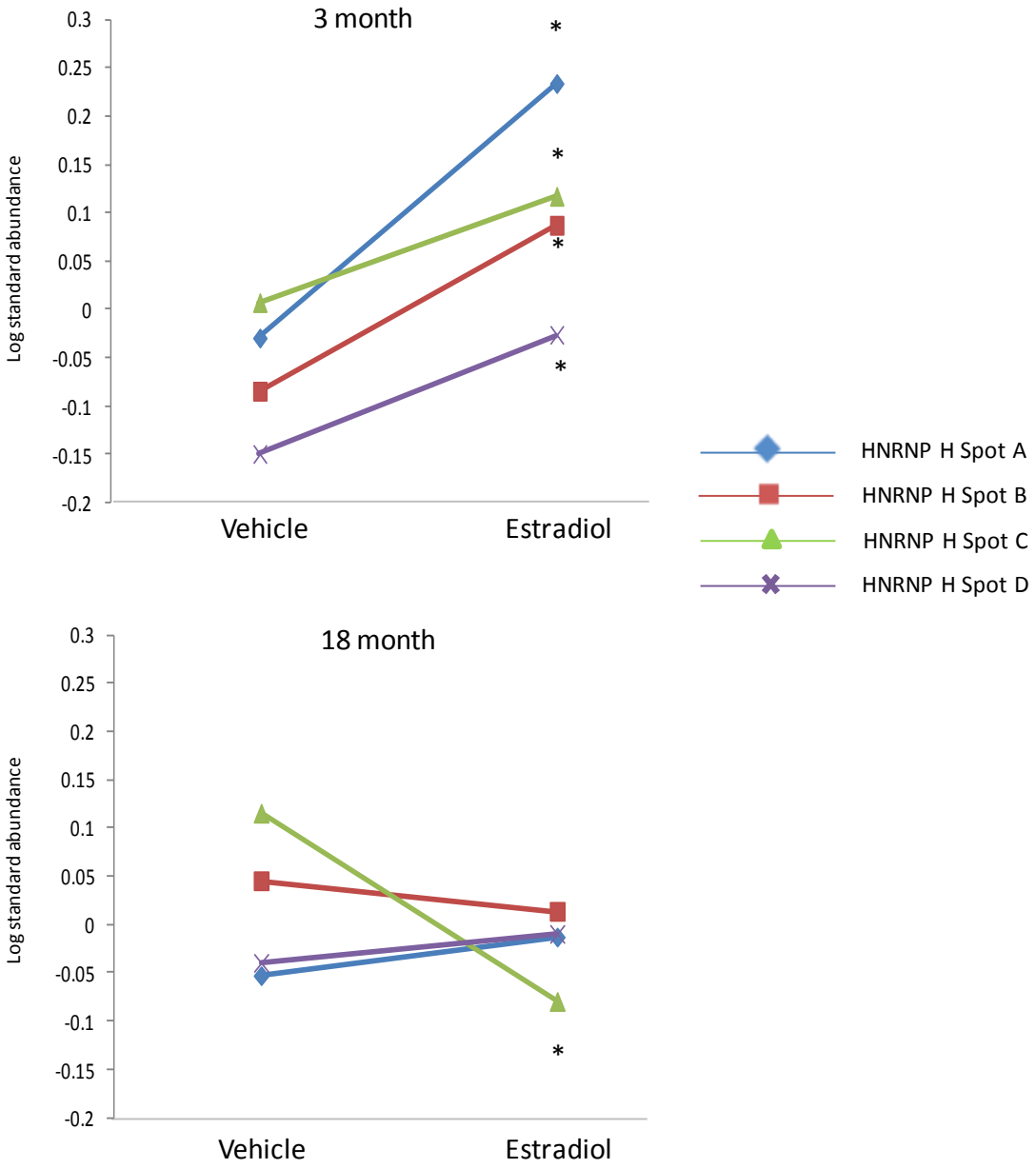


Figure 21C. DeCyder topographical, gel image analysis and average log standard abundance of heteronuclear riboprotein H (HnRNPH) in response to E_2 in young and aged animals. Graphs represent log transformed average abundance normalized to internal standard and matched to master gel. (Top 3-mo., Bottom, 18-mo.) Average calculated from 3 independent experiments with a biological variance of 4 pooled animals/experiment. (N=3, BV=15) * denotes significance from vehicle $p<0.05$.

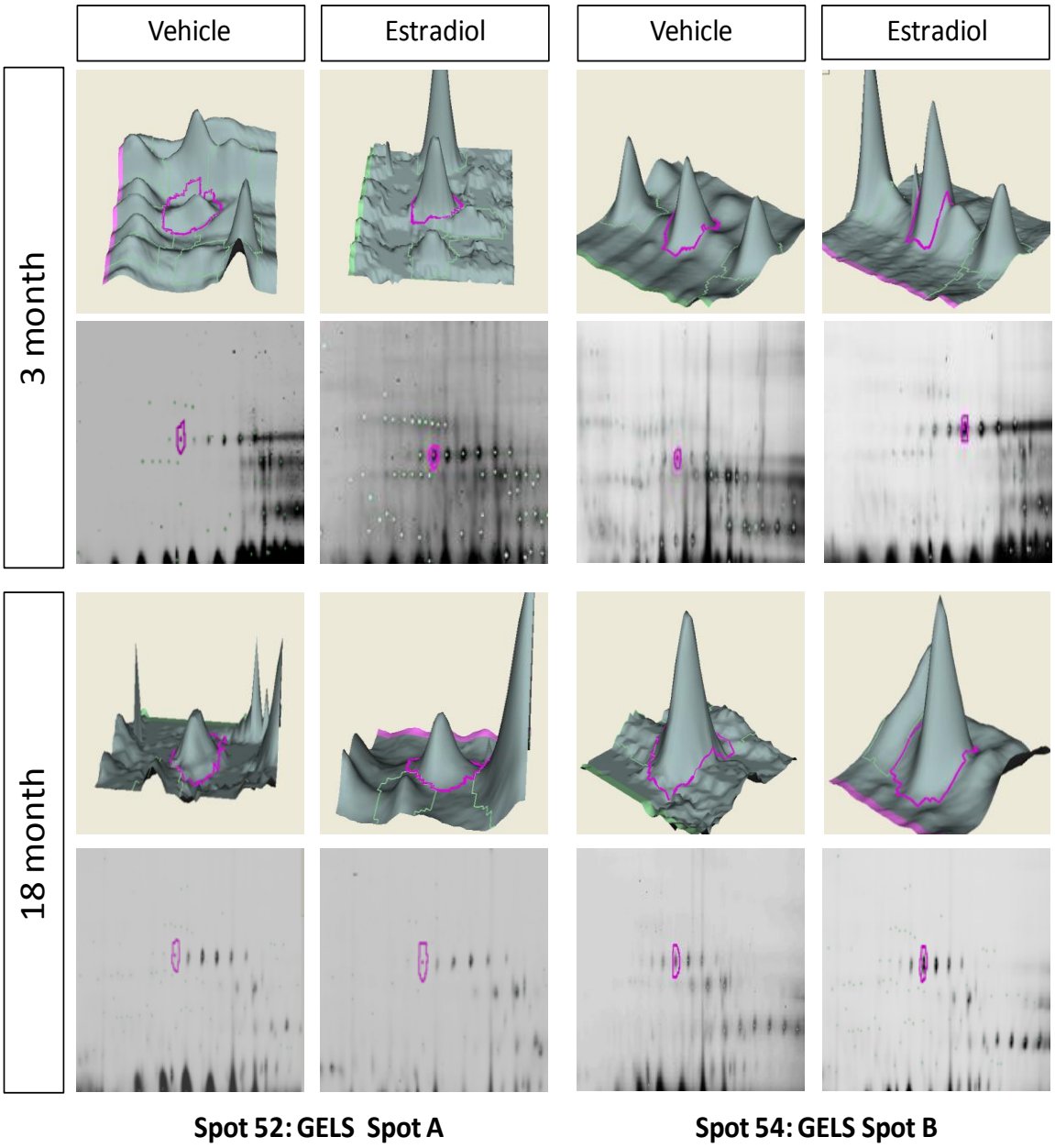


Figure 22A. DeCyder topographical, gel image analysis and average log standard abundance of gelsolin (GELS) in response to E_2 in young and aged animals. For each panel from top left to right: 3 month: YV representative topography, YE representative topography, YV representative gel image, YE representative gel image; 18 month: AV representative topography, AE representative topography, AV representative gel image, AE representative gel image.

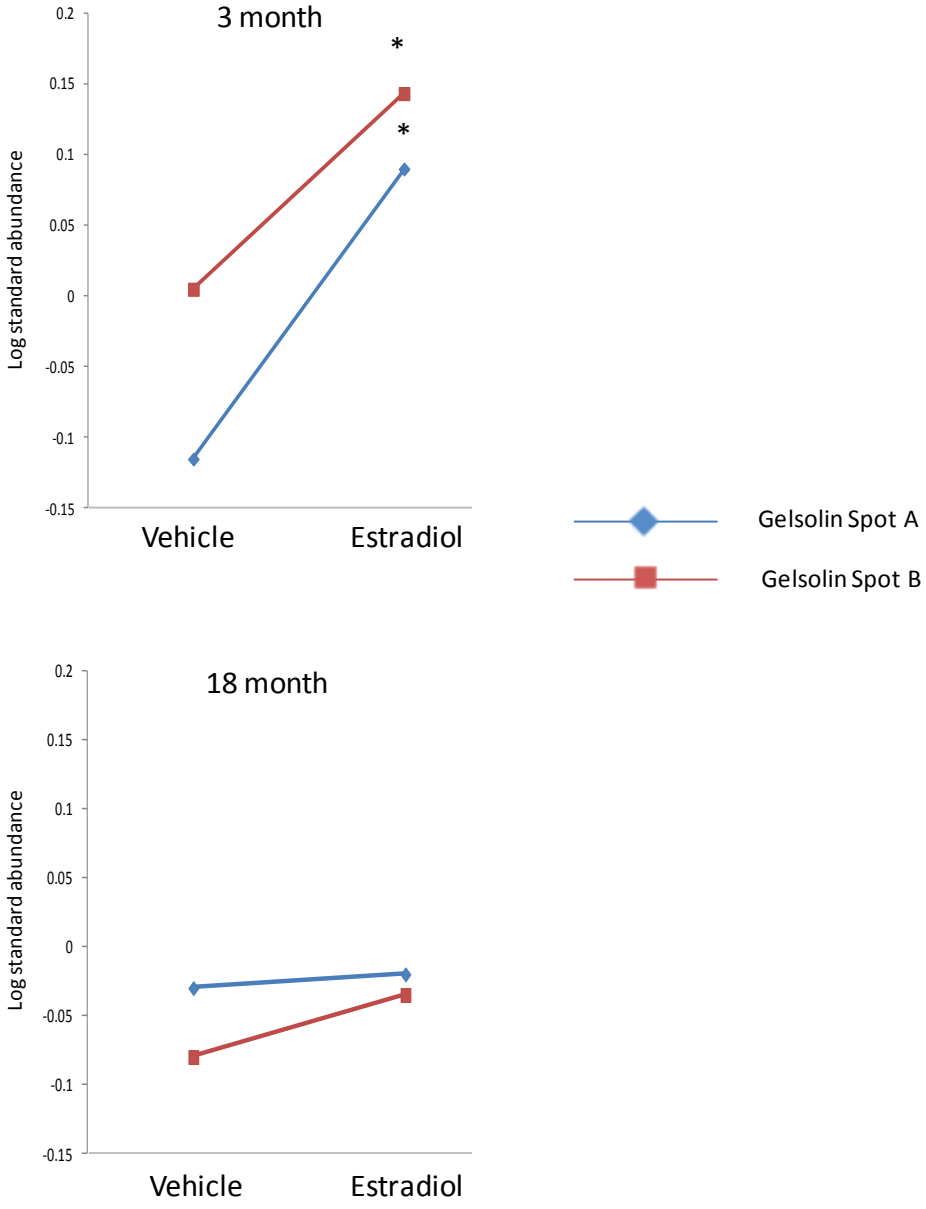


Figure 22B. DeCyder topographical, gel image analysis and average log standard abundance of gelsolin (GELS) in response to E_2 in young and aged animals Graphs represent log transformed average abundance normalized to internal standard and matched to master gel. (Top 3-mo., Bottom, 18-mo.) Average calculated from 3 independent experiments with a biological variance of 4 pooled animals/experiment. (N=3, BV=15) * denotes significance from vehicle $p<0.05$.

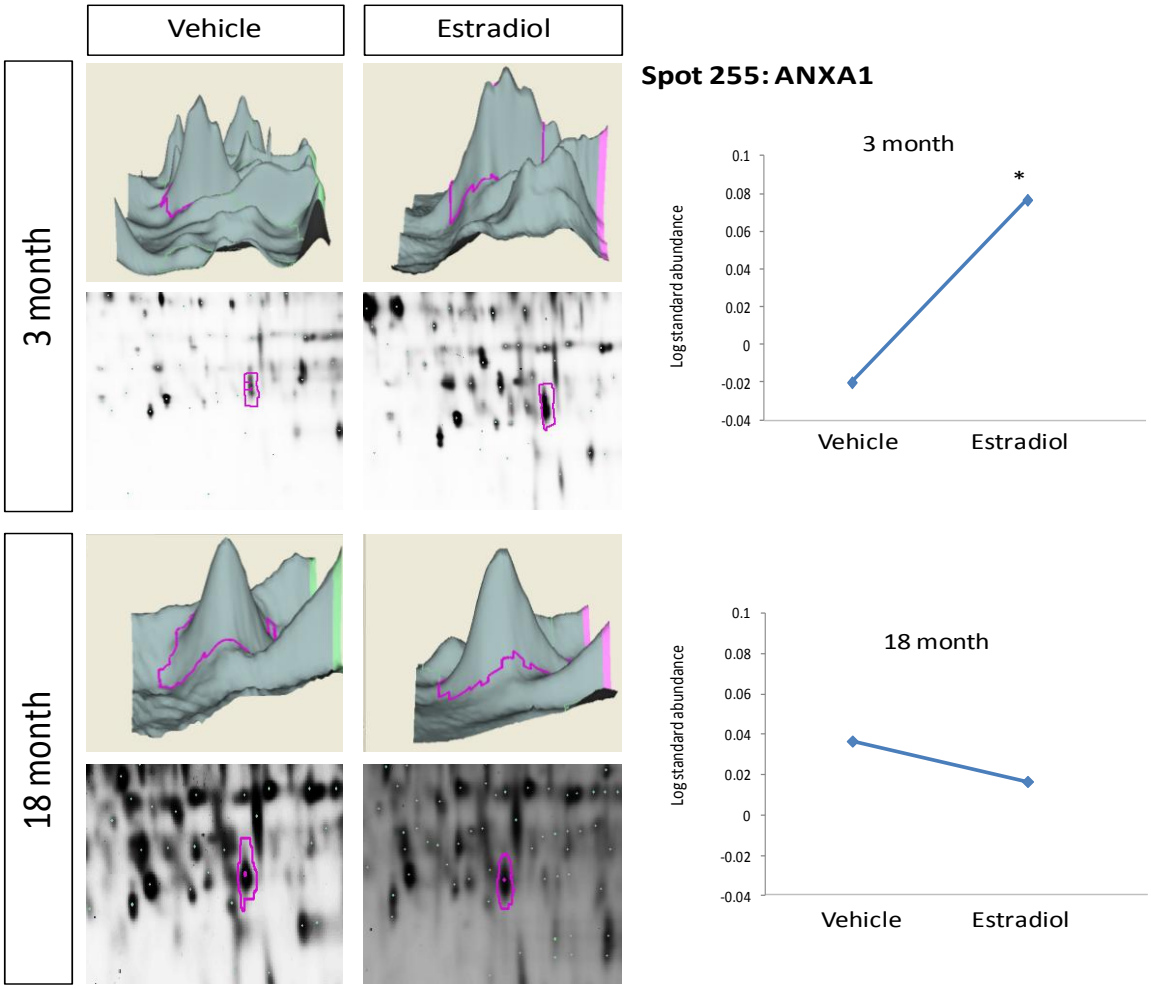


Figure 23. DeCyder topographical, gel image analysis and average log standard abundance of annexin 1(ANXA1) in response to E_2 in young and aged animals. For each panel from top left to right: 3 month: YV representative topography, YE representative topography, YV representative gel image, YE representative gel image; 18 month: AV representative topography, AE representative topography, AV representative gel image, AE representative gel image. Graphs represent log transformed average abundance normalized to internal standard and matched to master gel. (Top 3-mo., Bottom, 18-mo.) Average calculated from 3 independent experiments with a biological variance of 4 pooled animals/experiment. (N=3, BV=15) * denotes significance from vehicle $p<0.05$.

Spot clusters that decreased in E_2-treated young animals.

In addition to E_2-induced increases in ERβ protein associations, there were also proteins that showed a significantly decreased log standard abundance with ERβ following E_2 treatment. For example, BVA spot #141 (HSP70 spot A), #145 (HSP70 spot B) and #193 (HSP70 spot C) (Table 1; Fig. 24), corresponding to the spot cluster identified as HSP70, were all significantly decreased with E_2 treatment in young animals, yet the opposite effect was observed in aged animals for spot C, which was significantly increased (Fig. 24). HSP70 spots A and B remained unchanged following E_2 treatment in aged animals. Exhibiting a similar pattern, the BVA spot identified as α-enolase (ENO1, Table 1: BVA #218) was also significantly decreased with ERβ in response to E_2 treatment in young animals, and like HSP70 appeared to associate more readily with ERβ in aged animals treated with E_2 (Table 1, Fig. 25).

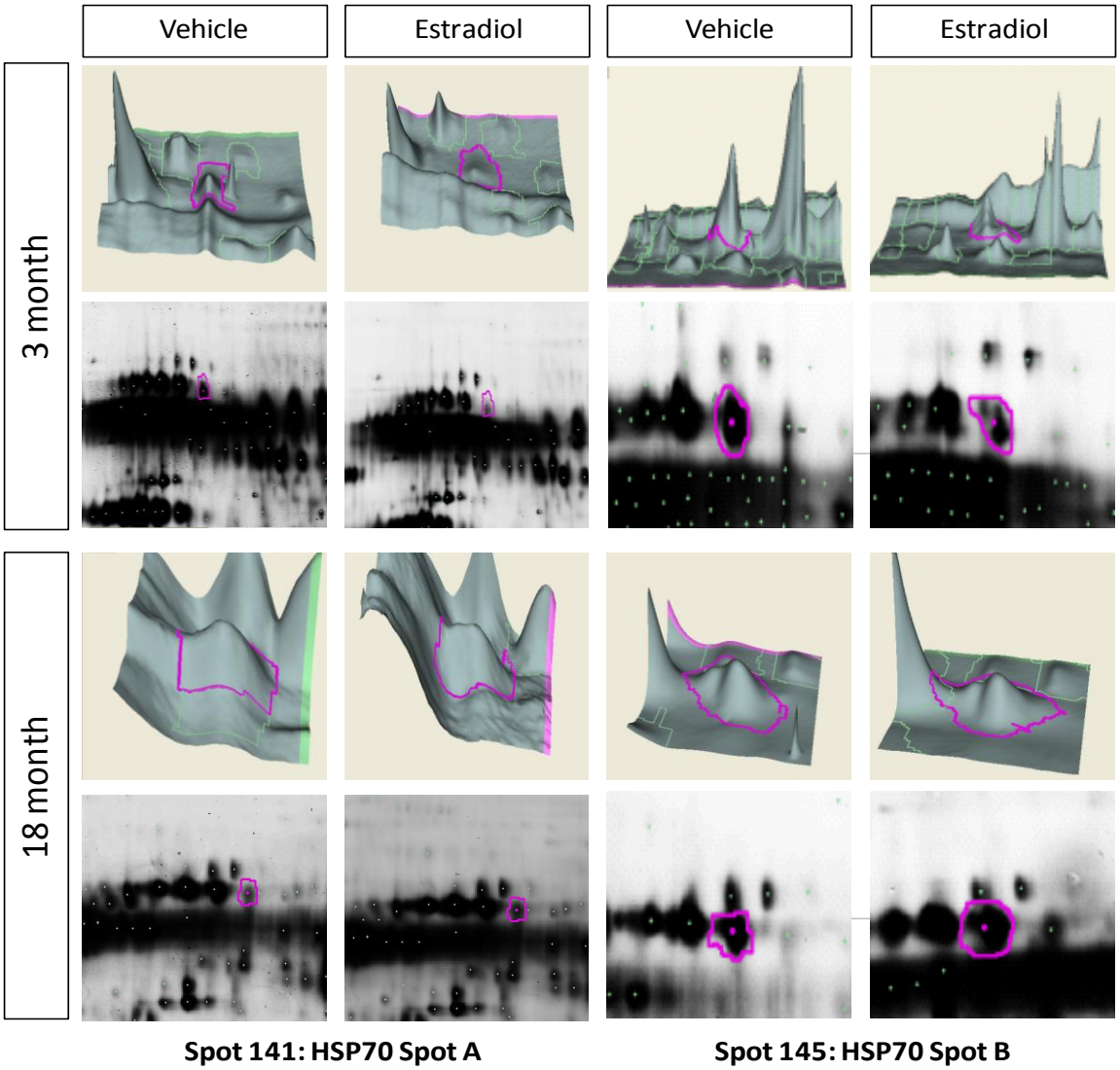


Figure 24A. DeCyder topographical, gel image analysis and average log standard abundance of heat shock protein 70 (HSP70) in response to E_2 in young and aged animals. For each panel from top left to right: 3 month: YV representative topography, YE representative topography, YV representative gel image, YE representative gel image; 18 month: AV representative topography, AE representative topography, AV representative gel image, AE representative gel image. Graphs represent log transformed average abundance normalized to internal standard and matched to master gel. (Top 3-mo., Bottom, 18-mo.) Average calculated from 3 independent experiments with a biological variance of 4 pooled animals/experiment. (N=3, BV=15) * denotes significance from vehicle $p<0.05$.

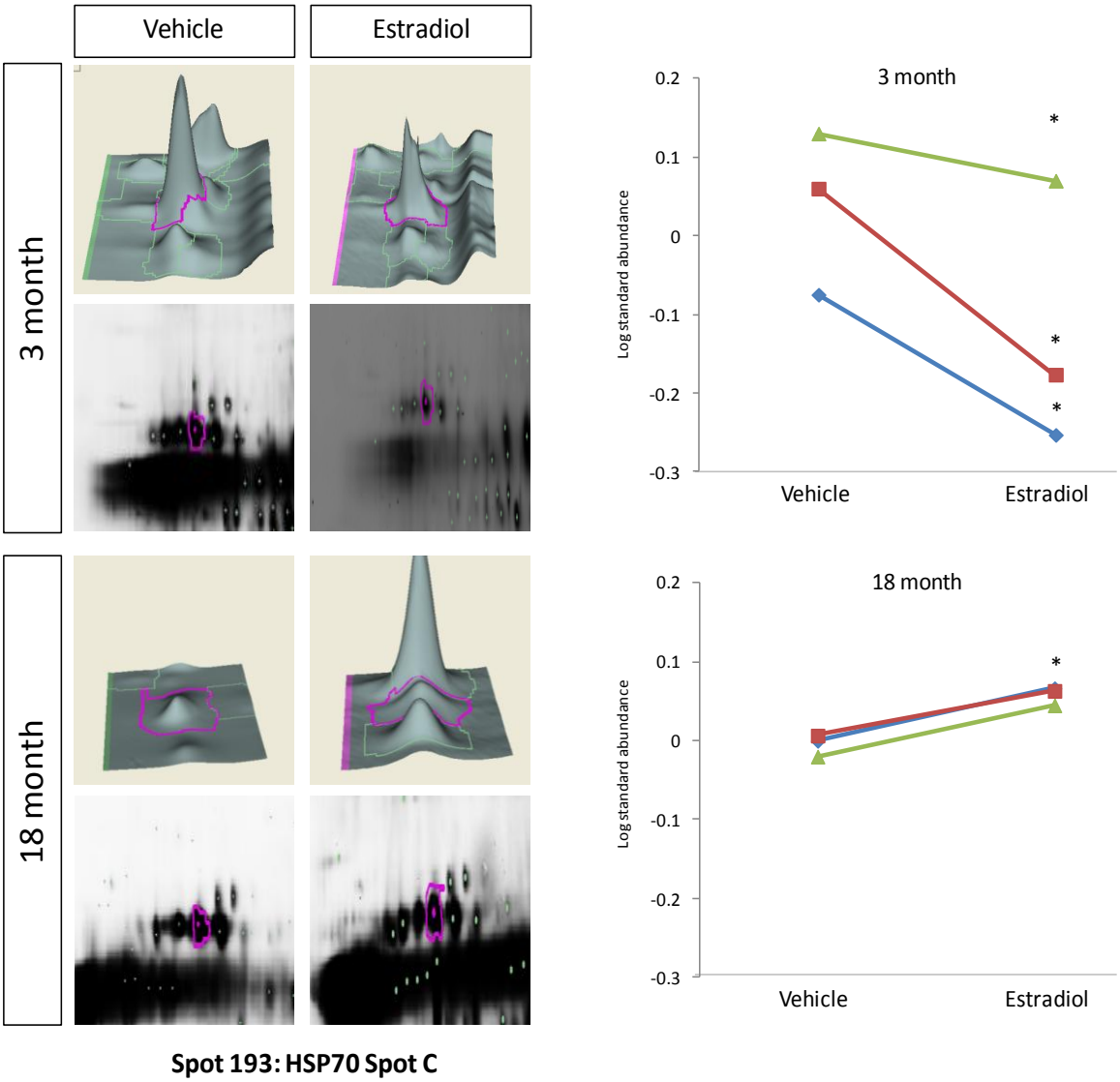


Figure 24B. DeCyder topographical, gel image analysis and average log standard abundance of heat shock protein 70 (HSP70) in response to E_2 in young and aged animals. For each panel from top left to right: 3 month: YV representative topography, YE representative topography, YV representative gel image, YE representative gel image; 18 month: AV representative topography, AE representative topography, AV representative gel image, AE representative gel image. Graphs represent log transformed average abundance normalized to internal standard and matched to master gel. (Top 3-mo., Bottom, 18-mo.) Average calculated from 3 independent experiments with a biological variance of 4 pooled animals/experiment. (N=3, BV=15) * denotes significance from vehicle $p<0.05$.

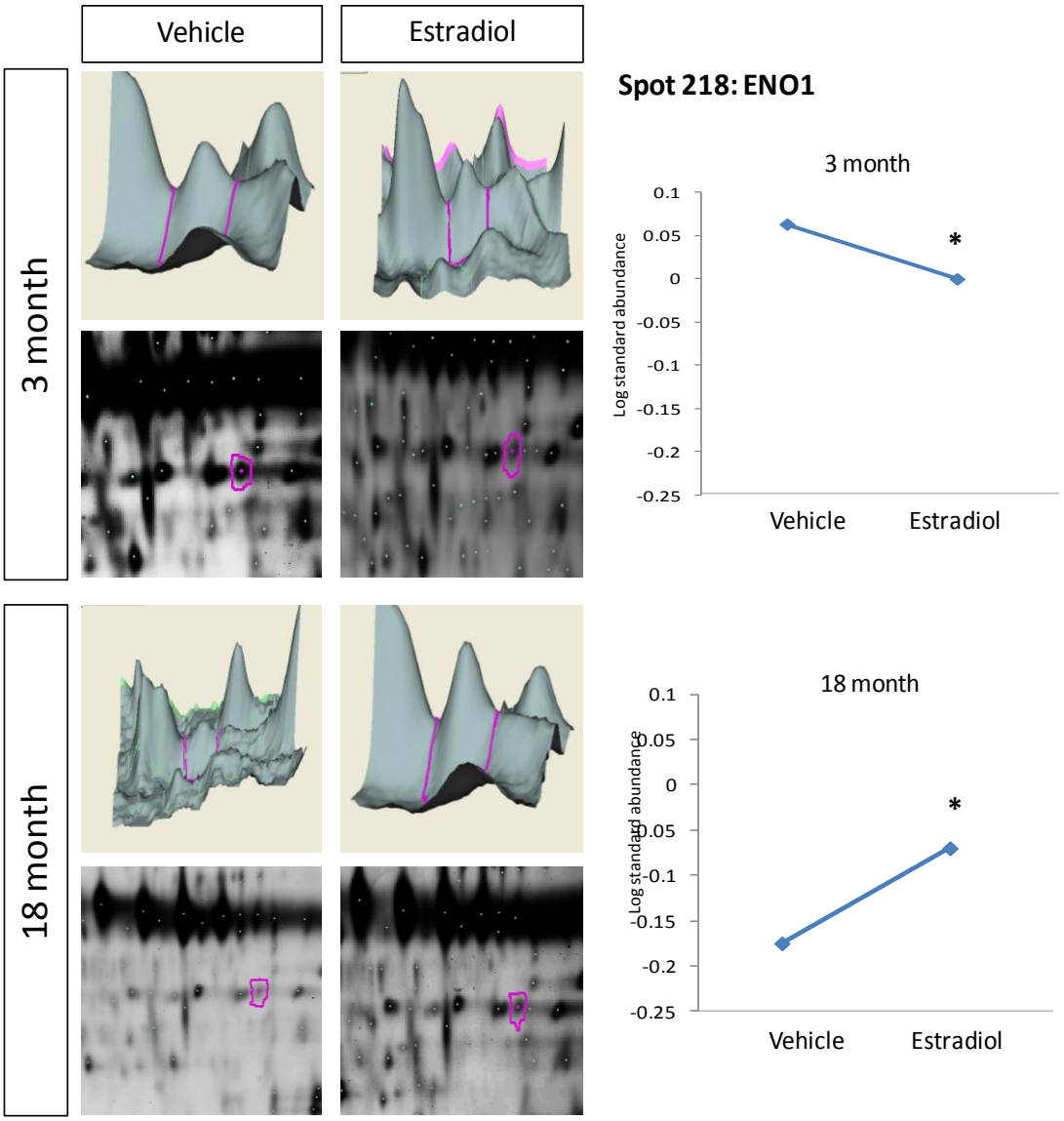


Figure 25. DeCyder topographical, gel image analysis and average log standard abundance of α-enolase (ENO1) in response to E_2 in young and aged animals. For each panel from top left to right: 3 month: YV representative topography, YE representative topography, YV representative gel image, YE representative gel image; 18 month: AV representative topography, AE representative topography, AV representative gel image, AE representative gel image. Graphs represent log transformed average abundance normalized to internal standard and matched to master gel. (Top 3-mo., Bottom, 18-mo.) Average calculated from 3 independent experiments with a biological variance of 4 pooled animals/experiment. (N=3, BV=15) * denotes significance from vehicle $p<0.05$.

The commonly considered housekeeping protein-deemed coactivator of AR, glyceraldehyde-3-phosphate (GAPDH) was also found to be associated with ERβ in both young and aged animals. E_2 treatment significantly decreased GAPDH in young, but not aged animals (Table, 1, Fig. 26). Alternatively, BVA spot #12, found in the group of spots identified as Valosin containing protein (VCP/p97) was significantly decreased with E_2 treatment in the young animals, and also tended to decrease with E_2 treatment in the aged animals (Table 1, Fig. 27). In order to further validate the quantitative changes observed using 2D-DIGE and DeCyder analysis, I performed western blot analysis on ERβ co-immunoprecipitated samples used for 2D-DIGE. As expected, VCP was decreased with E_2 in young animals, with a tendency to decrease with E_2 treatment in aged animals (Fig. 28), confirming the sensitivity and accuracy of the 2D-DIGE system when employed with LC-ESI-MS/MS.

Finally, E_2 treatment significantly decreased two protein spots (Table 1: BVA #79 and #351, Figs. 29 and 30) in young animals that were in the vicinity of the GELS cluster but were unable to be identified. These same spots were not significantly altered by E_2 in aged animals (Table 1, Figs. 29 and 30).

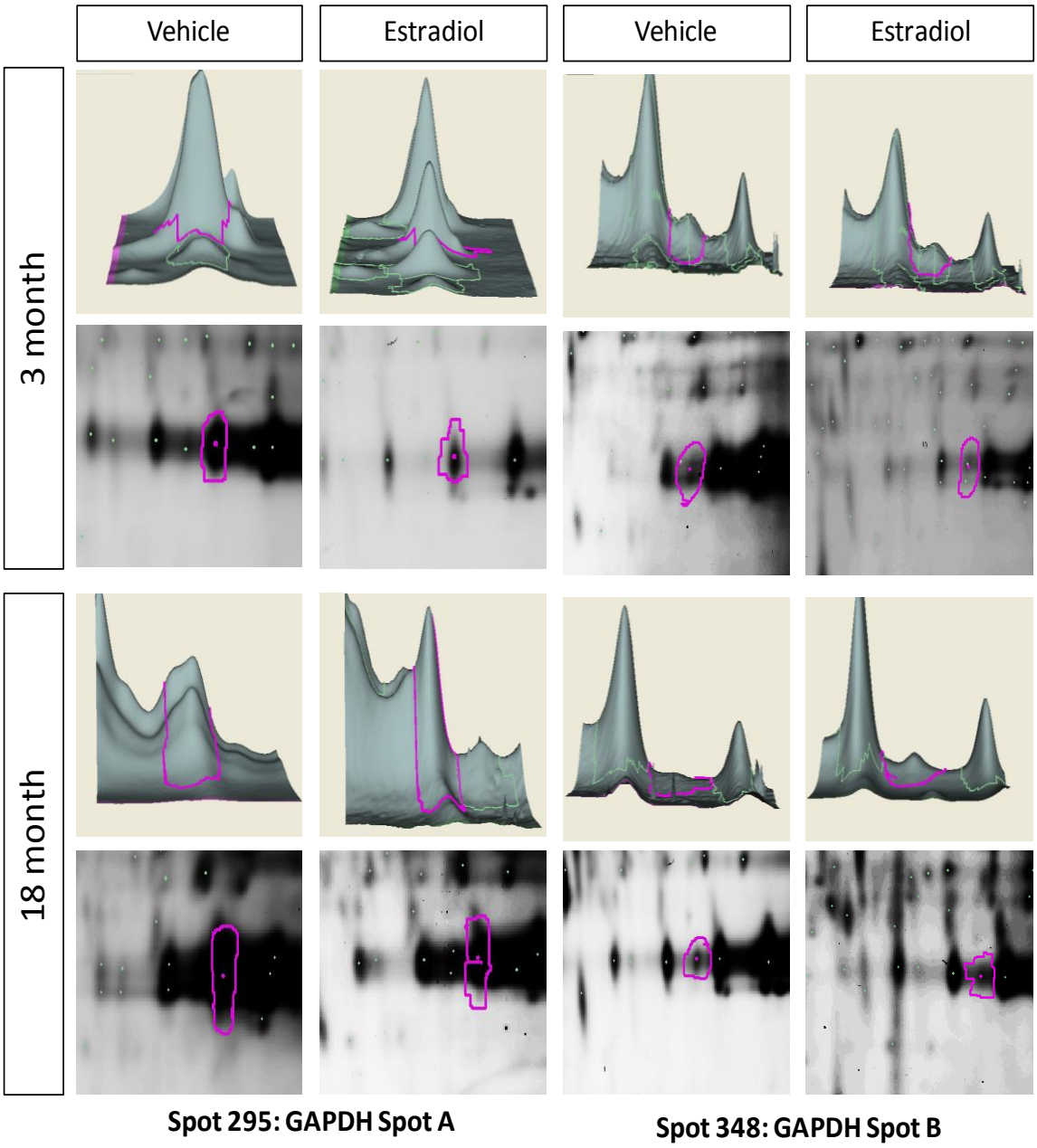


Figure 26A. DeCyder topographical, gel image analysis and average log standard abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in response to E_2 in young and aged animals. For each panel from top left to right: 3 month: YV representative topography, YE representative topography, YV representative gel image, YE representative gel image; 18 month: AV representative topography, AE representative topography, AV representative gel image, AE representative gel image.

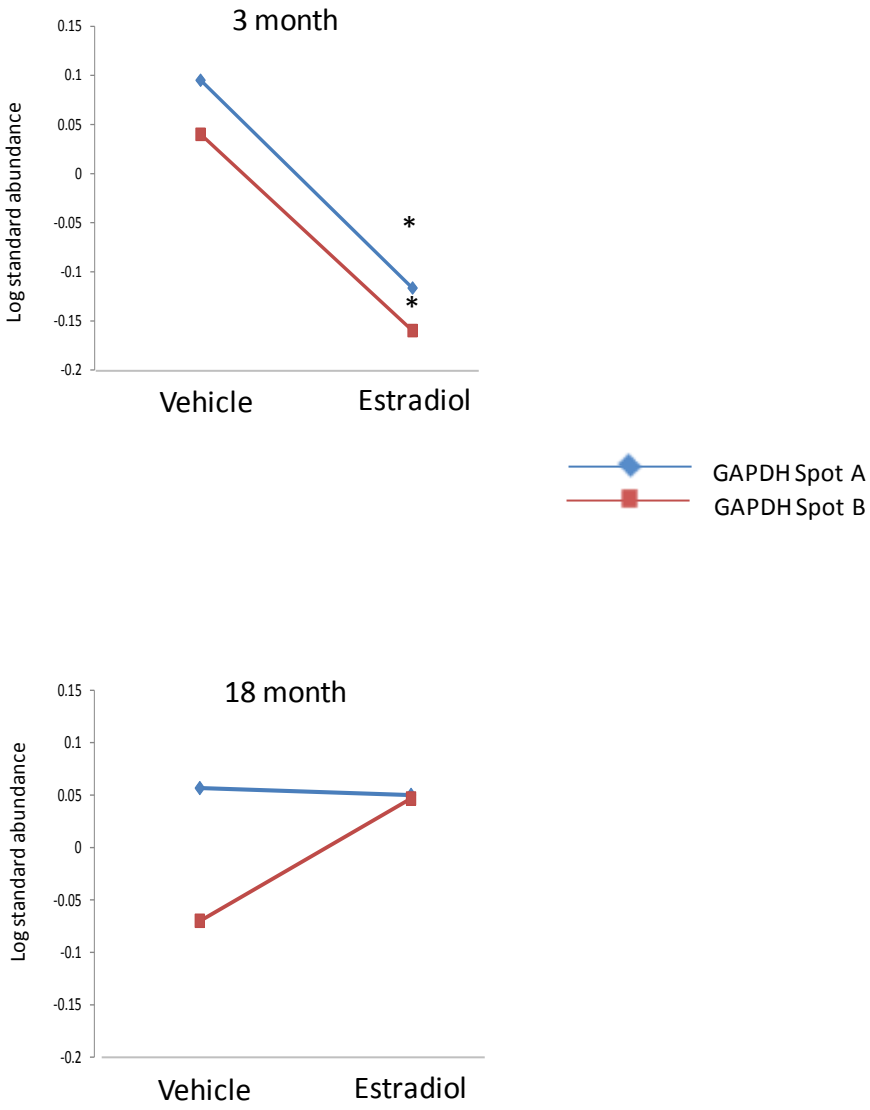


Figure 26B. DeCyder topographical, gel image analysis and average log standard abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in response to E_2 in young and aged animals. Graphs represent log transformed average abundance normalized to internal standard and matched to master gel. (Top 3-mo., Bottom, 18-mo.) Average calculated from 3 independent experiments with a biological variance of 4 pooled animals/experiment. (N=3, BV=15) * denotes significance from vehicle $p<0.05$.

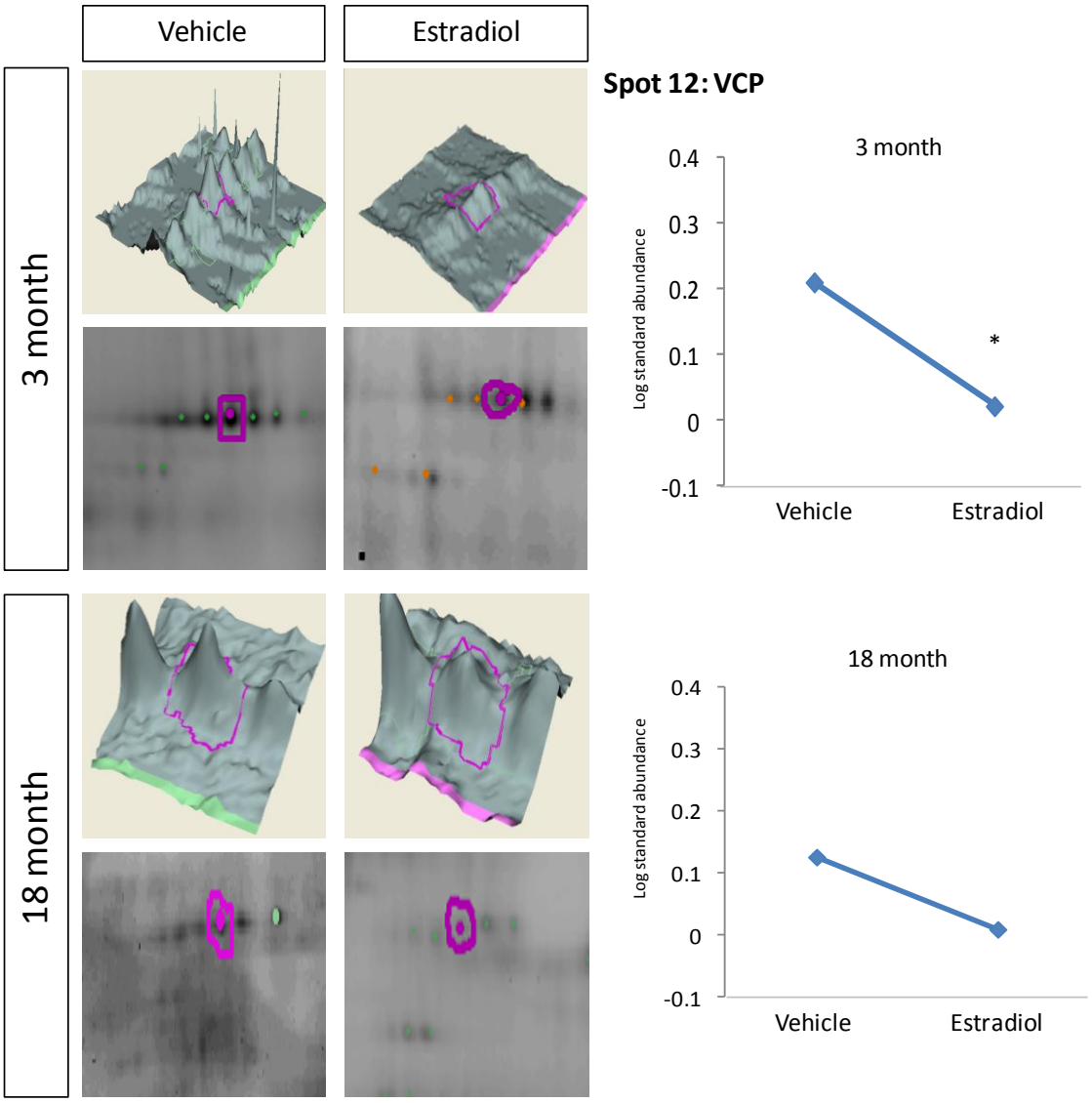


Figure 27. DeCyder topographical, gel image analysis and average log standard abundance of valosin containing protein/p97 (VCP) in response to E_2 in young and aged animals. For each panel from top left to right: 3 month: YV representative topography, YE representative topography, YV representative gel image, YE representative gel image; 18 month: AV representative topography, AE representative topography, AV representative gel image, AE representative gel image. Graphs represent log transformed average abundance normalized to internal standard and matched to master gel. (Top 3-mo., Bottom, 18-mo.) Average calculated from 3 independent experiments with a biological variance of 4 pooled animals/experiment. (N=3, BV=15) * denotes significance from vehicle $p<0.05$.

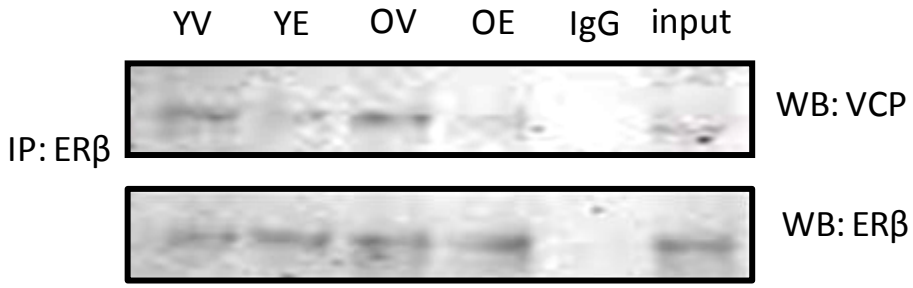


Figure 28: Confirmation of ERβ:VCP interaction. Co-immunoprecipitated nuclear extracts (YV, YE, AV, AE) were subjected to western blot analysis to confirm the interaction between ERβ and VCP.

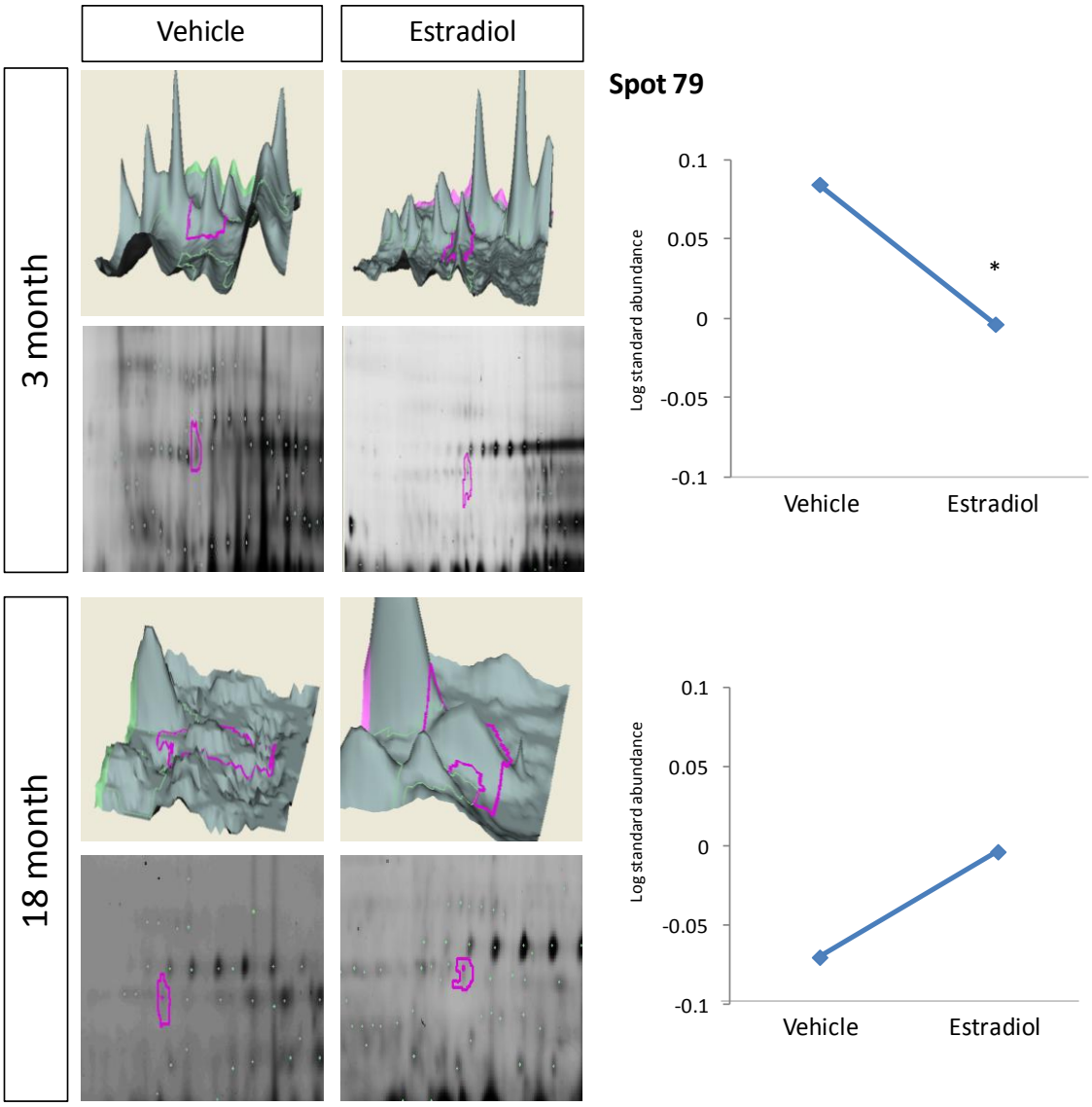


Figure 29: DeCyder topographical and gel image analysis of BVA Spot number 79. For each panel from top left to right: 3 month: YV representative topography, YE representative topography, YV representative gel image, YE representative gel image; 18 month: AV representative topography, AE representative topography, AV representative gel image, AE representative gel image. Graphs represent log transformed average abundance normalized to internal standard and matched to master gel. (Top 3-mo., Bottom, 18-mo.) Average calculated from 3 independent experiments with a biological variance of 4 pooled animals/experiment. (N=3, BV=15) * denotes significance from vehicle $p<0.05$.

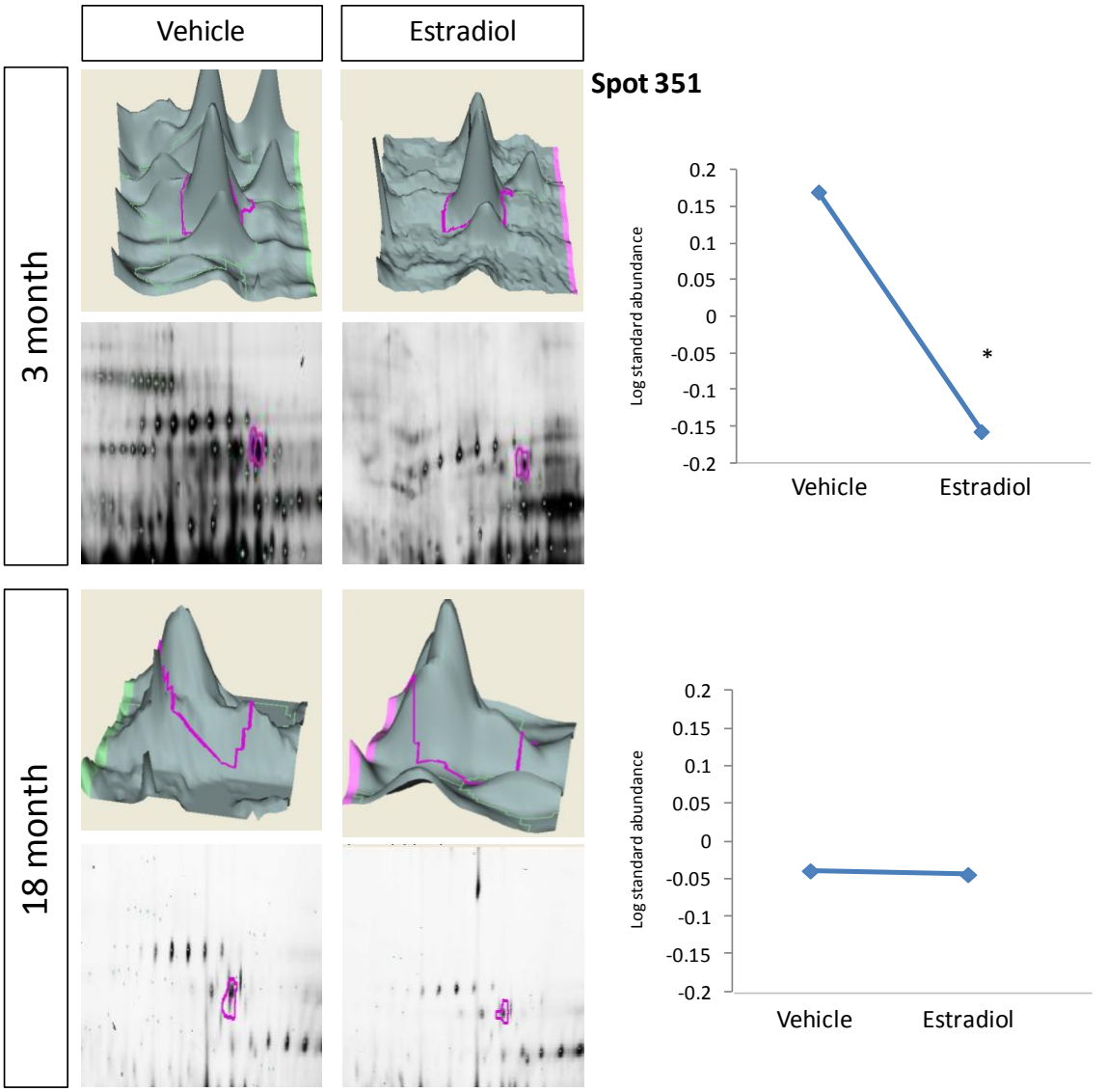


Figure 30: DeCyder topographical and gel image analysis of BVA Spot number 351. For each panel from top left to right: 3 month: YV representative topography, YE representative topography, YV representative gel image, YE representative gel image; 18 month: AV representative topography, AE representative topography, AV representative gel image, AE representative gel image. Graphs represent log transformed average abundance normalized to internal standard and matched to master gel. (Top 3-mo., Bottom, 18-mo.) Average calculated from 3 independent experiments with a biological variance of 4 pooled animals/experiment. (N=3, BV=15) * denotes significance from vehicle $p<0.05$.

Quantification of the effects of age and E_2 on protein expression

One possible explanation for changes in protein:protein interaction could be that there was a corresponding change in absolute protein expression levels of the partners in question. Therefore, I analyzed the subcellular expression levels of some of the proteins of particular interest that were identified as ERβ interacting proteins in the ventral hippocampus. I selected VCP, and GAPDH for their potential role in apoptosis and disease, GELS and HSP70 because they are known to interact with ERs, ENO1 as a novel ER interaction partner and HnRNP H because of its role in alternative splicing, a process known to increase with aging. Further, it is also possible that a change in nuclear/cytosolic shuttling could account for a change in protein associations; therefore the cytosolic fractions were examined as well.

First, I analyzed the expression levels of ERβ in the ventral hippocampus. Several studies have reported age-related changes in ERβ expression, however the reports are inconsistent and dependent on brain region (Wilson *et al.*, 2002; Chakraborty *et al.*, 2003b; Gundlah *et al.*, 2000; Sharma and Thakur, 2006; Zhang *et al.*). Our results showed a trend toward a decrease in ERβ expression in the ventral hippocampus with age, but no significance statistical difference was found using a 2-way ANOVA (Fig 31A, 31B). HSP70 levels followed a similar pattern, but again there were no significant changes in HSP70 cytosolic or nuclear expression. Interestingly, VCP was the only protein that showed a statistically significant increase as main effect of age in 18 month-old animals (Fig. 31A,B, $F(1,8)=0.0237$, $p<0.05$), however

there was no effect of E_2 and there was no interaction. Interestingly, this change in expression did not correlate with the interaction observed via 2D-DIGE between ERβ and VCP in aged animals, which showed a trend towards decreased association with ERβ (Table 1, Fig. 27). There was also a trend toward increased ENO1 expression in E2-treated aged animals, which corresponded to an observed increase in ERβ:ENO1 interaction (Figs. 25,31A, B). GAPDH and HnRNP H expression levels were unchanged by age and treatment. From these data it is clear that absolute protein expression levels are not solely responsible for changes in ERβ:protein interactions *in vivo*.

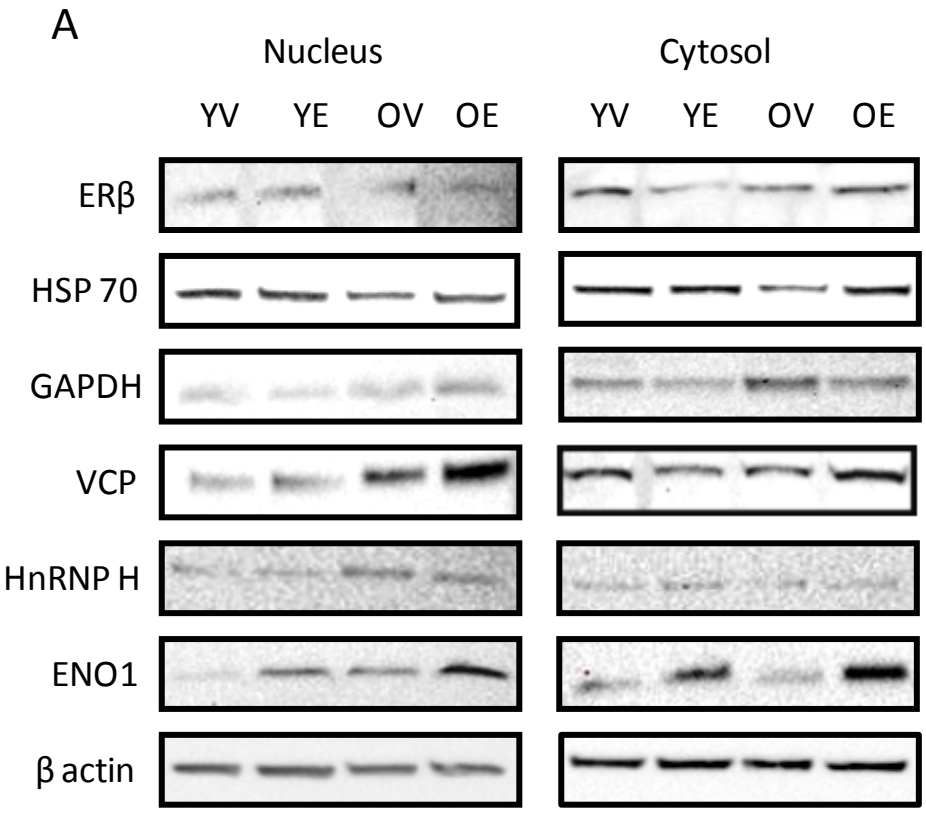


Figure 31A. Nuclear and cytosolic expression analysis of ERβ-interaction partners. A. Representative immunoblots for nuclear and cytosolic ERβ, HSP70, GAPDH, VCP HNRNP H, ENO1 normalized to β-actin.

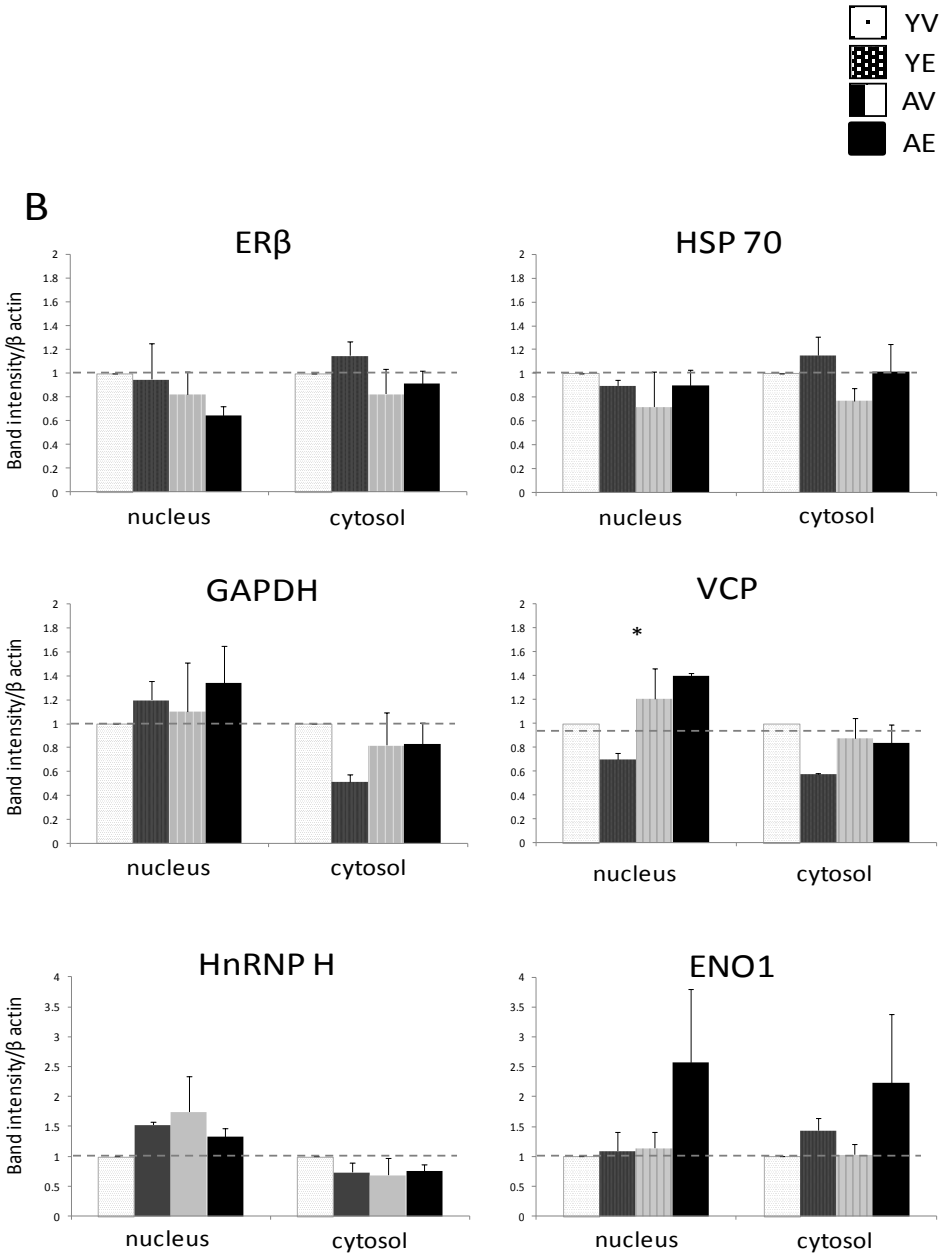


Figure 31B. Nuclear and cytosolic expression analysis of ERβ-interaction partners. B. Quantified densitometric analysis of protein expression calculated from at least 3 independent experiments (N=3). Letters denote significance between groups (2-wayANOVA, p>0.05).

Transcriptional effect of gelsolin knock-down on ERβ-mediated promoter activity

Dynamic protein:protein interactions are critical for cellular functions. In the nucleus, ERβ is well characterized as a transcription factor that regulates gene promoters by binding to specific enhancer elements. Gelsolin has recently been described as a transcriptional enhancer for nuclear receptors including ERα, but not ERβ at an estrogen response element (ERE)-mediated minimal promoter (Nishimura *et al.*, 2003). To test the functional consequence of a disruption in ERβ:gelsolin interactions we used siRNA to knockdown gelsolin in a neuronal-derived ERβ-expressing cell line. Importantly, 100 nM gelsolin siRNA reduced beta-actin expression; therefore a lower concentration of siRNA was used (50 nM, Fig 12A). Our results showed that gelsolin knockdown (50 nM) abolished ERβ-induced repression of an activator protein 1-mediated (AP-1) promoter (Fig. 12C), but not an ERE-mediated promoter.

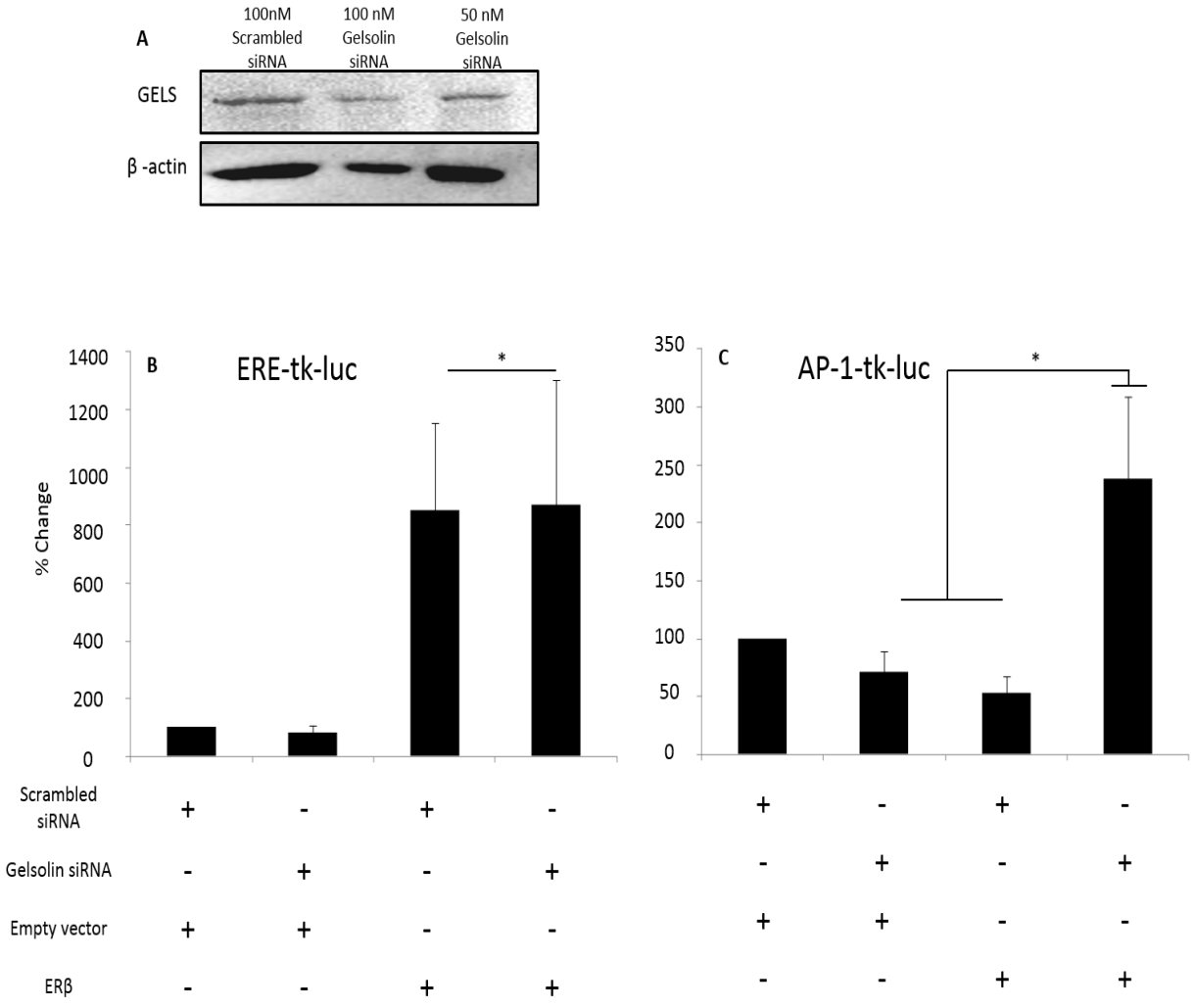


Figure 32. Effects of siRNA knock-down of Gelsolin on ERβ-mediated AP-1 and ERE promoter activity. HEK293T cells were transiently transfected with 50 nM Gelsolin siRNA or scrambled siRNA followed by cotransfection with an expression vector containing rERβ1 (150 ng) and (A) 150 ng tk-ERE-Firefly-luciferase or (B) tk-AP1-Firefly-luciferase reporter constructs plus 5 ng Renilla-luciferase control. Data represent the percentage change in dual luciferase ratio of relative light units (ERE or AP-1:Renilla). * denotes significance from empty vector control, $p<0.05$.

Cross linking with DTBP to obtain a chromatin bound fraction of nuclear ERβ

I also chose to examine the chromatin bound fraction of ERβ being pelleted in the nuclear extraction step and retain more protein interactions; a number of Crosslinking trials were employed. Ultimately, Crosslinking samples were crosslinked with dimethyl dithiobispropionimidate (DTBP), a membrane permeable, homobifunctional peptide cross-linker which reacts with primary amines of lyside residues and primary amines. Prior to nuclear extraction, tissue punches were incubated with DTBP for 45 minutes, and samples were then processed as described previously. Analytical gel replicates were ran followed by preparative gels, and then spot picking for MS/MS peptide identification was performed using the Ettan DIGE automated spot picker. Many proteins identified were typical transcriptional and coregulatory proteins that would normally be associated with ERβ, suggesting that while there was some success in retaining some potentially transient and chromatin-associated protein interactions. One limitation of utilizing the cross linker became evident in analysis of the images. Even with saturation labeling of cysteine- (verses lysine) reactive fluorescent dyes the gel replicates from these experiments were not highly reproducible (Supplemental figure). MS/MS results did not yield very high PEAKS scores, indicating the use of cautious interpretation of the identified peptides (See Tables 4 -19).

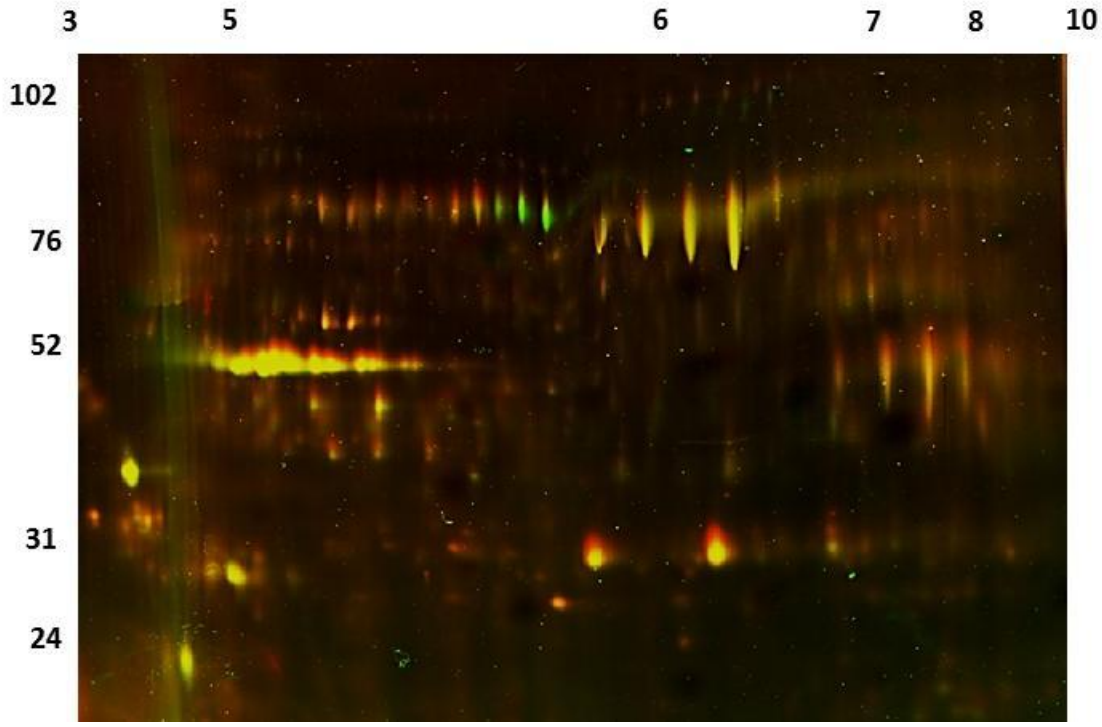


Figure 33. Representative image of DTBP crosslinked analytical gels. Brain tissue was incubated with DTBP for 45 minutes prior to nuclear extraction and co-immunoprecipitated for ERβ and subjected to 2D-DIGE as described previously.

Discussion

These data contribute novel findings that may aid in identifying alternate functions for ERβ in the brain. Moreover, the evidence presented herein lends support for the hypothesis that there is an intrinsic change in ERβ function upon the reintroduction of E_2 with advanced age. First, we provide evidence that shows novel age- and E_2-dependent interactions between ERβ and VCP, HnRNPs, ENO1, GAPDH, ANXA1 and ANXAV. Importantly, these changes do not appear to be influenced by subcellular localization of these proteins or absolute protein expression levels. Second, we confirmed and identified several known and novel ERβ:protein interaction partners, and we quantified changes in these interactions as a function of advanced age and E_2 treatment. Finally, these studies are the first to broadly characterize changes in ERβ protein interactions *in vivo* in the ventral hippocampus using co-immunoprecipitation and 2D-DIGE coupled with mass spectrometry.

The most compelling data from this analysis is the change in dynamic E_2-induced ERβ protein associations with age. Changes in ERβ protein:protein interactions supports clinical evidence for changes in estrogen signaling with age or E_2 deprivation; however this is the first study to provide evidence for a molecular mechanism that predicts a change in overall ERβ function as a consequence of age. Importantly, only 5 of the 19 ERβ protein interactions were altered by E_2 in aged animals, which contrasted markedly with the fact that all 19 ERβ protein interactions were affected in young animals. Overall, a very small percentage

(2.56%) of the confirmed protein spots changed significantly with age and E_2, attesting to the specificity of these results. These data also support the hypothesis that the receptivity of ERβ to E_2 is drastically altered by age, since the expression levels of these proteins did not significantly correlate with changes in protein:protein interaction. Changes in ERβ gene expression could contribute to altered E_2 receptivity, however in our study there were no significant changes in ERβ protein levels with age or E_2 treatment. These results add to a compilation of studies that show differential effects of advanced age or E_2 deprivation altered the gene expression of ERβ (Wilson *et al.*, 2002; Chakraborty *et al.*, 2003b; Gundlah *et al.*, 2000; Sharma and Thakur, 2006; Zhang *et al.*). In one study, E_2 deprivation or replacement decreased ERβ expression in 24-month but 18-month old rats (Zhang *et al.*). Other studies demonstrated E_2-mediated decreases in ERβ expression, but these reports are variable dependent upon endpoint measured (mRNA vs. protein) and brain region. Nevertheless, our results from the ventral hippocampus clearly demonstrate that E_2 significantly alters ERβ protein:protein interactions in an age-dependent manner regardless of ERβ protein expression levels.

The role of E2 in neuroprotection has been under investigation for some time, but delineating the exact actions that lead to a protective outcome has been difficult. The prevailing hypothesis in the field is one of a 'healthy cell bias', where the actions of E_2 are protective *prior* to insult. E_2 can be pro-apoptotic or anti-apoptotic depending upon cellular context and ER subtype. ERβ has been

characterized as predominantly anti-proliferative and pro-apoptotic in cancer models, in contrast to the proliferative and anti-apoptotic role of ERα. However, in the brain, both receptors demonstrate protection against various neurological insults such as ischemia and glutamate toxicity (Bryant and Dorsa, 2010; Dubal *et al.*, 2006). E_2 can induce anti-apoptotic factors such as Bcl-2 (Frasor *et al.*, 2003; Dubal *et al.*, 1999; Choi *et al.*, 2001; Bynoe *et al.*, 2000), and overexpression of Bcl-2 can induce nuclear localization factors such as ANXA1 (Ishido, 2005). Here I identified not only an increased interaction between ANXA1 and ERβ with E_2 administration, but I also demonstrated that this interaction was no longer affected by E_2 in aged animals.

Similarly, I showed that ANXAV interactions with ERβ were also changed with age; ANXAV is often used as a marker of apoptosis, due to its binding of cytosol-facing phosphatidyl serines in the cell membrane, however a role for nuclear annexins could be relevant to the neuroprotective actions of E_2. For instance, ANXAV can be induced to translocate to the nucleus by serum factors (Mohiti *et al.*, 1997), which could predict cellular senescence (Klement *et al.*, 2012). The samples that I obtained from the ventral hippocampus represent a diverse and heterogeneous population of cells including supporting glial cells that can senesce. Senescence in the brain has been postulated to contribute to pathological states such as Alzheimer's disease, and E_2 has been shown to protect against both senescence and dementia (Imanishi *et al.*, 2005c; Imanishi *et al.*, 2010; Imanishi *et*

al., 2005a; Imanishi *et al.*, 2005b; Bhat *et al.*, 2012; Raina *et al.*, 2001). Apart from a purported role in the activity of telomerase, it is not clear how E_2 could protect against senescence, but I speculate that the interaction between ERβ and ANXAV could contribute to the role of E_2 in senescence. Annexins, including annexin 11 (ANXA11), which was associated with ERβ, but unaffected by age or E_2, have been reported to localize to the nuclear envelope and may associate with microtubules at the nuclear membrane and assist in nuclear breakdown, potentially explaining the presence of structural proteins pulled-down in our results (Tomas and Moss, 2003). Interestingly, E_2 can also modulate the expression of members of the annexin family (Kawaminami *et al.*, 1998; Castro-Caldas *et al.*, 2001) further supporting an interplay between annexins and ERs. Taken together, these results suggest that protective aspects of E_2 signaling could be mediated through ER:ANXA interactions demonstrated here and in other reports (Nalvarte *et al.*; Ivanova *et al.*, 2011; Tarallo *et al.*, 2011), and unique to this study there is an age-related change in some of these associations with ERβ.

Like annexins, the multifunctional protein GAPDH is not typically considered a nuclear protein, but these data and other studies suggest it is possible to predict that a there is complex relationship between GAPDH, E_2 and ERs. Our results showed that ERβ:GAPDH interaction decreased following E_2 administration in young animals and was unaffected by E_2 in aged animals. Moreover, the ERβ:GAPDH was the only protein interaction that was altered (increased) by age alone. Not only

do these data indicate that there is likely to be an increase in the amount of ERβ:GAPDH in aged animals, but they also demonstrate that E_2 is ineffective at dissociating this increased interaction in aged animals. In the initial study that determined GAPDH was an AR coactivator, GAPDH did not enhance the transcriptional activity of ERα or GR at their respective response elements (Harada *et al.*, 2007), but GAPDH has a number of functions that are not well understood and may still bind ERβ when it is translocated to the nucleus (Sawa *et al.*, 1997; Ishitani *et al.*, 1998). S-nitrosylation (SNO) of GAPDH initiates apoptosis by translocating to the nucleus and interacting with Siah1 (an E3-ubiquitin ligase), also known as BAG-1. BAG-1 has been shown to interact with ERα and facilitate down-regulation of ERs over extended periods of E_2 deprivation (Zhang *et al.*). Overall, the role for a nuclear interaction between ERβ:GAPDH is not yet clear, but if these two proteins are playing a role in cell death, a change in this interaction could dysregulate the balance between E_2 neuroprotection and apoptosis in aged animals.

The possibility of S-nitrosylated (SNO) GAPDH and ERβ interactions underscores the probability that posttranslational modifications contributed to changes in the observed interactions in this study and warrants further investigation. Interestingly, the ERβ-selective agonist DPN induces SNO proteins as a cardioprotective mechanism in the heart (Lin *et al.*, 2009). Loss of SNO-associated proteins with age could ostensibly contribute a loss of cardioprotective effects of E_2 in older patients (Santhanam *et al.*, 2010). Moreover, S-nitrosylation of interaction

partners mediated through ERβ could result in the characteristic 'chain' patterns observed in the 2D-DIGE experiments. Other possible explanation for the chain patterns is carbamylation of proteins which can occur in urea-based buffers, or phosphorylation or other modifications that alter protein charge (McCarthy *et al.*, 2003). Protein modifiers such as p38 and SUMO are reported to both affect ERβ signaling and change with age (Suh, 2001; Li *et al.*, 2008; Akar and Feinstein, 2009), thus it is possible that modifications to ERβ or its interaction partners by these types of proteins could contribute to the observed effects.

Another novel finding from these results was the observed increase in nuclear VCP protein levels with age and the age-related changes in ERβ:VCP interactions. Similar to reported interactions between ERAP140 and ERβ, there was a trend towards decreased VCP:ERβ interaction with age, yet VCP nuclear expression paradoxically increased significantly as a factor of age. VCP is an AAA+ class of ATPase that has been recently implicated in diseases where polyglutamine-mediated protein accumulation is observed (Hirabayashi *et al.*, 2001), but the mechanisms involving VCP in these diseases have yet to be elucidated. In some instances, VCP has been shown to interact with polyglutamine tract proteins in the nucleus, potentially mediating aggregation of polyglutamine aggregates (Hirabayashi *et al.*, 2001). VCP can interact with nuclear receptor transcriptional complexes and suppress transcriptional processes (Koike *et al.*, 2010), but the exact function of VCP within a non-pathological nuclear protein complex is unknown

(Jung *et al.*, 2005). While the interaction between ERβ and VCP has not been fully characterized, the neuroprotective role of E_2 and the potential role of VCP in neurodegenerative diseases is an intriguing correlation that suggests changes in ERβ:VCP interactions with age might have significant functional consequences. Notably, nuclear ataxin-1, also identified as an ERβ interaction partner in this study has been implicated in polyglutamine-induced diseases (Klement *et al.*, 1998), but this interaction was unaltered by age or E_2 treatment. Our data are consistent with another report that showed an interaction between VCP and ERα when ERα was bound to a 9xERE (Nalvarte *et al.*), however this is the first report demonstrating an age-related change between VCP and ERβ *in vivo*.

Apart from non-traditional roles for ERs, I suggest the interactions between ERβ and transcriptional proteins could be affected by age as well. In this report, I demonstrated an age-related change in the association of the actin binding protein GELS and ERβ. GELS, also a known steroid hormone coregulator, enhances ER-mediated transcription (Nishimura *et al.*, 2003). As expected, E_2 increased the ERβ:GELS interaction in young animals, however there was no significant change in this interaction in aged animals (Fig 3C). This suggests that E_2 may not enhance gene transcription in aged animals the same way as it might in young animals, a finding which has also been demonstrated in ER-null animals (Han *et al.*, 2013). Furthermore, in this study there is evidence to suggest that the ERβ fraction isolated may be a part of non-DNA bound nuclear matrix associated complexes that direct

transcription. I identify a clear association between ERβ is the structural protein β-actin, which has also been demonstrated by others (Nalvarte *et al.*; Ivanova *et al.*, 2011; Tarallo *et al.*, 2011). β-actin was found to have an important role in the nucleus (Zheng *et al.*, 2009; Huang *et al.*, 2004; Hofmann *et al.*, 2004), providing a scaffold to assist in gene regulation and association with nuclear export as suggested by the leucine rich nuclear export signal within β-actin and studies demonstrating actin-mediated nuclear export of viral RNA (Hofmann *et al.*, 2001; Wada *et al.*, 1998). The identification of structural proteins may be through attachment of these proteins to ERβ involved in nuclear translocation near the nuclear envelope, but it is also postulated that actin and actin-binding proteins maneuver transcription associated proteins to position them for transcription.

Our intentional exclusion of chromatin from the samples, and our selection of protein spots on the 2D-gel that were limited to those common between 3 separate antibodies to ERβ was a highly conservative approach. Therefore, this approach likely excluded a number of putative interaction partners for ERβ that I was unable to characterize. Moreover, changes in individual neuronal populations (e.g., Pyramidal CA1, CA3, interneuron, etc.) may be obscured by examining the entire ventral hippocampus as I did in this study, however, the whole region was used to a) obtain enough protein for analytical and preparative gels, western blotting confirmation and expression analysis and b) gain a broad view of nuclear proteins associated with ERβ *in vivo.* Another exclusionary factor comes from the antibody

selected for ERβ co-immunoprecipitation, which was selected because following pull-down, a conservative number of protein spots were visualized after co-IP. I tested a total of three antibodies and found that the other two tested in this paradigm pulled down more proteins than the one I ultimately used to report these findings. In general, spots that did not exhibit significant overlap between the three antibodies tested were excluded from analysis and identification to avoid false positive interactions. However, this ultraconservative approach may have eliminated the discovery of additional ERβ:protein interaction partners, suggesting that the identified proteins in this study represent only a subset of ERβ-associated proteins. It is also important to note that the interactions described in this dataset may be direct or indirect. Protein interactions in the nucleus tend to be part of a larger complex, and based on our results some interactions may be mediated through proteins such as actin which was abundant in our samples and in other reports of a similar nature (Nalvarte *et al.*; Ambrosino *et al.*), however its abundance (co-immunoprecipitated with ERβ) was neither changed by age or E_2 administration. Thus, I hypothesize that the changes in interactions between ERβ and known actin associated proteins such as GELS and ENO1, which has been shown to modulate DNA methyltransferase (Tovy *et al.*) could be a function of changes in nuclear actin structure resulting in an altered transcriptional role of ERβ.

Other actin-bound nuclear proteins include the family of HnRNPs, which cooperate with actin to influence mRNA processing and splicing, and in this study I

demonstrated through co-immunoprecipitation, that ERβ might have a role in these processes. HnRNPs are molecular determinants of all facets of mRNA processing. Other HnRNPs have been shown to associate with ERα, but this study is the first to report an interaction between ERβ and HnRNP H (McNally *et al.*, 2006; Buratti *et al.*, 2004). Recently, ERs have been shown to participate on some level, in miRNA processing (Pak *et al.*; Yamagata *et al.*, 2009) and mRNA splicing (Masuhiro *et al.*, 2005), and recent evidence suggests that aging may lead to a global increase in alternative splicing (Tollervey *et al.*). HnRNPs including HnRNP H are often considered negative regulators of alternative splicing. In this report the ERβ:HnRNP H interaction is enhanced by E_2 in young animals, but decreased or unchanged by E_2 in aged animals, suggesting in aged animals the influence of E_2 over the actions of an ERβ:HnRNP H complex may be altered. Further investigation into ERβ:HnRNP interactions could help to explain E_2- and age-related changes in alternative splicing.

The data presented here fill a knowledge gap in the field regarding a) protein interactions with ERβ in the ventral hippocampus, and b) a possible mechanistic explanation for changes in E_2-mediated processes in aged individuals. Notably, Bert O'Malley's group recently identified the association of nuclear hormone receptors with upwards of 10,000 'coregulatory' proteins, however protein interactions with ERβ were not examined in that particular study and there was a lack of context as these experiments were performed *in vitro* (Malovannaya *et al.*). The interactions with ERβ described herein represent a novel fraction of proteins that may serve to

supplement the existing role of ERβ in mediating gene expression and possibly neuroprotection in the hippocampus. All in all, these novel ERβ:protein interactions require further in-depth study to elucidate the complete gamut of ERβ functions, and moreover how these functions may change with age and hormone replacement is essential to determine the neurological costs and benefits of hormone therapy.

CHAPTER V

FINAL DISCUSSION

Summary

The mechanisms responsible for estrogenic effects on physiological processes such as cognition, affect and even some aspects of homeostasis remain unclear. Even still, how age and E_2 deprivation alter expression patterns of ERs within the brain remain a mystery; however, there is some evidence that alternative splicing increases which may change the brain's receptivity to hormone, as some of the identified splice variants of ERβ do not bind E_2. Therefore, the goals of this project were to examine the functions of ERβ and ERβ splice variants to determine how changes in expression of these receptors may alter functions such as gene expression and protein:protein interactions that dictate general functionality of ERβ. In chapter III, the data show that hERβ splice variants are expressed in the aged human brain, and that the transcriptional actions of these splice variants are constitutive and not responsive to ligand. In chapter IV, the data quantify novel age- and E_2-dependent protein:protein interactions with ERβ which are influenced by absolute protein expression levels. Importantly, chapters III and IV are not mutually exclusive as the antibody used in chapter IV does not distinguish between splice variants. Therefore, the changes could be due to changes in splice variant

expression between young and aged animals. Ultimately, the data obtained from this dissertation demonstrates novel mechanisms for age-related changes in ERβ function that can be applied to older postmenopausal women seeking HT.

Key Findings

Chapter III: Characterization of hERβ splice variants

- Human ERβ splice variants are differentially expressed in human amygdala.
- Human ERβ splice variants bind a consensus ERE sequence *in vitro* regardless of E_2 binding.
- Human ERβ splice variants constitutively activate or repress ERE-luciferase and AP-1-luciferase promoter activity, respectively, in neuronal cells.
- Repression of the human Arginine vasopressin (hAVP) promoter by human ERβ splice variants is mediated by an AP-1 site in the proximal
- (> 1000bp) promoter region.
- Inhibition of p38, but not PI3K blocks repression of hAVP- and AP-1-promoter activity by hERβ splice variants in neuronal cells. Further, co-treatment of E_2 and p38 inhibitor enhances hAVP-mediated promoter activity.
- ER antagonist ICI 181 780 prevents only hERβ1-mediated effects on ERE-, AP-1 and hAVP-driven promoters, but co-treatment with p38 inhibitor and ICI 181 780 also blocks hERβ2-mediated repression of the hAVP promoter.

Table 3. *Summary of key findings*

Chapter IV: Age alters the dynamics of ERβ protein:protein interactions in the ventral hippocampus dependent upon 17β-estradiol

- 2.56% of protein interactions with ERβ (19/741) were either significantly increased or decreased by E_2 treatment in the ventral hippocampus of ovariectomized rats given 2.5μg/kg E_2 for 3 consecutive days.
- Four unique protein clusters were significantly increased by E_2 administration in young but not in aged animals which included spots identified as ANXAI, ANXA V, HnRNP H, and GELS. All of these proteins except for GELS are novel interaction partners for ERβ.
    - In a cluster of spots containing ANXAV 3 spots were increased by E_2 in young animals; in aged animals 1 spot was increased, 1 spot decreased and 1 spot was unaltered by E_2 .
    - In a cluster of spots containing HnRNP H 4 spots were increased by E_2 in young animals; in aged animals 1 spot was decreased, but 3 others were unaltered by E_2.
    - In a cluster of spots containing GELS 2 spots were increased by E_2 in young animals yet both were unaltered by E_2 in young animals.
    - ANXAI:ERβ was increased by E_2 administration in young animals, but decreased by E_2 in aged animals.

Table 3. *Summary of key findings*

Chapter IV

- The interaction between ERβ and 3 identified and 2 unidentified proteins was decreased significantly by E_2 administration in young animals, but not in aged animals including HSP70, ENO1, VCP, and GAPDH.
    - In a cluster of spots containing HSP70 3 spots were decreased by E_2 in young animals; in aged animals 1 spot was increased while the other 2 were unaltered by E_2.
    - One spot identified as ENO1 was decreased by E_2 in young animals but was increased by E_2 in aged animals.
    - In a cluster of spots containing VCP one spot was decreased by E_2 in young animals but unaltered by E_2 in aged animals.
    - In a cluster of spots containing GAPDH, 2 spots were decreased by E_2 in young animals but unaltered by E_2 in aged animals.
        - When comparing young vehicle treated samples to aged vehicle treated samples, the interaction between GAPDH and ERβ was decreased by age alone.
- The cytosolic and nuclear expression of ERβ, HnRNP H, ENO1, GAPDH and HSP70 were unaffected by aged or E_2 treatment, however nuclear VCP was increased as a main effect of age alone.

Table 3. *Summary of key findings*

Final Thoughts

Menopause and the ERβ-dominated brain

The data presented in Chapter 3 make significant contributions toward understanding the diverse actions of ERβ signaling in the brain. Importantly, I present an account of human ERβ splice variant expression in the human brain, and the constitutive transcriptional activities of hERβ splice variants. Second, I show that the hERβ splice variant activities are not governed by E_2; thus, the constitutive transcriptional activity of ERβ splice variants may be most evident when ERα is inactive due to a lack of E_2, for example during menopause. Ultimately, the expression and actions of ERβ splice variants, such as ERβ2, in the aged brain suggest that there may be an important role for ERβ in the brain, even in the absence of E_2.

Expression of ERs is a critical component to consider when studying ET in the menopausal brain. It is well established that E_2 down regulates ERα, however the effects of E_2 on ERβ expression are not as clear. The influence of E_2 over expression of ERβ1 has been highly controversial, but ERβ2 expression in the hippocampus increases dramatically with loss of ovarian hormones (Wang *et al.*). In one study, reinstatement of E_2 following a short-term (6 day) E_2 deprivation, caused a decrease in ERβ2 expression, contrary to long-term E_2 deprivation (180 days) after which administration of E_2 was unable to decrease ERβ2 levels (Zhang *et al.*). This study also demonstrated that ERβ2 expression increased between 6 and 9

month old cycling sham animals, suggesting that aging alone influences ERβ2 expression. In conjunction with studies that definitively show that ERα expression is decreased (Hatsumi and Yamamuro, 2006; Ellison-Zelski *et al.*, 2009), this study supports the claim that the aged, post-menopausal female brain is highly regulated by ERβ. Therefore, it is reasonable to speculate an increase in one or more ERβ splice variants would further tip the scales in favor of regulation by unliganded ERβ. Then, were E_2 to be reinstated under these conditions, the effects of E_2 would be very different than in a premenopausal, younger brain. These effects could vast if ERβ regulated genes are being activated while ERα, which requires E2, would be transcriptionally inactive during a period of hypoestrogenicity. Furthermore, E2 does play a role in epigenetics and a long period of hypoestrogenicity could lead to changes in DNA methylation that could influence transcription of E2-regulated genes upon reinstatement (Frick *et al.*, 2011).

Supplements to nuclear receptor signaling are relevant during menopause

ERs, and nuclear receptors in general, are bound by chaperone proteins prior to activation by ligand, or as we know now, phosphorylation. This may be important to estrogen receptivity in the aged brain. Chaperone proteins protect and prepare ERs for activation by ligand, and can even accompany proteins into the nucleus. For example, chaperone protein HSP70 participates in nuclear shuttling of cargo proteins (Shi and Thomas, 1992). There is no data regarding the function of chaperone proteins for ERβ splice variants, but since chaperones such as HSP70 and

HSP90 typically bind the LBD, it is unlikely that they associate in the same manner (Dittmar and Pratt, 1997). In chapter IV, the interaction between HSP70 and ERβ decreases with E_2 in young animals, as expected. However, in aged animals, ERβ:HSP70 interactions are unaltered by E_2 (Figure 34). These data could suggest that E_2 does not activate ERβ as well in aged animals, or even that there are splice variants within the population of ERβ being isolated. This could also indicate that ERβ:HSP70 interactions are more resistant to dissociation, but in any case, ERβ is more heavily associated with HSP70 in aged animals. I found that HSP70 expression was not significantly altered by age or E_2; however, in older animals there was a trend toward a decreased HSP70 in vehicle treated animals matching some reports that show expression of HSP70 can change with age (Sharma *et al.*, 2010; Heydari *et al.*, 1993; Pahlavani *et al.*, 1996; Heydari *et al.*, 1995; Heydari *et al.*, 1996). Also interesting, the localization of HSP70 in the nucleus is cell cycle dependent, with HSP70 accumulating during S phase (Moreau *et al.*, 1998; Milarski and Morimoto, 1986). While neurons are typically quiescent, glia and other supporting cells could contribute to the pool of nuclear HSP70 during S phase. Some studies even suggest that HSP70 has a DNA binding region that enhances ER:DNA interaction through DNA bending (Landel *et al.*, 1997). Hence, there is much left to study regarding classical HSP:ER interactions, with specific regard to splice variant interactions and how physiological processes such as menopause or aging changes in ER expression or PTMs.

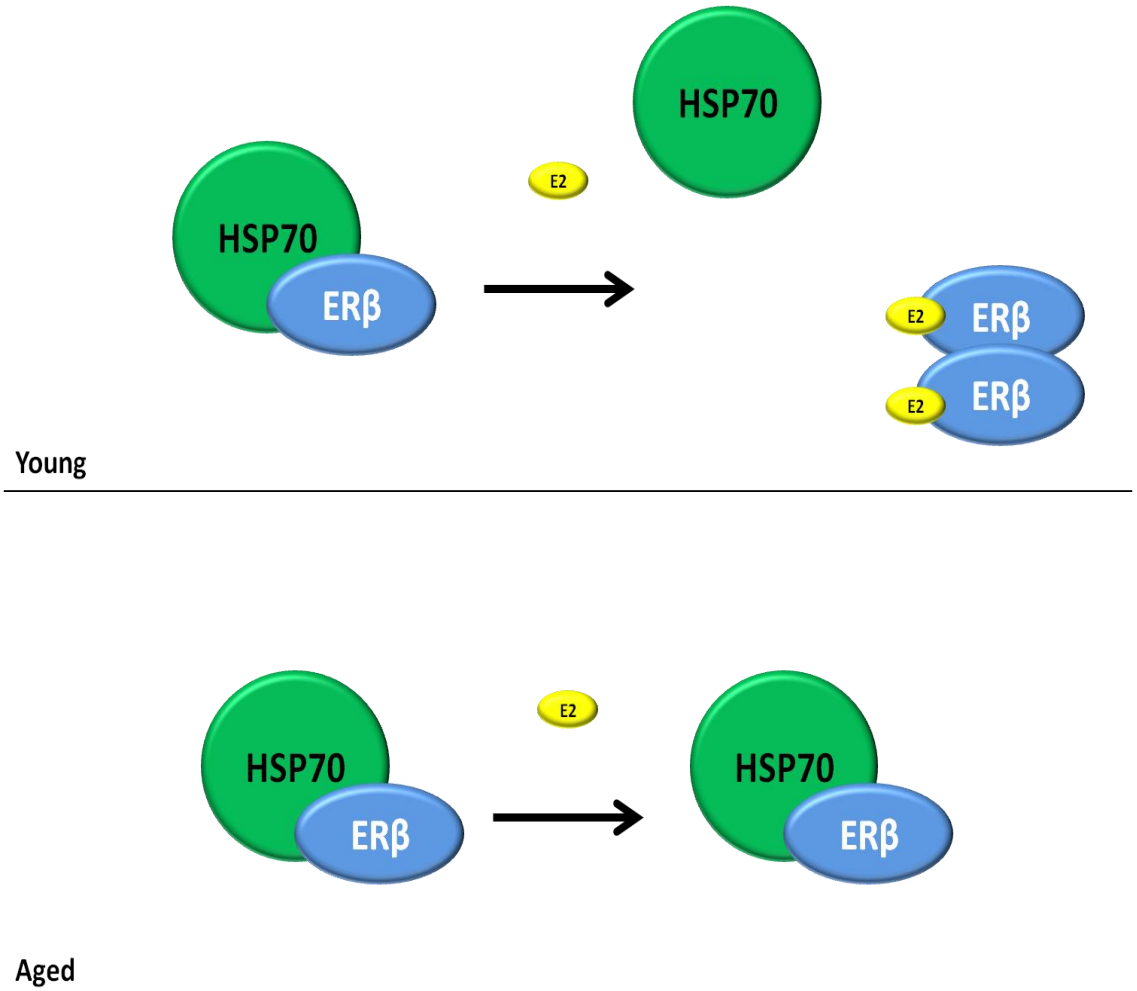


Figure 34. Model for age and E_2-dependent changes in HSP70:ERβ interactions. In 3-month old animals (Young, top), the interaction between HSP70 and ERβ is lost upon the addition of E_2. In 18-month old animals (Aged, bottom), E2 does not alter the interaction between ERβ and HSP70.

Furthermore, a broader picture of ERβ signaling in the aged brain would take into consideration possible age-related changes in expression and activities of not only ERs but also proteins that modulate ER-mediated activity. Here, I demonstrate how phosphorylation events play a critical role in ligand-independent gene transcription mediated by ERβ splice variants, and how inhibition of these signals can alter responses to ligand. In Chapter 3, the relevance of p38 as an important kinase signaling pathway in ERβ signaling is confirmed, adding to the work that suggests that p38 may be important for ligand-independent ERβ functions. Initial reports demonstrate how epidermal growth factor (EGF) can initiate a signaling cascade that induces ER translocation to the nucleus (Lin *et al.*, 2001), and later studies show that EGF signaling acts through MAPK/ERK to help ERβ recruit coregulatory proteins and facilitate gene transcription. I demonstrate that p38 inhibition specifically can block constitutive ERβ-mediated repression of an AP-1 driven promoter. It is possible that p38 may be affecting coregulatory or transcriptional proteins. However, there is some evidence to suggest that direct phosphorylation of ERβ is required for the receptor's transcriptional activity independent of ligand due to a p38 consensus sequence located on the N-terminus of ERβ and some ERβ splice variants (human, rodent and mouse). Interestingly, the p38 signaling can be influenced by E_2, indicating that there may be a regulatory loop for fine-tuning constitutive signaling of ERβ.

In chapter III, I show that p38 signaling is an important part of ERβ transcriptional functions, but in addition to phosphorylation, age-related changes in SUMO proteins and general sumoylation have been reported. While the abundance of SUMO and SUMO-related proteins appears to decrease with age in the male mouse brain, the number of sumoylated proteins in peripheral organs increases. The implications of sumoylation are broad, and for ERβ, sumoylation represses typical transcriptional activities, contrary to the activational effect of sumoylation on ERα. Changes in sumoylation of ERs with age and/or E_2 deprivation could have a significant impact on the protein:protein interactions of these receptors. Moreover, there are a number of ER PTMs that can influence receptor function (Le Romancer *et al.*, 2011). Further investigation into the role of ERβ-specific PTMs with respect to the aging brain and E_2 is essential to fully comprehend the neurobiological consequences of ER signaling.

ERβ, mood and cognition during menopause: proposed novel mechanisms involving neuroprotection and the stress response

Around the time of menopause, many women experience fluctuations in mood corresponding with a hormonal transition; since it is known that ER expression profiles change with E_2 and age, ERβ splice variant expression could be a contributing factor to changes in mood during this time. During the menopausal transition brief and prolonged periods of E_2 deprivation could create tumultuous effects of E_2 resulting from changes in ER expression. As stated previously, ERβ

splice variants and ERα can be regulated by E_2 levels. In particular, hippocampal ERβ2 expression can be significantly increased by periods of E_2 deprivation, but ERα levels decrease during extended E_2 deprivation (Zhang *et al.*). Many studies suggest that ERβ, more than ERα, can contribute to neurogenesis, increased cognition and improvements in behavioral outcomes for mood (Lund *et al.*, 2005; Imwalle *et al.*, 2005; Krezel *et al.*, 2001; Walf *et al.*, 2008b; Walf *et al.*, 2008a; Tomihara *et al.*, 2009; Day *et al.*, 2005). However, increased ERβ2 is correlated with learned helplessness, a depressive-like behavior and a decrease in neurogenesis in the hippocampus (Wang *et al.*). Therefore, it is reasonable to hypothesize that changes in ERα:ERβ1:ERβ2 stoichiometry could be responsible, in part, for mood dysregulation occurring at the menopausal transition.

Affective disorders develop from dysregulation of stress responses, resulting in exaggerated emotional responses, (Scott and Dinan, 1998; Pervanidou and Chrousos; Ikin *et al.*; Smith *et al.*, 1989; Newport *et al.*, 2003; Wood *et al.*; Steimer *et al.*, 2007). In this body of work I demonstrated the constitutive repressive actions of hERβ splice variants on the hAVP gene promoter. AVP expression activates the HPA axis and stress response in conjunction with CRH, thus in the menopausal brain increased expression of ERβ2 could mediate constitutive repression of AVP. Less AVP could contribute to a less reactive HPA axis and thus, a less anxious predisposition. Importantly, in Chapter III I show that p38 activity, reported to increase in the aged brain, serves as a regulator for ERβ2 action on the hAVP

promoter; therefore, increased p38 activity in an aged brain could reinforce ERβ2-mediated repression of hAVP expression, whereas in a younger brain ERβ2 activity would be tempered by decreased p38 signaling.

Regulation of the AVP promoter between rodent and human systems contrasts sharply and could have functional consequences for translating stress and emotion-based responses. In the rodent system, our lab and others have shown that rERβ constitutively activates the AVP promoter, as do rERβ splice variants. On the contrary, in Chapter III, I demonstrate constitutive repression of hAVP promoter activity by hERβ and hERβ splice variants. Using site directed mutagenesis to remove an AP-1 site, the repression of hAVP is reversed, but like rAVP, there are no EREs present on the promoter sequence, and thus ERβ-mediated activation of the rAVP and hAVP Δ AP-1 promoters are likely to be mediated through a non-ERE ERβ-selective response element. Regardless of mechanism, the fact that AVP is increased by ERβ in a rodent system and decreased by ERβ in a human system is an important consideration for interpretation of studies on E_2-mediated stress responses. Importantly, rERβ2 contains an 18 amino acid insert in the E domain that decreases the receptor's affinity for E_2, whereas hERβ2 has an altered F domain that occludes ligand binding altogether. This is of great importance when considering the implications of E_2 signaling when ERβ2 is more abundant than ERβ1. Hypothetically, were hERβ2 expressed when local synthesis of E_2 was occurring, hERβ2 would not be responsive, but in a rodent system rERβ2 might have some

responsiveness to E_2 production. These data serve as an important reminder that the relevance of a given model system should be closely examined, and processes considered 'highly conserved' do not always translate to other species, even for primitive neurological processes such as emotion and stress reactivity.

E_2 can enhance neurogenesis and protect against neuronal cell death, which is important for both cognition and mood; however, the mechanisms by which these processes occur and how they change with age is still of great intrigue. For a number of years one of the main purported neurological benefits of ET has been neuroprotection from stroke or neurological insult (Dubal *et al.*, 2001; Yang *et al.*, 2000); however, the mechanisms by which this occurs remain unclear. In models of middle cerebral artery occlusion (MCAO), E_2 reduces the size of infarction. Roberta Brinton and colleagues have supported the 'healthy cell bias' that suggests that preventative E_2 treatment will benefit and protect neurons as long as cells are not already in distress. Results from clinical studies such the Multi-Institutional Research in Alzheimer's Genetic Epidemiology (MIRAGE) and Kronos Early Estrogen Prevention Study (KEEPS) give merit to the healthy cell bias and that E_2 may be neuroprotective and beneficial in younger women; however, no clear mechanisms have been established to support these data (Henderson *et al.*, 2005; Brinton, 2005).

One possible contribution to changes in neuroprotection with menopause is variable expression of the ERβ splice variants. ERs can influence anti-apoptotic and pro-neurogenic factors in the brain that help support neurons (Marzioni *et al.*, 2012;

Choi *et al.*, 2001; Dubal *et al.*, 2006; Kwon and Magnuson, 2009) and relevant to this study, E_2 has been shown to enhance the protective actions of annexins and Bcl signaling (Castro-Caldas *et al.*, 2001; Nadkarni *et al.*, 2011) through unknown mechanisms. E_2 can induce anti-apoptotic Bcl-2 expression (Frasor *et al.*, 2003; Dubal *et al.*, 1999; Choi *et al.*, 2001; Bynoe *et al.*, 2000), and if ERβ splice variants can constitutively regulate these gene promoters, as they do for AVP, there is likely to be an altered effect of the expression of these genes around the time of menopause. Furthermore, ERβ and ERβ splice variants require protein:protein interactions to exert their effects on target gene promoters and other cellular processes. It is possible to determine the functional consequences of menopausal changes in ERβ splice variant expression by using knowledge regarding the structural properties of each receptor and their interaction partners. Through gene regulation and other mechanisms it is very likely that ERβ splice variants contribute to a mechanistic explanation for changes in ER-mediated neuroprotection at the time of menopause.

Correlations between changes in ERβ protein:protein interactions and changes in neuroprotection around the time of menopause

In chapter IV, I identify a number of novel protein interactions with ERβ in the ventral hippocampus, a region important for both affect and cognition. Furthermore, I demonstrate that the magnitude of these interactions change with age in response to E_2 change. Upon examination of the functions of the identified

ERβ protein interaction partners, I propose that a number of these interactions could contribute to mechanisms of neuroprotection. Age-related changes in interactions between ERβ VCP, ANXAI, ANXAV, or GAPDH could all reasonably contribute to E2-mediated neuroprotection.

I identify a novel interaction between ERβ and VCP, which appears in nuclear aggregatates in neurodegenerative diseases. Interestingly, the ERβ:VCP interaction decreases with E_2 in young animals, yet VCP nuclear expression is increased by age alone. This age-related increase in nuclear VCP suggests that age alone could increase nuclear aggregation of this protein. Further, if E_2/ERβ is neuroprotective in young animals, one could speculate that E_2 decreases aggregation of VCP in young, but not in aged animals, leading to an increased incidence of neurodegenerative disease. VCP interacts with nuclear coregulatory proteins such as BRCA and SRC-1 (Zhang *et al.*, 2000; Jung *et al.*, 2005), therefore its association with these complexes and possibly ERβ could sequestering VCP and prevent aggregation in young animals. However, a lack of E_2 induced association with ERβ could be one contributing factor to an increase in incidence of nuclear protein aggregates and neurodegenerative disease. Thus, the interaction between ERβ and VCP could represent a preemptive, protective role for ERβ in the brain.

Another preventative measure to block cell death involves vigilant DNA repair and maintenance and could represent another role for ERβ in neuroprotection. In chapter IV, I identify and quantify an interaction between ERβ

and ANXA1. ANXA1 has DNA helicase activity which suggests a role for ANXA1 in DNA repair (Hirata and Hirata, 2002; Hirata and Hirata, 1999). ANXA1 has been found in the nucleus of cancer cells, and has also been implicated in proliferation (Kim *et al.*, 2003; Liu *et al.*, 2003). Further, another study suggests that ANXA1 has a role in microglial clearance of apoptotic cells. All of these processes seem like likely targets for ERβ to exert effects on neuroprotection through ANXA1. While E_2 has been implicated in DNA repair, in one study ERα enhanced DNA repair through association with 3-methyladenine DNA glycosylase (Likhite *et al.*, 2004), whereas in another study, ERβ when associated with insulin receptor substrate 1 (IRS-1) homologous recombination DNA repair was inhibited (Wilk *et al.*, 2012; Urbanska *et al.*, 2009). Also, ERβ is typically thought to be anti-proliferative, but can contribute to neurogenesis. Interestingly, ANXA1:ERβ interaction is increased by the administration of E_2 to young animals, but in aged animals this effect is no longer induced by E_2. These results may be indicative of a change in the function of ERβ regarding DNA repair or proliferation with age, both of which are consequences of nuclear ANXA1 activities. While no direct link exists apart from the interaction presented here, the role of ERβ:ANXA interactions could be an integral part of cellular maintenance, protection and perhaps even neurogenesis regulated by E_2, and further, changes in these interactions with age could have a significant impact when considering HT in older patients.

Annexins in general have been implicated in cell death and may also interact with ERβ to modulate the effects of E_2 on cell death. Typically, annexins are not commonly thought of as nuclear proteins, but localization of annexins to the nucleus confers cellular senescence and cell death. Localization of ANXAV at the nuclear envelope has been noted as a marker for cellular senescence (Klement *et al.*, 2012), and ANXAV is a common tool for determining cell death through its ability to bind cytoplasmic facing phosphatidylserines on the cellular membrane (Koopman *et al.*, 1994). Membrane proteins found within or around the nucleus are, in some cases, internalized by mechanisms similar to that of EGFR (Lin *et al.*, 2001). Internalization could provide a mechanistic explanation for how proteins like annexins might relocate to the nucleus. Notably, increased tyrosine kinase activity, downstream of EGFR, causes nuclear localization of ANXAV to the nuclear membrane (Mohiti *et al.*, 1997). Also, ERβ is constitutively activated by increased EGFR activity, thus the likelihood of a nuclear interaction between ERβ and ANXAV would be increased in young animals based upon increased expression. E_2 has been suggested to reduce cellular senescence through increasing telomerase expression and activity (Imanishi *et al.*, 2010; Imanishi *et al.*, 2005a; Imanishi *et al.*, 2005b) and can be proliferative and anti-apoptotic or anti-proliferative and pro-apoptotic, dependent largely upon the actions of ERα and ERβ, respectively (Marzioni *et al.*, 2012; Attia and Ederveen, 2012; Dubik and Shiu, 1992). In breast cancer, interestingly, long-term E_2 deprivation can cause E_2 to induce apoptosis (Lewis *et*

al., 2005). The interaction between ERβ and ANXAV identified in this work could contribute to the mechanisms by which ERβ blocks cellular senescence.

One completely novel ERβ-interaction partner identified in chapter IV is α-Enolase (ENO1). ENO1 has a newfound role in the nucleus as an inhibitor of DNA methyltransferase (DNMT) activity and can bind DNA. Decreased DNMT activity is observed in aging and senescence. In chapter IV, the ERβ:ENO1 interaction is decreased by E_2 in young animals and completely reversed by E_2 in aged animals. It is interesting to speculate that ERβ could be playing a role in cellular senescence with ENO1 as well. Interestingly, cytosolic and nuclear ENO1 expression trended toward an increase in aged animals given E_2 treatment, and with significance, these data would correlate with an increased ERβ:ENO1 interaction with age. Hypothetically, E2 could be potentiating ENO1-mediated DNMT inhibition through ERβ – leading to cellular senescence. ENO1 also exists as an alternatively spliced variant called c-myc binding protein (MBP). Importantly, α-Enolase and not MBP was identified through peptide fingerprinting. One interesting new finding is that ENO1 expression is greatly increased in ER+ breast carcinoma, and MBP is not. In fact, increased MBP expression correlates with a better prognosis (Ray and Steele, 1997; Contino *et al.*, 2013; Lo Presti *et al.*, 2010). Taken together, while the functional consequences of an interaction between ERβ:ENO1 remain unknown, the change in this interaction would surely have opposite consequences with E2 administration between young and aged animals.

The age related changes of the ERβ:ANXAV interaction reported in this work are variable and the consequences are unknown. All of the spots identified in the cluster identified as ANXAV were increased by E2 in young animals. In aged animals, however, one of the protein spots increased with E_2, while another decreased with E_2 treatment in aged animals, and a third spot increased with E_2. The spot patterns (Figure 20) are suggestive of a shift in post-translational modifications; however, no modifications to ANXAV have been discovered. It is possible that carbamylation of this protein produced protein spots that could be seen as artifacts, but the shift in spot pattern appears to be vertical indicating a shift in molecular weight, usually due to an ubiquitin modification, not carbamylation. Nevertheless, the relationship between ERβ and annexins remains uncharacterized, but could have a role in cellular senescence. Overall, interactions between ERβ and nuclear annexins could work in concert toward neuroprotection. However, this interaction is changed by age when E_2 is administered *in vivo,* therefore any functional consequences of such an interaction would be altered as well.

Like annexins, GAPDH is not typically considered a nuclear protein, but it is possible to speculate that a nuclear interaction between ERβ and GAPDH may influence apoptosis and neuroprotection. Nuclear GAPDH is usually an indicator of apoptosis (Ishitani *et al.*, 1998; Sawa *et al.*, 1997). Further, S-nitrosylation (SNO) of GAPDH initiates apoptosis by translocating to the nucleus and interacting with Siah1 (BAG-1) (Hara *et al.*, 2005). BAG-1 is an E3-ligase that can aid in ubiquitination and

ultimate proteasomal degradation of ERα (Zhang *et al.*). While BAG-1 interactions with ERβ have not been shown, data from the cross linking studies (Appendix A, Table 9) supported the interaction between ERβ and a number of E3-ligases. It is possible that the ERβ:GAPDH interaction occurs though one of these connections. In these experiments, ERβ:GAPDH decreases with E_2 administration in young animals and is unaffected by E_2 in aged animals. It is interesting to speculate that if the interaction between ERβ and GAPDH facilitates apoptosis, then E_2 could regulate this process through ERβ in young animals. Otherwise, an E3-ligase could target both proteins for degradation, and as postulated for VCP, ERβ could contribute to sequestering GAPDH, thus blocking its nuclear apoptotic function. Consequently, a loss of this E_2 induced interaction could dysregulate the balance between E_2 neuroprotection and apoptosis in aged animals.

Nuclear actin aids traditional and non-traditional ERβ interactions that are altered by E2 in the aged brain

ERβ has been clearly defined as a transcription factor, but non-transcriptional roles for ERβ have been the subject of debate. However, the discovery of nuclear actin has been helpful in connecting alternative functions for nuclear receptors with their usual transcriptional roles due to the involvement of actin in transcription and translation. From the studies presented in chapter IV, Identify a number of novel ERβ-associated proteins including nuclear actin and actin-associated proteins. Thus, by examining the transcriptional functions of

nuclear actin and proteins that bind actin in the nucleus, I am able to speculate on potential novel functions for actin bound ERβ.

Another proposed mechanism for ER-mediated neuroprotection is through the interaction between ERs and various actin-binding scaffolding proteins such as modulator of nongenomic activity of estrogen receptor (MNAR) and p130Cas, which facilitate E_2-activated kinase cascades. Here I report an interaction between ERβ and GELS, an actin-binding protein found in the nucleus. While GELS can serve as a coactivator for nuclear receptors such as AR and ER, the mechanism by which this occurs are not known. It has been suggested that actin and actin binding proteins interact with transcription factors and nuclear receptors to help create a dynamic stage upon which transcription can be performed (Miyamoto and Gurdon, 2012; Miyamoto *et al.*, 2011b; Miyamoto *et al.*, 2011a; Miyamoto and Gurdon, 2011). Actin-binding proteins such as gelsolin are proposed to assist in positioning transcription factors in proximity to target genes (Figure 35). Typically, E_2 enhances an interaction between coactivator proteins and ERs, therefore it is possible that in young animals GELS is acting as a coactivator and preferentially associating with ERβ when E_2 is present. However, in aged animals, this E_2-induced increase in association is lost, suggesting a change in the ERβ:actin dynamics and coactivator activity of GELS.

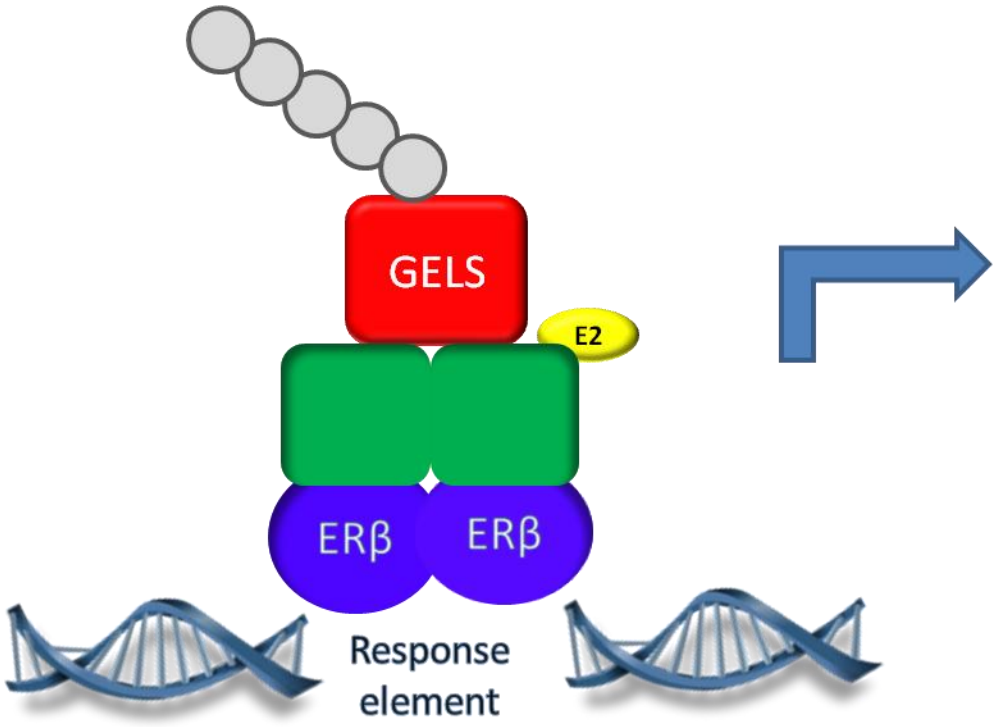


Figure 35. Proposed model for GELS:ERβ interactions. Gelsolin (GELS) is an actin (grey circles) binding protein that helps direct transcription in the nucleus. Changes in the interaction between GELS and ERβ with age and E_2 treatment suggest that GELS may play a role in E_2 gene regulation. Green box- transcription factors.

Also commonly bound to actin are heteronuclear riboproteins (HnRNPs), splicing factors that may contribute to a splicing feedback loop with ERβ. HnRNPs were found to be more associated with ERβ in young animals and less associated in aged animals in response to E_2. Regardless of nuclear or cytoplasmic localization, HnRNPs, utilize actin to maneuver mRNA into position for processing (Pahlich *et al.*, 2009). Studies suggest that HnRNPs can block alternative splicing, and data from

our group and others suggests that E_2 deprivation is involved in alternative splicing of ERβ (unpublished data, (McNally *et al.*, 2006)) . HnRNPs have been depicted as inhibitors of alternative splicing because they can enhance exon exclusion, and in this case E_2 might no longer prevent alternative splicing through decreased ERβ:HnRNPH interaction in the ventral hippocampus of aged rodents. This hypothesis fits with an increase in rodent ERβ2 in the hippocampus of aged animals subjected to estrogen deprivation, as rERβ2 contains an included exon that encodes its 18-amino acid insert. Furthermore, preliminary data from our lab also supports a regulatory loop between ERβ-mediated splicing of its own transcript through a splicing factor, Nova1. Increases in alternative splicing with age have been proposed to be deleterious (Tollervey *et al.*, 2011). Thus, a change in ERβ interaction with HnRNPH in aged animals could contribute to some of the negative neurological effects of aging and/or ET after a long period of estrogen deprivation.

Iimplications for ERβ in the periphery

ERβ is most known for its roles in non-reproductive systems ranging from the colon to the brain. There are cardioprotective effects of E_2 in the heart, both anti- and pro-tumorigenic properties of E_2 in various cancers, and proliferative effects in bone and breast to name a few. Overall, the WHI studies gave insight into the role of E_2 signaling during and after menopause suggesting a broad change in way the body receives and processes E_2 after deprivation and with advanced age. From these studies many models have been developed and there is a wealth of

knowledge that could be translated and applied between the brain and peripheral organ systems.

The WHI studies demonstrated that a change in E_2 signaling is not just apparent in the brain, but also in cardiovascular systems. Premenopausal women are much less likely to experience cardiovascular disease than men, however postmenopausal women are not protected (Atilla *et al.*, 2001). DPN, an ERβ-selective ligand induces S-nitrosylation of proteins in the heart, hypothesized to be one mechanism of cardioprotection (Lin *et al.*, 2009). SNO-associated proteins decrease with age, and it has been further hypothesized that this is an important factor as to why postmenopausal women have a greater chance of experiencing cardiovascular disease than premenopausal women (Santhanam *et al.*, 2010).

The WHI studies were suspended abruptly due in part, to an increase in the incidence of invasive breast cancer. ERβ splice variants may be of particular interest in cancers in spite of a lack of evidence that implicates ERα splice variants in the progression of breast cancer (Madsen *et al.*, 1995; Madsen *et al.*, 1997), ERβ promotes apoptosis and not proliferation in colon and breast carcinomas, thus the presence of ERβ splice variants could reasonably serve to create diversity in estrogenic signaling by acting as a counterbalance to ERα. In breast cancer cells, unique ER splice variants have been found, suggesting again that blocking the role of ligand-activated ERs could allow for the expression of alternative variants that may possibly act independent of ligand (Poola *et al.*, 2002). The expression of such

variants would warrant deeper investigation as unliganded ERβ can regulate overlapping and distinct classes of genes from E_2-activated ERβ (Vivar *et al.*, 2010). Although no such experiment has been performed for individual ERβ splice variants, it is tempting to speculate, based upon data from this project, that ERβ splice variants are likely to regulate a set of genes that would overlap with a ligand-independent class of ERβ1-mediated genes.

Moreover, the therapeutic use of selective estrogen receptor modulators (SERMS) such as tamoxifen (TAM) has been a breakthrough for the treatment of ER positive (ER+) breast cancers, but ERβ splice variants may be unresponsive to these treatments. It is fortunate that the expression of ERβ2 in breast cancer corresponds with a favorable prognosis (Sugiura *et al.*, 2007), because there is no pharmacological modulator of ERβ2 activity. To the same extent, the use of aromatase inhibitors would be ineffective for targeting human ERβ splice variants. In Chapter III, transcriptional actions of ERβ2 are not blocked by the full antagonist (ICI 182 780, or fulvestrant), however upon the inhibition of p38 activity, ICI blocks ERβ2 repression of the hAVP promoter. This finding could translate to an important drug interaction with anti-estrogens in the event that p38 inhibitors are cleared for therapeutic use to treat inflammatory issues such as arthritis and neuropathic pain (Anand *et al.*, 2011); this finding could be critical since there is no current method for modulating the activity of ERβ2.

Importantly, anti-estrogen therapy such as TAM can be long-term, lasting up to 10 years, but the long-term effects of tamoxifen, especially on neurological processes has not been adequately addressed (Davies *et al.*, 2012). Reports from Adjuvant Tamoxifen Longer Against Shorter (ATLAS), a randomized clinical trial that demonstrated the benefits of longer TAM treatment, have suggested that TAM may have significant negative effects on memory and hippocampal volume (Eberling *et al.*, 2004). TAM is not a full 'anti-estrogen', since it can have agonistic effects in the brain, therefore the consequence of long-term TAM treatment could be very different from long-term ET. It goes without saying that the effects of these treatments would likely depend upon the age and menopausal status of patients for all the reasons discussed here relevant to ER expression profiles, E_2 receptivity and protein:protein interactions that lead to gene expression and other cellular and behavioral outcomes.

Future Directions

The studies presented here represent a significant contribution to the study of estrogen receptor actions in the brain around the time of menopause. Further, these data support the hypothesis that inherent structural and molecular components contribute to changes in ERβ action with age and in the absence of E_2. While the data presented are substantive, there are many new possible lines of investigation brought about from this work.

First, the evaluation of the constitutive actions of the human ERβ splice variants requires context. Within in this body of work there is evidence for the expression of these splice variants in the aged human brain, however mapping of the human splice variants in the brains of pre-, peri- and postmenopausal women is a key component to understanding how ET or even anti-estrogens will be processed and utilized by the aged brain. If ERβ2 is upregulated after an extended period of estrogen deprivation as in the rodent hippocampus (Wang *et al.*, 2012), then one could expect those brain regions to be less responsive to E_2. To target those regions, it would be prudent to determine how the hERβ splice variant functions in neural contexts to determine whether allosteric modulators or other pharmacological agents could be useful in the place of ET.

There is some evidence to suggest that the ERβ splice variants could interact with a different set of proteins. It is also important to determine how the protein interaction partners of ERβ would differentially interact with alternative splice variants of ERβ. One caveat to this idea is that the human ERβ splice variants, are dissimilar in sequence alterations. This could create differential interactions due to alterations to the AF-2 domain in the human splice variants and an unmodified AF-2 region in the rodent variants. Limitations to this type of investigation are that performing this type of experiment in human tissue would be impractical and there are no antibodies designed specifically to the human splice variants. However, experiments with the rodent ERβ2 specific antibody would be appropriate and very

informative. Further, since the antibody used for the experiments described in this document are directed against the N-terminus, this antibody could have potentially included all of the rodent splice variants examined in Chapter 3. ERβ was identified via western blot around an isoelectric point of 7.5-9.0, thus further investigation into ERβ splice variants would likely require a narrowed isoelectric focusing around this range. In fact, most of the proteins identified had an isoelectric point between 4-8. More proteins could be identified using alternate isoelectric focusing ranges.

Characterizing the interactions between ERβ and proteins identified in Chapter 4 would be a large undertaking; however such work is critical to determine the relevance of changes in these interactions with age. The interactions were identified as the result of co-immunoprecipitation experiments, thus any ERβ:protein interactions discovered could be direct or indirect. The first step in characterizing these interactions would be to determine whether ERβ interacts directly with the protein in question. These experiments could be performed using yeast-two-hybrid experiments, however using a measure of proximity (i.e., bioluminescence/fluorescence resonance energy transfer assays) may also be a useful tool as protein:protein interactions can depend upon cellular context and modifications to the proteins.

Post-translational modifications such as phosphorylation of ERβ and associated proteins can change their inherent functionality by altering charge and ultimately protein:protein interactions. Aging alters the availability of proteins such

as activated p38 (Li *et al.*, 2011), and estrogen deprivation could have a combined effect on these processes. It would be informative to identify the PTMs of protein spots that are significantly affected by age. For example, multiple protein spots identified as HnRNP H, and not all spots displayed the same interaction profile with ERβ in response to aging and E_2. This could be indicative of modified versions of this protein that preferentially associate with ERβ. On the contrary, another direction for this line of research could be examining how modified ERβ receptor protein differentially interacts with effector proteins by creating mutant proteins lacking phosphorylation or sumoylation sites. Another interesting avenue of exploration is how S-nitrosylation (SNO) of proteins identified in Chapter 4 could alter their interaction with ERβ. Further, there is evidence to suggest that changes in SNO proteins with age in the brain could be a result of hormone deprivation and replacement (Nakamura *et al.*, 2013), therefore, interrogation of SNO proteins in the same paradigm could be of particular interest in neuroprotection.

The possible extrapolations of the work done in this dissertation are vast due to the uncharacterized nature of a) the human ERβ splice variants, with particular regard to the actions of these receptors in the aged brain during and after extended estrogen deprivation and b) the mass proteomics experiment performed identifying many novel potential interaction partners for ERβ in the aged brain. This work leads to more questions regarding ERβ signaling in the menopausal brain and

suggests that the molecular mechanisms governing these processes are complex and less understood than previously thought.

Take Home Message

The work presented in this dissertation supports the idea that ERβ signaling is drastically altered by age and during periods of estrogen deprivation, such as menopause. The function of human ERβ as a ligand-inducible transcription factor is dependent upon alternative splicing. This should be taken into consideration when considering HT and further the use of anti-estrogen therapies or therapeutic kinase inhibitors should be evaluated on an individual basis. In addition, *in vivo* studies of the rodent hippocampus suggest that some protein:protein interactions with ERβ in response to E_2 after a brief period of E_2 deprivation are significantly changed with age. This supports the idea that ERβ function, or the ERβ interaction partners identified are altered with age in a way that would change such an interaction. Taken together, the data presented here provide the initial rationale for potential mechanisms that lead to changes in E_2 signaling during menopause (Figure 36). More importantly, this suggests that ET in post-menopausal women could have neurological effects compared to younger peri-menopausal women. Taken together, the work presented in this document lends support to the hypothesis that there are inherent changes in ERβ function with age and in the absence of E_2 that aberrant effects of ET resulting advanced age should be taken into consideration.

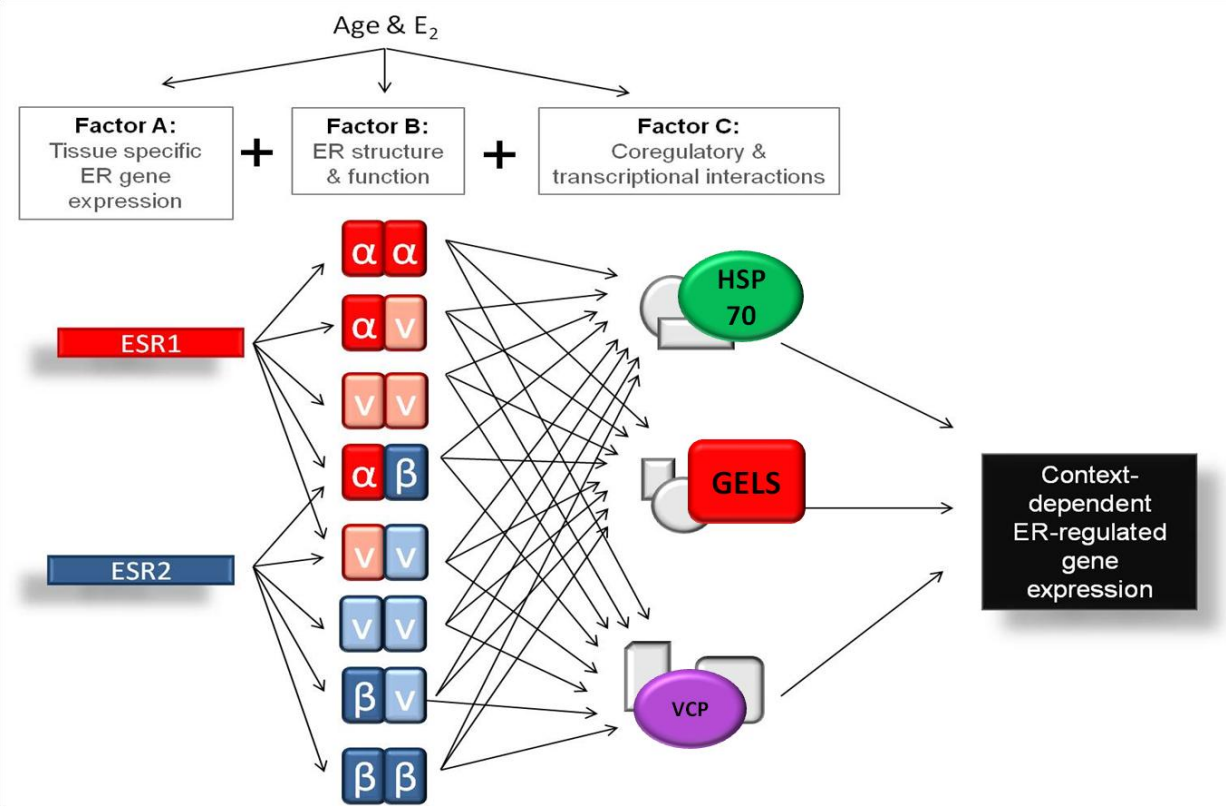


Figure 36. Model for the influence of age and E_2 over ER-mediated cellular processes. Alternative splice variants of ERα and ERβ require various protein:protein interactions to regulate E_2-mediated cellular responses. Age and E_2 exposure changes interactions between ERβ and HSP70/GELS/VCP and other proteins which could serve as a mechanistic explanation for age-related changes in the molecular actions of E_2.

CHAPTER VI

GENERAL METHODS

Chapter III

Human tissue

The amygdala of 3 human subjects (female) obtained through the Netherlands Brain Bank. The absence of neuropathological changes was confirmed by systematic neuropathological investigation by a neuropathologist (Dr. W. Kamphorst, Free University Amsterdam). Total RNA isolation was performed on sonicated tissue samples using Trizol reagent (Invitrogen Inc., Carlsbad, CA) according to the manufacturer's directions. Following RNA isolation, 0.5 μg total RNA was reverse transcribed using the First Strand Synthesis SuperMix for qRT-PCR (Invitrogen Inc., Carlsbad, CA).

Primer sequences

Plasmid expression vectors (pcDNA 3.0; Invitrogen, Carlsbad, CA, USA) containing inserts for human ERβ1, ERβ2, ERβ4 and ERβ5 were used as previously reported: ER-β1 forward, 5′-GTC AGG CAT GCG AGT AAC AA-3′; ER-β1 reverse, 5′-GGG AGC CCT CTT TGC TTT TA-3′; ER-β2 forward, 5′-TCT CCT CCC AGC AGC AAT CC-3′; ER-β2

reverse, 5′-GGT CAC TGC TCC ATC GTT GC-3′; ER-β4 forward, 5′-GTG ACC GAT GCT TTG GTT TG-3′; ER-β4 reverse, 5′-ATC TTT CAT TGC CCA CAT GC-3′; ER-β5 forward, 5′-GAT GCT TTG GTT TGG GTG AT-3′; ER-β5 reverse, 5′-CCT CCG TGG AGC ACA TAA TC-3′; GAPDH-F: 5′-TCC CTG AGC TGA ACG GGA AG-3′; GAPDH reverse, 5′-GGA GGA GTG GGT GTC GCT GT-3′ (University of Cincinnati, Cincinnati, OH, USA) and have been extensively characterized (8).

Cell culture

The cell lines used for all transient transfections were HT-22 mouse hippocampus-derived neuronal cells (generously provided by Dr D. Schubert, Salk Institute, San Diego, CA, USA) or human neuroblastoma-derived SK-N-SH cells (American Type Culture Collection, Manassas, VA, USA). HT-22 and SK-N- SH neuronal cells were maintained in phenol red-free minimal essential medium (MEM) (SK-N-SH) or MEM with Earle's salts, respectively. The medium contained 4.5% glucose and L-glutamine (Invitrogen) and was supplemented with 1x non-essential amino acids, and 10% fetal bovine serum (FBS) or dextran charcoal-stripped FBS (Hyclone Laboratories, Logan, UT, USA). Cells were grown to 70% confluency and all transient transfection experiments were performed within ten passages.

Transient transfections

HT-22 cells were plated at a density of 0.2 x 10^5 cells / well in 96-well plates and allowed to grow to 70–80% confluency until 24 h before transfection. SK-N-SH cells were plated at a similar density and allowed to grow to 70–80% confluency until 48 h before transfection. Immediately before transfection, the media was removed, and cells were washed once with 1x PBS. Regular media was replaced with media containing 10% charcoal dextran-stripped FBS to eliminate the presence of exogenous steroids or growth factors. Transfections were carried out using a lipid-mediated transfection reagent in accordance with the manufacturer's instructions (Fugene6; Roche Molecular Biomedical, Indianapolis, IN, USA). Cells were then incubated with the transfection media complex in stripped media containing the empty vector, hERb1, 2, 4 or 5 expression vectors for 12 h, which was then replaced with phenol red-free Dulbecco's modified Eagle's medium containing dextran charcoal-stripped fetal bovine serum. After a total of 24 h in stripped media, vehicle, hormone and / or kinase inhibitor treatments were given for an additional 12 h. The hormone compounds were diluted in 100% EtOH and used at a final concentration of 100 nM in 0.001% EtOH: E_2 (Sigma-Aldrich Co. St Louis, MO, USA), 5α-androstane-3β, 17β-diol (3β-diol) and ICI 182 780 (Steraloids, Newport, RI). The p38 inhibitor SB 202190 was diluted in nuclease-free water and phosphoinositide 3-kinase (PI3K) inhibitor LY 294002 was diluted in 100% EtOH; both were used at a final concentration of 10 lM.

Reporter constructs

The ERE-tk-luciferase reporter (generously provided by Dr P. Budworth, Case Western Reserve University, Cleveland, OH, USA) contains two copies of the vitellogenin ERE sequence coupled to the minimal tk-Firefly luciferase promoter and sub cloned into pGL2-Basic plasmid (Promega, Madison, WI, USA). The AP-1-tk-Firefly luciferase promoter (generously provided by Dr C. Clay, Colorado State University, Fort Collins, CO, USA) contains three copies of the AP-1 sequence (TGACTCA) coupled to the minimal tk-Firefly luciferase promoter and sub cloned into pGL2-Basic plasmid. The human arginine vasopressin (AVP) promoter-Firefly luciferase reporter construct was purchased from Switch Gear Genomics (Menlo Park, CA, USA) and contains a 929-bp insert upstream from the transcription start site of the human AVP promoter in the pSGG_prom plasmid vector. The Renilla luciferase pGL4 reporter construct (Promega) was used as an internal control for calculating plasmid transfection efficiency.

Luciferase assays

Control reporter (Renilla luciferase) and reporter (Firefly luciferase) activity was measured a total of 36 h post-transfection using the Dual Luciferase Reporter Assay system (Promega) in accordance with the manufacturer's instructions. Relative light units for each construct were measured using the Synergy HT multimode plate reader (BioTek Instruments Corp., Winooski, VT, USA) and represented as a ratio of

Firefly : Renilla. Luciferase substrates (100 ul / well) were added to cells using an automatic injector system. All constructs were transfected in replicates of six wells within each assay, and each transfection assay was repeated in a minimum of three independent experiments. Independent experiments were compared by calculating the percentage change from empty vector controls. Furthermore, each experiment was performed using a minimum of three different preparations for each plasmid reporter construct and expression vector. Differences among hormone treatment groups for individual receptors were analyzed by one-way ANOVA followed by Tukey's honestly significant difference test. Post-hoc comparisons between control groups (empty-vector + vehicle; receptor + vehicle) were analyzed using Student's t-test. $P < 0.05$ was considered statistically significant. All transfection data are represented as the percentage change compared to vehicle-treated, promoter + empty vector controls.

Electromobility shift assay (EMSA):

Human ERβ expression vector plasmids (1μg each) were used to synthesize receptor proteins in vitro using the TnT-coupled rabbit reticulocyte lysate system (Promega) with T7-RNA polymerase according to manufacturer's directions.

Oligonucleotides:

Double-stranded oligonucleotides containing the vitellogenin consensus ERE sequence were 32-P end-labeled with T4 polynucleotide kinase. The percentage of

32-P incorporation was determined and labeled probes with greater than 50% 32-P incorporation were used for EMSAs.

Gel electrophoresis

Receptor protein lysates were incubated with 100 nM E_2 or 0.001% ethanol (vehicle control) for 18hours before gel electrophoresis. Following ligand-binding, receptor lysates were incubated with 1x gel shift binding buffer [20% glycerol, 5 mM MgCl2, 2.5 mM ethylenediaminetetraacetic acid, 2.5 mM dithiothreitol, 250 mM NaCL, 50 mM Tris-HCL, 0.25 mg / ml poly(dI-dC)poly(dIdC)] for 10 min. Specific binding reactions were also incubated with 500-1000 fold excess of unlabelled ERE oligonucleotide. Nonspecific binding was tested using the 32P-SP1 oligonucleotide (data not shown). After an initial 10-min incubation, 32-P-ERE was added and incubated for an additional 20 min. DNA–protein complexes were resolved on a 6% Novex DNA retardation bis-acrylamide gel (Invitrogen) for 20 min at 250 V. Gels were dried on a vacuum gel dryer at 80 °C for 2 h before autoradiography.

Autoradiography and analysis

Dried gels were exposed to X-ray film (Biomax MS; Eastman Kodak Company, New Haven, CT, USA) for 12 h at 70°C. Gel bands were scanned and optical density was quantified using IMAGE J (NIH, Bethesda, MD, USA). Relative densitometry of replicate gels were averaged and data are reported as the mean SD density of pixels.

Site-directed mutagenesis

Site-directed mutagenesis was used to delete the putative AP-1 regulatory site from the human AVP promoter. The imperfect AP-1 site, TGACTCC, located –611 bp upstream of the transcription start site, was deleted by directing primers to the region (forward: 5'-CCTCTCATTCTGTGTCCCTACGACGGCGG-3'; reverse: 5'-CCGCCGTCGTAGGGACACAGAATGAGAGG-3') using the Quik Change XL system (Stratagene, La Jolla, CA, USA) in accordance with the manufacturer's instructions.

Chapter IV

Animals

Female Fisher 344 rats (3 mo (N = 40)- and 18-mo (N= 39)) were obtained from the NIH aging colony (Taconic) and allowed to acclimate for 7 days prior to treatments. Next, all animals were bilaterally ovariectomized (OVX) and allowed to recover post-OVX for 7 days. Briefly, rats were deeply anesthetized under isofluorane gas and the ovary and distal end of the uterine horn were pulled from the body cavity through a 1cm incision made through the skin and body wall. The horn was clamped with a hemostat and ligated proximal to the clamp. The ovary and distal uterine horn were then removed to ensure that all potential ovarian sources of E_2 were eliminated, thereby creating a surgically-induced model of menopause. After 7 days post-OVX the animals received once/day subcutaneous injections of 2.5ug/kg

17β-estradiol or safflower oil (vehicle) for 3 consecutive days. Animals were sacrificed by rapid decapitation 24 hours after the last injection and trunk blood and brains were collected further analysis.

Estradiol enzyme-linked immunoassay

Circulating17β-estradiol was measured by using an enzyme-linked immunoassay system (EIA, Cayman Chemical). Briefly, trunk blood was collected in tubes coated with 20-50 units of porcine heparin (Sigma) per ml of blood collected. Blood was then centrifuged at 4000 x g for 7 minutes and plasma was removed subjected to immunoassay per manufacturer's instructions. The limit of detection for the assay was 6.6 pg/ml. Plasma E_2levels were determined to be 53.67 (SEM+/- 7.24) pg/ml in young animals and 50.56 (SEM+/- 8.78) pg/ml in aged animals, within the physiological range for post-menopausal patients receiving hormone replacement therapy (17-75pg/ml) (Schmidt *et al.*, 1994).

2D Sample preparation

Brains were rapidly frozen using isopentane and stored at −80°C until further processing. Briefly, frozen brains were sectioned at 200 μm on a freezing microtome and the ventral hippocampus was microdissected using a 0.75 mm Palkovit's brain punch tool (Stoelting Co., Woodale, IL). The specificity of the microdissection was confirmed using The Rat Brain in Stereotaxic Coordinates, Fourth Edition Atlas (G.

Paxinos and C. Watson) and are as follows: From bregma -4.16 - -5.80mm, DV 6.0-9.0mm AP 3.0-6.0mm. (Banasr *et al.*, 2006). Punches were pooled (4 animals/sample) and placed in CERI solution of non-denaturing NE-PER Nuclear Protein Extraction Reagents (Thermo Scientific Pierce), supplemented with 7x EDTA-free Complete Mini Protease inhibitors (Roche). Nuclei were subjected to lysis and insoluble material including DNA was pelleted and excluded from the soluble portion of the extracts. Nuclear extracts were subjected to co-immunoprecipitation for ERβ (Ab288, Clone 14C8, Abcam (1ug/100ug protein) overnight. Subsequently, antibody and extracts were incubated with magnetic beads for 10 minutes at room temperature (Millipore Protein G) and after antibody binding, beads were washed 3x with 1X PBS prior to elution with 1.25 M Glycine. Two additional antibodies were tested using the same paradigm including α-ERβ LBD (1ug/100ug protein) (Saji *et al.*, 2000) and H-150 (1ug/100ug protein , Cruz Biotechnology). Protein spots that were common between all three antibodies were considered specific, whereas those that did not overlap were excluded from the final analysis. In addition, a control rabbit-anti-IgG antibody was used under the same experimental paradigm to identify non-specific spot patterns. Following co-IP, samples were prepared for isoelectric focusing using the 2-D Cleanup system (GE Healthcare).

CyDye labeling

7.5µg from each sample was combined and aliquoted into an internal standard to correspond with each sample being compared (N=3 for each group). Each standard (7.5 µg) and sample (7.5 µg) was reduced using 2nmol TCEP (tris(2-carboxyethyl)phosphine) for 1.5h at 37ºC in the dark. Then, all samples and standards were labeled with 4nmol Cy5 and Cy3 DIGE Fluor saturation dyes, respectively (GE Healthcare), for 30 minutes at 37ºC in the dark. Saturating dyes are an advantage over minimal dyes due to labeling of ~ 98% of cysteine sulfhydryls (compared to ~6% of lysines) resulting in maximum sensitivity. The reaction was stopped by adding equal volume 2x Rehydration buffer (UTC (7 M Urea, 2 M Thiourea, 4% w/v CHAPS) with Pharmalytes (2%v/v final) and DTT (130 mM final)).

Isoelectric focusing & SDS-PAGE

Each dyed sample and corresponding standard (15µg of protein: 7.5µg Cy3 labeled pooled internal standard and 7.5µg Cy 5 labeled experimental group) were incorporated into a rehydration buffer (UTC with 0.5%v/v IPG buffer 3-11NL, 15mgl/ml Destreak Reagent) and applied to a 24cm 3-11NL Immobiline Drystrip and subjected to active rehydration (10h at 50V) followed by an optimized run program: 1) Step: 500V for 500Vh, 2) gradient: 1000V for 1000Vh 3) gradient 8000V for 16500Vh 4) Step: 8000V for 42000Vh (75uA limit at 15ºC, 61000 total

Vh). After the 1st dimension strips were equilibrated in 1% w/v DTT, 2.5%w/v iodacetamide and a brief 1X SDS Running buffer wash before being resolved on a 12% SDS-PAGE at 2W/gel (limit: 500V, 40mA/gel) for 17:30h.

Imaging and analysis

Gels were imaged on the Typhoon 9400 (Cy5: ex: 633nm em: 670nm BP 30, Cy3: ex: 532nm em: 580nm BP 30 100pixels, 450PMT) prior to analysis with DeCyder Analysis software (GE). Using Differential in-gel Analysis (DIA) each gel was analyzed individually for processing up to 1500 spots, using standard spot exclusion for the following properties: slope >1, area <200, volume <2500, peak height <16 >10,000. All gels were analyzed together using the Biological Variance Analysis (BVA) module.

Spot Analysis and statistics

The BVA module was used to compare replicate gels and perform inter-gel statistical analysis and will be referred to from this point forward. The BVA module accounts for the spots identified and confirmed in each gel's DIA workspace, and automatically selects a master gel (gel displaying the most confirmed spots) to match and compare each replicate gel against. Each protein spot was matched individually by examining each gel, using match vectors and creating landmark spot affirm accurate spot matching. Standard abundance quantifies a given protein spot

based upon protein spot volume, area and background. Each spot is then normalized to its own internal standard and log transformed to perform statistical tests. Each gel represents the pooled internal standard (equal amounts of protein from each experimental replicate) compared to samples from young vehicle (YV) treated animals, young estradiol (YE) treated animals, aged vehicle (AV) and aged E_2 (AE) treated animals. Each gel was performed via 3 independent experiments (i.e, Experiment 1: YV1 v. internal standard, Experiment 2: YV2 v. internal standard, etc.). Each sample (i.e., YV1, YV2, YV3) was representative of 4 pooled ventral hippocampus taken from different animals, thereby contributing to a biological variance of 12 animals/group, n =3. Statistical significance for 2D-spot analysis was determined using Decyder software by calculating an average log standard abundance for each group being tested (i.e., YV v. YE); thereby the statistical significance can be determined by using 1-way ANOVA ($p>0.05$). Notably, statistical significance was equivalent using 1-way ANOVA or student t-test.

Spot picking

After electrophoresis and analysis of analytical gels, a preparative gel representing ~400ug of co-immunoprecipitated protein was used to pick spots for peptide identification via tandem mass spectrometry. Gels were fixed and post-stained with Sypro Ruby and/or Coomassie G250 to visualize protein spots for excision. While individual protein spots were analyzed through BVA, due to the small size of protein

spots, and low visibility of some post-stained spots, groups or 'chains' of similar spots were picked and pooled. Spots from preparative gels were picked using the Ettan DIGE automated spot picker, and residual gel spots were excised using a sterile glass Pasteur pipette. Reference markers were placed at 3.5 cm and 10 cm from the edge of glass plates following treatment fixative treatment with Bind Silane (8 % Ethanol (v/v), 0.002% Acial Acetic acid, 0.0001% Bind Silane). Spot picking parameters that were customized from standard settings include: Jazz 1.3 mm, 50ul aspiration volume, 51ul dispense volume.

In-gel digestion of peptides

Mass spectrometry and peptide fingerprinting analysis were performed at the Midwest Center for Proteomics under the direction of Dr. Marc Glucksman (Rosalind Franklin University Chicago Medical School). After spot excision, proteins within the gel plugs were washed 2x with 1:1 v/v of 0.1 NH4HCO3 for 15 minutes. The wash solution was replaced with LC/MS grade acetonitrile (ACN) to fully cover the gel plugs (~2x the plug volume). Once the gel plugs aggregated ACN was replaced with a rehydration solution of 0.1M Nh4HCO3 for 10 minutes. After drying the plugs in a vacuum centrifuged proteins were reduced with 10m< dithiothreitol (DTT) and alkylated with 55 mM iotacedamide in 0.1M NH4HCO3. Following another wash in NH4HCO3, the peptides in the plugs were subjected to tryptic digest for 24 hours at 37^0C. Peptides were recovered by adding 10mL of 25mM

NH4CO3 and 5mls of 5% formic acid and CAN. Desiccated peptides were resolved in a formic acid:water:ACN:trifleuroacetic acid mixture (0.1:9.5:5:0.01) (Yang *et al.*, 2009).

Identification of proteins with LC-ESI-MS/MS

Reconstituted peptides were separated with a reversed-phase column (C-18 PepMap100, LC Packings/Dionex, Sunnyvale CA, USA), described previously (Yang *et al.*, 2009). The eluate was introduced onto a QSTAR XL mass spectrometer (Applied Biosystems and Sciex, Concord Ontario, Canada) by electrospray ionization. Candidate peptides were identified via half-second MS scans (300-1500 Thompson), collecting up to five 1.5s tandem MS scans (65-1500 Th). Each ion was assigned a charge between 12-14, and the dynamic exclusion was 40. Identification of proteins was completed using PEAKS software and rat databases from NCBI (http://www.ncbi.nlm.nih.gov/RefSeq/). The False Discovery Rate (FDR) for the PEAKS program was set to 60 providing a conservative estimate of proteins identified within a given spot or chain of spots. Proteins identified with a PEAKS score of 60 or above are listed in Table 1. In the event that multiple proteins were identified for a group of spots picked, the predominant peptide match with a PEAKS score of 60 or above, matched for size and isoelectric point was selected.

Western blotting

Co-immunoprecipitated proteins were obtained as described above, added to a denaturing 4X laemelli buffer, and boiled at 95^0C for 5 minutes. Samples were resolved on 4-20% SDS-PAGE gels (Pierce) for 1.5 hours at 90V and transferred to 0.045µm PVDF membranes overnight at 10mA/gel. Membranes were blocked with 5% bovine serum albumin (BSA) for 1 hour before the addition of 1^0antibody in 1% BSA and 0.01% NaN3 for 1.5 hours. All antibodies were used at a 1:1000 dilution: VCP (Pierce, PA5-17486), ERβ (Santa Cruz, Sc-8974x), ENO1 (Santa Cruz, sc-15343), GAPDH (Santa Cruz, sc-25778) HnRNPH (Santa Cruz, sc-15387) HSP70 (GenTex, GTX-104126) β-actin (Cell signaling, 4970S). Blots were washed 3X with TBST for 5 minutes prior to application of 1:4000 goat α-rabbit-HRP (1 hr.; Santa Cruz, sc-2004). Blots were washed 3X with TBST and imaged on the Biorad Chemidoc XRS+ imager using ECL Chemiluminescent substrate (Pierce). Densitometry was performed using ImageLab software and statistical significance (via 2-way ANOVA and Tukey post-hoc analysis) was calculated using an average of 3 or more independent blots using samples from different animals ($n=3$, $p>0.05$). To confirm the presence of ERβ on the 2D gels, samples were labeled (Cy3) and resolved and visualized on a 2D gel as described previously. Then a portion of the gel narrowed for molecular weight and isoelectric range of ERβ (MW 55kDa, pI~8.8) was transferred onto a PVDF membrane. The membrane was imaged as described previously, and then probed with primary α-ERβ antibody (Sc-8974x) and

secondary goat α-rabbit-Cy5 (GE Healthcare, PA-45011V) and imaged accordingly (Fig. 34).

APPENDIX A

SUPPLEMENTARY DATA

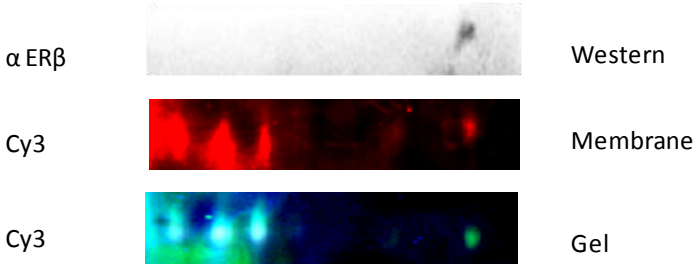


Figure 37. Identification of ERβ by 2D-DIGE and western blotting. Pooled nuclear extracts immunoprecipitated for ERβ were labeled (Cy3) and resolved and visualized on a 2D gel as described previously. Then a portion of the gel narrowed for molecular weight and isoelectric range of ERβ (MW 55kDa, pI~8.8) was transferred onto a PVDF membrane. The membrane was imaged and probed with primary α-ERβ antibody and secondary goat α-rabbit-Cy5.

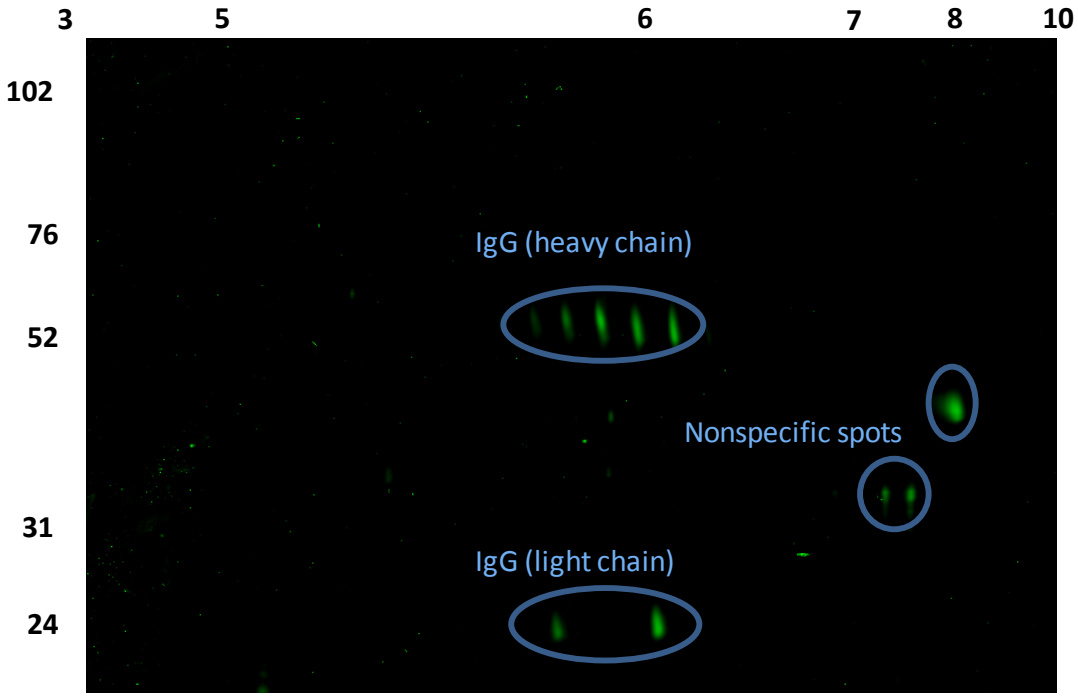


Figure 38. Representative image of non-specific proteins bound to rabbit IgG. Pooled nuclear extracts from YV, YE, AV and AE were incubated with rabbit IgG, co-immunoprecipitated, and subjected to 2D-DIGE as described previously (Chapter VI, Methods). Spots identified here were visually matched with experimental groups and excluded from further analysis.

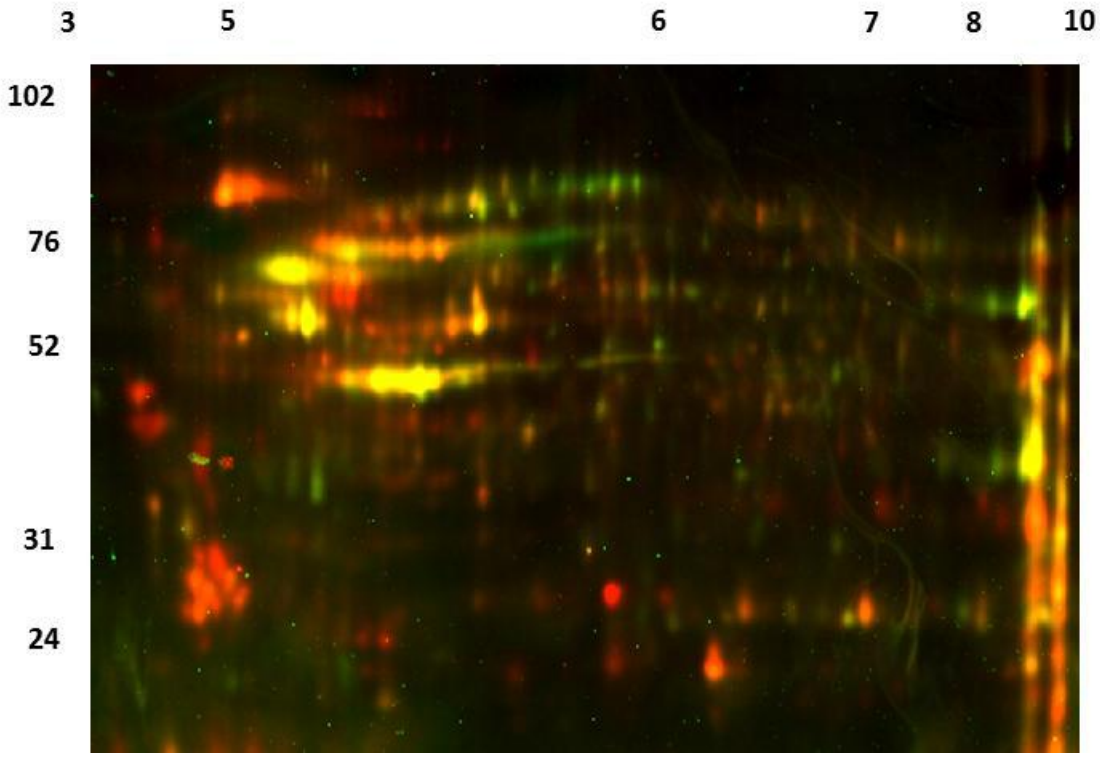


Figure 39: Representative image of proteins co-immunoprecipitated with 2 different α-ERβ antibodies. Pooled nuclear extracts from YV, YE, AV and AE were incubated with α-ERβ antibodies LBD (Green) and Abcam 14C8, (Red), co-immunoprecipitated, and subjected to 2D-DIGE as described previously (Chapter VI, Methods). The antibody that immunoprecipitated the least amount of spots was used and non-overlapping spots were excluded from analysis.

Epigenetic enzymes					
Accession No.	Molecular weight (Da)	PEAKS score	% Coverage	ID	Function
gi|149055327	139191	13.7	0.64	5-methyltetrahydrofolate-homocysteine methyltransferase	DNA methyltransferase
gi|293348935	1595372	13	0.52	myeloid/lymphoid or mixed-lineage leukemia 2	histone methyltransferase
gi|149039889	283429	5.3	0.77	nuclear receptor binding SET domain protein 1	histone methyltransferase
gi|157817995	166822	5.5	0.71	DOT1-like, histone H3 methyltransferase	histone methyltransferase
gi|157822347	262495	5.3	0.84	histone-lysine N-methyltransferase, H3 lysine-36 and H4 lysine-20 specific	histone methyltransferase
gi|157818737	326472	10.9	0.27	probable histone-lysine N-methyltransferase ASH1L	histone methyltransferase
gi|157818797	33853	19.2	3.7	rRNA/tRNA 2'-O-methyltransferase fibrillarin-like protein 1	histone acetlytransferase
gi|27674605	246319	11.9	0.44	mortality factor 4 like 2-like	histone acetyltransferase
gi|157822215	39903	5.7	3.06	chromodomain protein, Y chromosome-like 2	histone acetyltransferase

Table 4. Epigenetic enzymes co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

Transcriptional proteins					
Accession No.	Molecular weight (Da)	PEAKS score	% Coverage	ID	Function
gi|157786842	43248	6.3	4.06	TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor	RNA polymerase
gi|149067363	93190	7.6	2.54	similar to RNA polymerase III subunit RPC2	RNA polymerase
gi|293350969	243382	5.9	0.55	mediator of RNA polymerase II transcription, subunit 12 homolog	RNA polymerase
gi|187469033	60068	5	2.45	Taf15 protein RNA polymerase	RNA polymerase
gi|6978509	310533	7	0.35	adenomatous polyposis coli (APC)	RNA polymerase
gi|157819657	125683	6.4	1.48	AF4/FMR2 family member 4 (Aff4)	RNA polymerase binding protein
gi|6981636	75896	8.2	3.68	transcription factor 12 (HTF-4)	transcription factor
gi|27545350	44417	5.1	3.41	YY1 transcription factor	transcription factor
gi|139001696	247749	14.2	0.66	YY1AP-related protein1	transcription factor
gi|57340270	201319	14.2	0.82	GON4L isoform B	transcription factor
gi|12831205	96718	8.2	0.8	Brain and muscle ARNT-like 1 (BMAL1)	transcription factor
gi|297206838	63515	7.2	1.05	Brain and muscle ARNT-like 2(BMAL2)	transcription factor
gi|55741510	81819	5.3	1.34	CCR4-NOT transcription complex subunit 10 CNOTA	transcription factor
gi|207540	42404	14.8	1.92	POU domain, class 3, transcription factor 2	transcription factor
gi|21362908	24247	13.4	4.13	Runt-related transcription factor 22	transcription factor
gi|136462	38554	8.1	1.88	Homeobox protein Nkx-2.1	transcription factor
gi|400922	28349	5.3	2.81	General transcription factor IIF subunit 2 (TFIIF-beta)	transcription factor
gi|157818503	253819	6	0.47	transcription factor HIVEP3	transcription factor
gi|149061039	174679	5.1	0.83	chromodomain helicase DNA binding protein 7	transcription factor
gi|149049417	113418	6.9	1.1	chromodomain helicase DNA binding protein 4	transcription factor
gi|157821521	303448	5.9	0.63	chromodomain helicase DNA binding protein 6	transcription factor
gi|157821651	73207	6.3	0.91	GTP-binding protein GUF1 homolog	transcription factor
gi|1710720	49078	7.5	1.97	Retinoic acid receptor RXR-beta	nuclear receptor
gi|164663891	55995	6.3	1.38	nuclear receptor subfamily 1 group D member 1 isoform 2	nuclear receptor
gi|11024654	27407	10.9	2.93	prolactin-8A4 precursor (PR8A4)	nuclear receptor
gi|157823029	39818	5.7	3.25	sex comb on midleg-like protein 4	polycomb protein
gi|293340288	71982	15.9	1.4	suppressor of zeste 12 homolog	polycomb protein
gi|293342784	84623	5.5	1.05	enhancer of polycomb homolog 1-like	polycomb protein
gi|293343448	6060695	9.5	1.88	enhanced at puberty (EAP)	coregulator
gi|157816927	61194	11	1.07	glucocorticoid modulatory element-binding protein 1 GMEB1	coactivator
gi|149025968	42692	13.9	1.3	lymphoid enhancer binding factor 1 (LEF1)	coactivator
gi|293355685	1158603	6.8	1	glucocorticoid receptor DNA-binding factor 1	corepressor
gi|19705547	56450	10.7	0.97	nucleus accumbens-associated protein 1	corepressor
gi|149060097	144461	6.6	1.12	similar to BCL6 co-repressor-like 1	corepressor
gi|149041925	44530	6.3	2.07	ladybird homeobox 1 homolog corepressor 1	corepressor
gi|149052886	272492	6.9	1.34	nuclear receptor co-repressor 1 (NCoR1)	corepressor
gi|3023901	127307	5.5	2.54	Protein hairless corepressor	corepressor
gi|149066585	49767	5.7	2.82	Ngfi-A binding protein 2 (NAB2)	corepressor
gi|189083863	44471	17.1	2.29	tumor protein 63 isoform f	corepressor
gi|293354700	127061	5	1.46	SWI/SNF related, matrix associated, a, member 1	chromatin remodeling
gi|109503770	194761	5.8	0.47	polybromo 1-like isoform 2	chromatin remodeling
gi|157817412	207493	8.3	0.63	AT rich interactive domain 1A (SWI-like)	chromatin remodeling
gi|149043863	103715	5.8	1.28	AT rich interactive domain 5B (Mrf1 like)	chromatin remodeling

Table 5. Transcriptional proteins co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

DNA replication/repair proteins					
Accession No.	Molecular weight (Da)	PEAKS score	% Coverage	ID	Function
gi|157819081	96570	5.5	0.8	Ewing tumor-associated antigen 1	DNA damage
gi|157818805	112826	12	1.46	DNA cross-link repair 1A	DNA damage
gi|157822211	133912	8	0.83	CST complex subunit CTC1	DNA repair
gi|109499818	57227	8.2	2.71	apurinic/apyrimidinic endonuclease 2 (APEX nuclease 2)	DNA repair
gi|27229310	71934	5.6	3.64	Werner helicase interacting protein 1	DNA repair
gi|198278575	33016	21.5	3.09	BRCA1/BRCA2-containing complex (BRCC3)	DNA repair
gi|6978829	63592	6.1	1.97	Fanconi anemia, complementation group C	DNA repair
gi|293348540	123631	7.6	1.91	DNA-directed RNA polymerase III B-like	DNA polymerase
gi|149032985	343173	6.4	0.62	REV3-like DNA polymerase	DNA polymerase
gi|4103934	123247	6.3	2.9	replication factor C	DNA replication
gi|157816943	91977	5.4	2.17	minichromosome maintenance complex component 8	DNA replication
gi|149028522	68350	18.7	1.47	minichromosome maintenance deficient 7	DNA replication
gi|171847062	38619	6.7	2.01	Rtel1 protein	DNA helicase
gi|261337192	95497	8.3	0.85	probable ATP-dependent RNA helicase DDX23	DNA helicase
gi|293344978	119731	9.1	2.64	DNA replication helicase 2	DNA helicase

Table 6. DNA replication and repair proteins co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

Other DNA binding proteins					
Accession No.	Molecular weight (Da)	PEAKS score	% Coverage	ID	Function
gi|157786594	107992	19	0.93	chromosome transmission fidelity protein 18 homolog	DNA binding
gi|281332168	266116	5	0.74	SON DNA-binding protein isoform 1	DNA binding
gi|62079099	50104	23.5	2.53	origin recognition complex subunit 5	DNA binding
gi|149063915	135288	6.9	0.57	apoptotic chromatin condensation inducer 1	RNA splicing/DNA binding
gi|6981166	65960	7.3	1.89	pleiomorphic adenoma gene-like 1	zinc finger
gi|157786742	67197	15.2	1.45	hypermethylated in cancer 2 protein	zinc finger
gi|51948514	79010	5.2	2.87	gametogenetin binding protein 2 ZNF403	zinc finger
gi|149055493	63469	33.5	2.21	similar to ZNF6 protein	zinc finger
gi|157821929	55995	5.8	1.6	zinc finger protein 275	zinc finger
gi|68163463	58538	14.6	2.32	gypsy retrotransposon integrase-like protein 1	zinc finger
gi|58219528	48299	9.2	4.46	zinc finger CCCH domain-containing protein 15	zinc finger
gi|71043914	72438	7.9	1.74	zinc finger protein 90	zinc finger
gi|157819879	113826	5.3	1.54	teashirt zinc finger homeobox 3	zinc finger
gi|293346501	54615	7	1.2	zinc finger protein 334	zinc finger
gi|198041681	198996	8	0.34	zinc finger protein 294	zinc finger
gi|219280810	108721	5.3	0.84	zinc finger protein 26	zinc finger
gi|62078923	88025	6.1	2.45	zinc finger protein DZIP1L	zinc finger
gi|149015883	175028	5.7	0.5	similar to zinc finger protein 407	zinc finger
gi|149032531	171510	10.8	0.63	GLI-Kruppel family member GLI3	zinc finger
gi|293343415	112355	10.1	0.84	zinc finger, CCHC domain containing 14	zinc finger
gi|149048469	90476	10.7	0.97	zinc finger and BTB domain containing 10	zinc finger
gi|157821667	58556	5.5	1.01	zinc finger protein 703	zinc finger
gi|293347374	2276152	34.4	0.53	zinc finger protein 462	zinc finger
gi|290560659	150770	7.6	1.63	zinc finger protein 609	zinc finger
gi|293343704	75545	7.1	1.49	zinc finger protein 347-like	zinc finger
gi|56001099	222575	7.2	0.35	monocytic leukemia zinc finger protein	zinc finger
gi|293345274	1391432	6.7	0.2	zinc finger homeobox 4	zinc finger

Table 7. Other DNA binding proteins co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

RNA binding/translation associated proteins					
Accession No.	Molecular weight (Da)	PEAKS score	% Coverage	ID	Function
gi|81295381	85966	5.3	1.66	nucleolar RNA helicase 2	RNA helicase
gi|109478564	69641	8.7	2.9	DEAD-box protein abstrakt-like	RNA helicase
gi|77539760	117946	5.4	1.54	superkiller viralicidic activity 2-like 2	RNA helicase
gi|81170402	58246	8.5	1.35	Cyclin-L2	RNA splicing
gi|109471441	66276	6.1	1.35	Negative elongation factor C/D	translation
gi|55250714	83457	6.4	1.33	G elongation factor	translation
gi|157817837	175435	11.6	0.51	eukaryotic translation initiation factor 4 gamma 3	translation
gi|149025194	12836	5.3	5.08	eukaryotic translation initiation factor 2B	translation
gi|59808927	29274	24	2.29	Histidyl-tRNA synthetase 2-like	translation
gi|293339967	30237	5	2.33	ribosomal protein L28-like	ribonucleoprotein
gi|57114334	60963	6.3	2.22	ribosomal L1 domain containing 1	ribonucleoprotein
gi|58865844	52673	5	1.35	RNA-binding protein FUS	ribonucleoprotein
gi|50925625	37277	5.9	2.47	Bystin-like	ribonucleoprotein
gi|149052044	83702	11.8	1.32	transducin (beta)-like 3	ribonucleoprotein
gi|293352379	95259	5.5	1.25	PAB-dependent poly(A)-specific ribonuclease subunit 3 isoform 1	ribonucleoprotein
gi|62643818	117895	8	1.06	integrator complex subunit 3 (INT1)	SnRNP
gi|290560677	39820	20.5	9.44	U7 snRNA-associated Sm-like protein LSm11	SnRNP
gi|293351499	145467	6	0.69	gem associated protein 5	SnRNP
gi|62078733	34870	5.5	4.1	MAK16 homolog	RNA processing
gi|37654330	268183	5.3	0.69	LRRGT00057	RNA binding
gi|109478606	216888	28.2	0.78	dicer 1, ribonuclease type III	mirna processing
gi|215277019	85898	6.1	1.15	polyribonucleotide nucleotidyltransferase 1	mRNA degradation
gi|169642585	41520	7.2	2.35	Pnpt1 protein	Ribonuclease
gi|293343704	75545	7.1	1.49	zinc finger protein 347-like	zinc finger
gi|56001099	222575	7.2	0.35	monocytic leukemia zinc finger protein	zinc finger
gi|293345274	1391432	6.7	0.2	zinc finger homeobox 4	zinc finger

Table 8. RNA binding/translational proteins co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

Protein modifying proteins					
Accession No.	Molecular weight (Da)	PEAKS	% Coverage	ID	Function
gi|33636730	36046	6.9	2.53	BTB/POZ domain-containing protein TNFAIP1	E3 Ubiquitin ligase
gi|149020185	97449	5.6	0.83	ring finger protein 20	E3 Ubiquitin ligase
gi|282154815	530370	7.4	0.39	BIR repeat-containing ubiquitin-conjugating enzyme	E3 Ubiquitin ligase
gi|157821023	80178	5.5	1.79	E3 ubiquitin-protein ligase LNX	E3 Ubiquitin ligase
gi|112984060	42872	16.4	2.1	E3 ubiquitin-protein ligase RNF133	E3 Ubiquitin ligase
gi|84781733	1573715	6.4	0.13	E3 ubiquitin-protein ligase UBR4	E3 Ubiquitin ligase
gi|164565379	113460	5.6	0.72	E3 ubiquitin-protein ligase BRE1A	E3 Ubiquitin ligase
gi|157819469	194704	6.7	1.41	E3 ubiquitin-protein ligase SHPRH	E3 Ubiquitin ligase
gi|293340606	5592572	9.3	0.33	ring finger protein 213	E3 Ubiquitin ligase
gi|56090373	19288	26.2	4.24	E3 ubiquitin-protein ligase RNF181	E3 Ubiquitin ligase
gi|62641247	199847	45.2	1.45	retinoblastoma binding protein 6 isoform 2	E3 Ubiquitin ligase
gi|189217530	95373	7.6	1.44	anaphase-promoting complex subunit 2	E3 ubiquitin ligase
gi|159024658	68818	5.5	2.07	ligand of numb-protein X 1	E3 ubiquitin ligase
gi|149044545	81201	29.5	1.51	ubiquitin specific peptdiase 1	deubiquitinase
gi|62543525	87329	29.5	1.4	ubiquitin carboxyl-terminal hydrolase 1	deubiquitinase
gi|198278575	33016	26.3	3.09	lys-63-specific deubiquitinase BRCC36	deubiquitinase
gi|71051126	24147	7.7	5.26	Pias4 protein	E3 SUMO ligase/corepressor
gi|293340888	85726	6	1.33	Sumo1/sentrin/SMT3 specific peptidase 5	De-Sumoylation
gi|157786920	116872	5.5	0.87	sentrin-specific protease 7	De-Sumoylation
gi|51948388	26541	5.2	3.93	calcyclin-binding protein	protein-protein interaction
gi|293339931	461266	5	0.35	polycystin-1	protein-protein interaction
gi|196115075	105487	5.1	0.92	armadillo repeat gene deleted in velo-cardio-facial syndrome	protein-protein interaction
gi|24308490	63262	6	1.76	kelch-like protein 12	protein-protein interaction
gi|84781638	65840	7	3.4	Kelch-like protein 25	protein-protein interaction
gi|149041207	15848	5.1	4.32	N-acetyltransferase 5 (ARD1 homolog)	acetlytransferase
gi|220838	57282	7.2	3.14	dihydrolipoamide acetyltransferase	acetlytransferase
gi|149021096	11251	5.1	14.95	N-myristoyltransferase 2	myristoltransferase

Table 9. Post-translational modifying protein co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

Chaperone proteins					
Accession	Molecular weight (Da)	PEAKS	% Coverage	ID	Function
gi|157823115	16580	44.9	14.94	prefoldin subunit 2	chaperone
gi|62079115	88870	43.8	1.43	DnaJ (Hsp40) homolog, subfamily C, member 16	chaperone
gi|25742760	58889	5.1	1.99	anti-mullerian hormone	chaperone
gi|47087121	70549	19.6	3.9	heat shock 70kD protein 3	chaperone
gi|11177910	69529	24.5	8.53	heat shock protein 2	chaperone
gi|347019	70928	49.3	15.48	dnaK-type molecular chaperone hsp72-ps1	chaperone
gi|13242237	70871	49.3	15.48	heat shock protein 8	chaperone
gi|76096320	74612	12.1	1.47	Usher syndrome 1C binding protein 1	chaperone
gi|71795646	36035	5.7	1.58	C1GALT1-specific chaperone 1	chaperone
gi|1778213	60897	47	6.28	chaperonin 60	chaperone

Table 10. Chaperone proteins co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

Cell signaling proteins					
Accession No.	Molecular weight (Da)	PEAKS	% Coverage	ID	Function
gi|81871416	93782	9.4	1.42	phosphatidylinositol-glycan-specific phospholipase D	Cell signaling
gi|56905	56623	25.9	13.69	phospholipase C	Cell signaling
gi|149066123	54047	6	1.44	similar to sphingomyelin phosphodiesterase 3	Cell signaling
gi|18143335	41456	5.1	2.51	cyclic nucleotide phosphodiesterase 7B3	Cell signaling
gi|21070934	77276	5.6	0.87	calcium-binding and coiled-coil domain-containing protein 1	cell signaling
gi|157821639	24466	18.3	3.85	coiled-coil domain-containing protein 25	cell signaling
gi|67846086	46462	8.6	2.52	coiled-coil domain-containing protein 71	cell signaling
gi|293360473	5077295	25.4	2.23	coiled-coil domain containing 38	cell signaling
gi|50400206	69589	5.2	1.56	probable Coiled-coil domain-containing protein 8	cell signaling
gi|293347562	134158	19.1	0.74	SCL-interrupting locus protein homolog	cell signaling
gi|274323811	137238	32.4	0.68	insulin receptor substrate 2	cell signaling
gi|293341722	194550	6.1	0.68	Bcl3 binding protein-like isoform 1	cell signaling
gi|25742751	62196	13.7	2.1	collapsin response mediator protein 1 (CRMP-1)	cell signaling
gi|293347161	104481	5.1	0.96	epidermal growth factor receptor pathway substrate 8	cell signaling
gi|194474088	114450	9.5	3.04	caspase recruitment domain-containing protein 10	Cell signaling scaffold
gi|61212441	150347	5.5	1.33	CASK-interacting protein 1	Cell signaling scaffold

Table 11. Cell signaling proteins co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

Kinase/Phosphatase					
Accession No.	Molecular weight (Da)	PEAKS	% Coverage	ID	Function
gi|149025173	132526	5.8	1.1	similar to YLP motif containing protein 1 (Nuclear protein ZAP3)	Nucleoside kinase
gi|16758824	88235	5.2	1.39	MAP/microtubule affinity-regulating kinase 1	protein kinase
gi|157821269	80949	5.5	1.95	p21 (CDKN1A)-activated kinase 7	protein kinase
gi|157822407	191194	8.5	1.38	microtubule-associated serine/threonine-protein kinase 2	protein kinase
gi|149042377	27173	7.9	4.26	protein kinase, X-linked	protein kinase
gi|9507127	129744	12.8	1.09	SRC kinase signaling inhibitor 1	protein kinase
gi|293350832	150363	5.5	0.6	mitogen-activated protein kinase kinase kinase 15-like	protein kinase
gi|157821531	86546	5.1	1.33	serine/threonine-protein phosphatase with EF-hands 2	protein kinase
gi|149019691	241330	18.4	0.52	protein kinase, DNA activated	protein kinase
gi|149031715	149862	5.6	1.4	Rho-associated coiled-coil forming kinase 1	protein kinase
gi|71361669	235308	11.1	0.92	citron Rho-interacting kinase	protein kinase
gi|27923854	44874	10.7	3.53	Aurora kinase A	protein kinase
gi|293356892	29784	5.5	4.85	myristoylated alanine-rich C-kinase substrate	protein kinase
gi|33469057	53179	11.5	2.1	calcium/calmodulin-dependent protein kinase type 1G	protein kinase
gi|149026195	46019	8.4	3.77	protein kinase, cAMP dependent, catalytic, beta	protein kinase
gi|300253230	138561	5.5	1.46	phosphorylase b kinase regulatory subunit alpha	protein kinase
gi|149025911	127470	5.1	0.78	alpha-kinase 1	protein kinase
gi|66730484	105474	7.2	1.67	serine/threonine-protein kinase TAO3	protein kinase
gi|34576547	62170	5.7	0.89	serine/threonine-protein kinase SRPK3	protein kinase
gi|293340144	106170	15.8	1.55	Unc-51 like kinase 2	protein kinase
gi|157824055	75584	6.3	1.83	cytoplasmic tyrosine-protein kinase BMX	protein kinase
gi|149046617	141018	5.2	0.63	membrane associated guanylate kinase, WW and PDZ domain containing 2	protein kinase
gi|15100164	55735	12.3	1.46	RAC-alpha serine/threonine-protein kinase	protein kinase
gi|149031390	26020	7.7	5.02	protein kinase, AMP-activated, gamma 2 non-catalytic subunit	protein kinase
gi|19705447	58501	5.1	1.43	CDC-like kinase 3 CLK3 protein	protein kinase
gi|157822535	115364	5.4	0.86	serine/threonine-protein kinase LATS2	protein kinase
gi|293348895	281047	6.7	0.4	leucine-rich repeat kinase 2	protein kinase
gi|149060087	48515	6.2	2.16	oculocerebrorenal syndrome of Lowe	protein phosphatase
gi|149023335	64786	6	1.39	cell division cycle 25 (CDC25)	protein phosphatase
gi|109464300	283413	11.2	0.34	microtubule associated serine/threonine kinase family member 4	protein phosphatase
gi|73920066	105245	11.3	1.62	Type II inositol-3,4-bisphosphate 4-phosphatase	protein phosphatase
gi|56560	132601	5	0.85	Receptor-type tyrosine-protein phosphatase C	protein phosphatase
gi|148277511	81008	6.2	1.55	receptor-like protein tyrosine phosphatase kappa	protein phosphatase
gi|158631175	162183	6.2	0.76	protein tyrosine phosphatase, receptor type, K	protein phosphatase
gi|169642485	89662	9.6	1.22	Protein phosphatase 1, regulatory subunit 9B	protein phosphatase
gi|293350479	11249	6.3	11.43	dual specificity phosphatase 3	protein phosphatase
gi|19424260	64287	6	1.39	M-phase inducer phosphatase 2	protein phosphatase

Table 12. Kinases & Phosphatases co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

GTPase					
Accession No.	Molecular weight (Da)	PEAKS	% Coverage	ID	Function
gi|149047303	21512	31.4	4.17	Ras –related protein RAB28	GTPase
gi|8394142	25068	36.6	4.07	Ras –related protein RAB27A	GTPase
gi|16758202	24620	7.5	4.13	Ras –related protein RAB27B	GTPase
gi|68566301	83908	6.3	1.72	Dynamin-1-like protein	GTPase
gi|66730266	33394	17	2.73	GTPase, IMAP family member 7	GTP binding protein
gi|154800420	65267	7	3.99	guanine nucleotide binding protein-like 3 (nucleolar)-like	GTP binding protein
gi|164565387	47551	17.5	2.18	TBC1 domain family member 14 isoform 1	GTPase interacting
gi|293347992	106815	35	0.73	ArfGAP with SH3 domain, ankyrin repeat and PH domain 2	GTPase activating protein
gi|7767545	191114	5.3	0.6	Kalirin-7c isoform	GTPase activating protein
gi|293357218	96904	5.3	1.51	T-cell activation Rho GTPase-activating protein-like	GTPase activating protein
gi|149054448	71947	8.1	1.42	Rho GTPase activating protein 27	GTPase activating protein
gi|149043416	155745	5.6	1.2	synaptic Ras GTPase activating protein 1 homolog	GTPase activating protein
gi|293358233	70283	5.5	1.56	Rho GTPase-activating protein 18-like	GTPase activating protein
gi|37542279	201912	8.9	0.77	serine-rich synapse associated protein SERSAP1	GTPase activating protein
gi|149068733	135676	6.5	1.08	centaurin	GTPase activating protein
gi|149042860	166621	8.3	1.21	similar to P-Rex1	guanidine exhange factor (GEF)
gi|197927178	238942	8.5	0.76	dedicator of cytokinesis 8	guanidine exhange factor (GEF)
gi|28212262	58390	8.6	3.65	synembryn-B	guanidine exhange factor (GEF)
gi|149068757	9153658	10.7	1.68	Rho guanine nucleotide exchange factor (GEF) 17	guanidine exhange factor (GEF)
gi|31342051	201973	23.5	0.45	brefeldin A-inhibited guanine nucleotide-exchange protein 2	guanidine exhange factor (GEF)
gi|149059004	72557	5.2	2.48	RAS protein-specific guanine nucleotide-releasing factor 2	guanidine exhange factor (GEF)
gi|293340103	923560	10.3	0.29	obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	guanidine exhange factor (GEF)
gi|60552083	41165	7.1	1.87	Rgs14 protein	GDP-dissociation inhibitor
gi|109479608	54473	9.8	1.48	regulator of G-protein signaling 6 isoform 1	GDP-dissociation inhibitor

Table 13. GTPases & related proteins co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

Cell cycle/cell death					
Accession No.	Molecular weight (Da)	PEAKS	% Coverage	ID	Function
gi|281371331	341969	8.2	0.7	centromere protein F	mitosis
gi|293357587	1289001	5.2	0.24	centromere protein E	mitosis
gi|51871605	103334	20	1.97	centromere protein C 1	mitosis
gi|109483746	161000	5.6	0.71	protein QN1 homolog	cell division
gi|109464316	159274	14.7	1.14	NLR family, apoptosis inhibitory protein 2	antiapoptotoc
gi|293356522	30021	6.4	3.77	cytochrome P450, family 2, subfamily c, polypeptide 24	apoptosis
gi|157817294	219689	7.3	0.61	CASP8-associated protein 2	apoptosis
gi|293344027	27261	5.6	3.6	kallikrein related-peptidase 14	cell death

Table 14. Cell cycle & cell death related proteins co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

Structural/binding proteins					
Accession No.	Molecular weight (Da)	PEAKS	% Coverage	ID	Function
gi|40849886	520015	25.9	0.57	plectin 1	structural binding protein
gi|149038136	78004	7.4	1.66	polyamine modulated factor 1 binding protein 1	structural binding protein
gi|149038012	9067370	5.5	1.9	similar to FH1/FH2 domain-containing protein	structural binding protein
gi|21326463	201924	8.9	0.77	signal-induced proliferation-associated 1-like protein 1	structural binding protein
gi|293340060	92774	5.1	1.28	apical protein of Xenopus-like	structural binding protein
gi|47477769	282378	35.6	1.92	Spna2 protein	structural binding protein
gi|17380501	284636	35.6	1.9	Spectrin alpha chain, brain	structural binding protein
gi|157817081	105286	7.8	3.25	catenin alpha-2	structural binding protein
gi|11496271	115480	5.5	1.15	CAP-GLY domain containing linker protein 2 (CLIP2)	structural binding protein
gi|62078611	64645	25	2.17	CAP-Gly domain-containing linker protein 4 (CLIP4)	structural binding protein
gi|149063106	115817	5.5	1.15	cytoplasmic linker 2	structural binding protein
gi|338818272	619608	6.2	0.33	Microtubule-actin cross-linking factor 1	structural binding protein
gi|293344918	324879	12.3	1.29	pericentrin-like	structural binding protein
gi|300795283	164631	5.6	0.67	Shroom4	structural binding protein
gi|149023883	608652	16.8	0.45	similar to microfilament and actin filament cross-linker protein isoform b	structural binding protein
gi|149026252	78198	51.4	2.75	nexilin	structural binding protein
gi|13432197	78564	5.5	1.2	Microtubule-associated protein tau	structural binding protein
gi|149044038	75112	31.2	2.56	similar to KARP-1 binding protein 1	structural binding protein
gi|58743349	18593	5	4.55	protein FAM89A	structural binding protein
gi|293341673	133415	6.1	0.61	centrosomal protein 135-like	structural binding protein
gi|157821671	122267	7.3	0.79	protein cordon-bleu	structural binding protein
gi|293341687	337367	6.3	0.33	furry homolog isoform 1	structural binding protein
gi|109506053	118243	11.1	0.74	anillin, actin binding protein	structural binding protein

Table 15. Scaffolding proteins co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

Structural/binding proteins II					
Accession No.	Molecular weight (Da)	PEAKS	% Coverage	ID	Function
gi|21392397	109188	6.7	0.83	filamin-interacting protein S-FILIP	structural binding protein
gi|293344591	539182	6.5	0.18	AHNAK nucleoprotein isoform 1 actin binding	structural binding protein
gi|157818987	55313	5	1.95	NCK-interacting protein with SH3 domain	structural binding protein
gi|305682553	94280	13.8	1.05	GAS2-like protein 2	structural binding protei
gi|300796937	108388	8.2	1.3	espin	structural binding protein
gi|293341301	203493	6.3	1.5	Nck-associated protein 5	structural binding protein
gi|22219452	45308	5.8	5.99	WAS/WASL interacting protein family, member 3	structural binding protein
gi|293340174	512803	13.7	0.47	dynein, axonemal, heavy polypeptide 9 isoform 2	structural/motor protein
gi|312147379	338196	7	0.36	laminin subunit alpha-1 precursor	nuclear matrix
gi|300798041	403781	6.9	0.27	laminin subunit alpha-5 precursor	nuclear matrix
gi|68341941	60299	11.7	1.12	importin subunit alpha-6	nuclear matrix
gi|158508582	267303	5	1.14	translocated promoter region	nuclear pore binding protein
gi|157823031	118926	7.8	0.74	importin-4	nuclear pore
gi|157820325	110214	8.8	0.72	exportin-2	nuclear pore
gi|16758020	204157	6.8	0.48	nuclear pore membrane glycoprotein 210 p	nuclear pore
gi|16758020	204157	8.8	0.9	nucleoporin 210	nuclear pore
gi|58865420	93302	6.7	1.59	nucleoporin 93kDa	nuclear pore
gi|13928704	228963	6.3	0.66	myosin, heavy chain 10, non-muscle	nuclear matrix
gi|205830436	223320	5	1.03	myosin heavy chain IIa	nuclear matrix
gi|106879208	222878	6.5	1.26	myosin-XVI	nuclear matrix
gi|149060618	87034	6.3	1.5	myosin-4	nuclear matrix
gi|6636340	147842	7.7	1.54	myosin, light polypeptide kinase	nuclear matrix
gi|157819015	259309	13.1	0.89	procollagen, type VII, alpha 1	structural
gi|149019071	188004	17	0.64	procollagen, type XII, alpha 1	structural
gi|149034013	63508	10.3	4.12	procollagen, type IX, alpha 3	structural
gi|149057578	165977	7.8	1.18	procollagen, type IV	structural
gi|14389299	53733	18.9	12.02	vimentin	structural
gi|11968118	53457	6.1	3.41	desmin	structural
gi|149047823	850767	15.3	0.33	nebulin	structural

Table 15. Scaffolding proteins co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

Membrane associated proteins					
Accession No.	Molecular weight (Da)	PEAKS	% Coverage	ID	Function
gi|149058905	28378	6.1	3.57	spermatogenesis associated 9	membrane protein
gi|19705483	107729	9.1	1.35	calsyntenin 2	membrane protein
gi|157822997	32262	5.9	4.79	fat-inducing transcript 1	membrane protein
gi|149027462	273594	7.5	0.28	insulin-like growth factor 2 receptor	membrane protein
gi|300798434	310876	5.1	0.54	tenurin 4	membrane protein
gi|58200463	5627	8.4	18	pecanex 1	membrane protein
gi|149022983	101530	18.9	3.26	dispatched homolog 2	membrane protein
gi|157823021	93830	6.3	0.89	proline-rich transmembrane protein 4	membrane protein
gi|19071449	62975	15.7	1.06	organic anion transporter K8	membrane protein
gi|293343910	558899	28.2	0.42	ryanodine receptor 1, skeletal muscle	membrane protein
gi|157818225	145075	7.3	0.46	contactin-associated protein-like 4 precursor	membrane protein
gi|58865568	76061	22.2	2.69	C2 domain-containing protein 2-like	membrane protein
gi|24308466	86930	6	1.27	integrin, beta 3 precursor	membrane protein
gi|149046639	305888	19.7	0.29	piccolo	membrane protein
gi|157817085	169650	14	0.38	brain-specific angiogenesis inhibitor 2 precursor	membrane protein
gi|54400718	37178	5.1	1.45	growth hormone-inducible transmembrane protein	membrane protein
gi|91982740	167829	8.1	0.61	cystic fibrosis transmembrane conductance regulator ABC ion transporter	membrane protein
gi|25742799	96072	21.4	0.84	toll-like receptor 4	membrane receptor
gi|157822681	37282	6.8	3.27	ephrin B2	membrane receptor
gi|149023913	55286	5.7	2.75	similar to Ephrin type-A receptor 10	membrane receptor
gi|157151704	109883	9.3	2.24	Eph receptor B1	membrane receptor
gi|149043433	97451	8.2	0.69	glutamate receptor, metabotropic 4	GPCR
gi|293361565	82778	12.4	1.61	G protein-coupled receptor 115	GPCR
gi|47577953	35839	16.3	2.22	olfactory receptor Olr111	GPCR
gi|47576521	35010	10.3	2.91	olfactory receptor Olr878	GPCR
gi|47576703	35577	10.8	6.33	olfactory receptor Olr171	GPCR
gi|20806161	149445	20.3	0.82	probable G-protein coupled receptor 116 precursor	GPCR
gi|156119581	88506	7.3	1.4	vomeronasal 2 receptor 37	GPCR
gi|15826856	96898	8.9	2.41	potassium voltage-gated channel, subfamily Q, member 3	ion channel
gi|21217559	70191	12	2.04	Shaw-related voltage-gated potassium channel protein 2	ion channel
gi|548372	166070	5.1	1.35	Glutamate [NMDA] receptor subunit epsilon-2	ion channel
gi|56748617	219731	5	0.51	Sodium channel protein type 10 subunit alpha	ion channel
gi|1586352	225528	5	0.5	voltage-gated Na channel	ion channel
gi|293344668	196286	6.1	0.64	transient receptor potential cation channel, subfamily M, member 3	ion channel

Table 16. Membrane associated proteins co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

Membrane associated proteins II					
Accession No.	Molecular weight (Da)	PEAKS	% Coverage	ID	Function
gi|149065431	72166	8	1.07	chloride channel 1, isoform CRA_c	ion channel
gi|21217559	70191	17.8	2.04	potassium voltage-gated channel subfamily C member 2 isoform b	ion channel
gi|149034091	61309	5.9	2.33	glutamate receptor, ionotropic, delta 1	ion channel
gi|157823621	190135	5.6	0.52	plexin-D1	synaptic protein
gi|300798121	133541	5.3	1.65	leucine-rich repeat-containing protein 9	synaptic protein
gi|293346681	143830	5.3	0.95	calcium-dependent secretion activator 1-like isoform 5	synaptic protein
gi|5262748	192385	15.8	0.44	Proline rich synapse associated protein 2	synaptic protein
gi|149067246	95411	5.9	1.51	SCY1-like 2	vesicular protein
gi|17939356	75901	6.3	1.19	synaptotagmin-like protein 4	vesicular protein
gi|18543333	172592	16.3	2.33	synaptonemal complex protein 2	vesicular protein
gi|149054043	77750	10.7	1.76	SNAP25-interacting protein	vesicular protein
gi|157821387	161270	7.9	0.64	early endosome antigen 1	vesicular protein
gi|149046329	15354	20.6	5.67	similar to lysozyme-like	vesicular protein
gi|109460813	49910	5.6	3.52	similar to unc-93 homolog A	vesicular protein
gi|149032835	102712	5.4	0.74	similar to intracellular protein transport like	vesicular protein
gi|6693834	127055	10.7	1.11	SNIP-a SNAP25-interacting protein	vesicular protein
gi|149062471	30008	61.1	3.83	syntaxin 3, isoform	vesicular protein
gi|6981078	273397	25.8	0.48	cation-independent mannose-6-phosphate receptor precursor	lysozomal protein
gi|197246879	72397	14.7	1.59	Vps35 protein	lysosomal protein
gi|205360969	232155	9.8	0.43	similar to vacuolar protein sorting 13C protein	lysosomal protein
gi|149024662	127459	5.4	1.69	kinesin family member 1B	transport
gi|293344149	150577	21.2	1.12	kinesin family member 7	transport
gi|149017591	141963	9.7	0.56	kinesin family member 21A	transport
gi|4033695	14544	5	3.91	Gastrotropin	transport
gi|71051731	22873	8.4	5.66	Slc38a10 protein	transport
gi|149064373	128964	9.3	1.2	Treacher Collins Franceschetti syndrome 1	nuclear trafficking
gi|293345113	401733	8.6	0.38	cardiomyopathy associated 5	anchoring protein
gi|1389903	54838	5.9	2.94	5HT3 receptor	neurotransmitter receptor
gi|10566951	66728	7.1	2.97	s-gicerin/MUC18	extracellular glycoprotein
gi|149025851	231829	5.7	0.29	ATP-binding cassette, sub-family A (ABC1), member 4	transmembrane glycoprotein
gi|16758300	102942	15.1	0.99	ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1)	transmembrane glycoprotein

Table 16. Membrane associated proteins co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

Metabolic proteins					
Accession No.	Molecular weight (Da)	PEAKS	% Coverage	ID	Function
gi|4699607	30379	8.4	4.73	Chain A, Dienoyl-Coa Isomerase	metabolism
gi|7387724	27246	7.3	4.21	3-hydroxyacyl-CoA dehydrogenase type-2	metabolism
gi|157823599	47731	6	3.73	alpha-1,2-mannosyltransferase ALG9	metabolism
gi|12018256	36202	7.6	3.98	delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase	metabolism
gi|157818651	20804	5.3	5.91	methyl-CpG binding domain protein 3-like 1	metabolism
gi|58865680	64417	15.4	1.91	carboxylesterase 7	metabolism
gi|149041705	69193	13.9	1.46	acyl-CoA synthetase bubblegum family member 1	metabolism
gi|16758230	54610	5.9	2.24	malonyl-CoA decarboxylase	metabolism
gi|123797828	56782	7.8	1.98	Acyl-CoA-binding domain-containing protein 5	metabolism
gi|12018256	36202	5.6	3.98	enoyl coenzyme A hydratase 1, peroxisomln	metabolism
gi|6015047	36172	5.6	3.98	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase	metabolism
gi|149024283	17830	7.4	7.23	3-hydroxy-3-methylglutaryl-Coenzyme A lyase	metabolism
gi|16758804	276097	5.1	0.57	acetyl-Coenzyme A carboxylase beta	metabolism
gi|17028103	92480	6.6	1.53	ATP-citrate lyase	metabolism
gi|19705501	25720	11.9	6.15	dicarbonyl L-xylulose reductase	metabolism
gi|149031335	24893	23.4	4.33	melanoma antigen	metabolism
gi|16758040	398989	8.4	0.25	cubilin precursor	metabolism
gi|206213	62305	5.4	7.14	R-pyruvate kinase	metabolism
gi|157818293	46330	9.2	5.3	methylmalonic aciduria (cobalamin deficiency) cblA type	metabolism
gi|88853855	42095	5.8	2.69	GDP-mannose 4,6 dehydratase	metabolism
gi|109467565	100662	9	2.29	similar to N-deacetylase/N-sulfotransferase	metabolism
gi|293349371	3071589	8.7	1.8	carbohydrate sulfotransferase 2	metabolism
gi|157820677	117351	5.9	0.58	beta-1,4-N-acetyl-galactosaminyl transferase	metabolism
gi|62078631	18329	6.8	6.06	UDP-N-acetylglucosamine transferase subunit ALG13 homolog	metabolism

Table 17. Metabolic proteins co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

Metabolic proteins II					
Accession No.	Molecular weight (Da)	PEAKS	% Coverage	ID	Function
gi|169642225	56318	7.8	1.99	Acbd5 protein	metabolism
gi|157822785	37543	5.8	2.35	D-aspartate oxidase	metabolism
gi|6980956	61416	10.9	5.38	glutamate dehydrogenase	metabolism
gi|20978407	146920	8.7	0.75	Aldehyde oxidase	metabolism
gi|149024594	74516	7.8	1.38	5,10-methylenetetrahydrofolate reductase (NADPH)	metabolism
gi|1289336	52724	8.3	3.83	glycine-, glutamate-, thienylcyclohexylpiperidine-binding protein	metabolism
gi|11560131	31426	8.1	2.81	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	metabolism
gi|149038763	54094	5	1.83	prosaposin	metabolism
gi|40849880	46123	6.6	2	I-branching beta-1,6-acetylglucosaminyltransferase family polypeptide 1	metabolism
gi|6980956	61416	10.9	5.38	glutamate dehydrogenase	metabolism
gi|4699607	30379	5.6	4.73	Chain A, Dienoyl-Coa Isomerase	metabolism
gi|32527761	7378	5	24.62	Ac2-281	nucleotide binding
gi|67078508	76758	6	1.44	GMP synthase Glutamine amidotransferase	nucleotide metabolism
gi|199562203	63125	5.4	1.47	5'-nucleotidase domain containing 3	nucleotide metabolism
gi|194328668	84126	13.5	2.77	oxidation resistance protein 1 isoform 2	oxidative stress response
gi|114145534	43080	5.3	1.53	methylthioadenosine phosphorylase precursor	polyamine metabolism
gi|157821815	327157	8.2	0.38	low density lipoprotein-related protein 1B	lipoprotein
gi|188536057	177429	14	1.31	low-density lipoprotein receptor-related protein 5 precursor	lipoprotein
gi|149037498	143132	6.1	1.56	high density lipoprotein binding protein	lipoprotein
gi|81894378	536031	28.8	0.34	Apolipoprotein B-100	lipoprotein
gi|198278475	39679	10.2	3.84	apolipoprotein L3	lipoprotein
gi|38649090	41292	6	4.29	Hdlbp protein	lipoprotein
gi|149064942	31946	11.1	2.78	paraoxonase 1	lipoprotein associated
gi|77736615	66709	15.4	1.04	polypeptide N-acetylgalactosaminyltransferase 4 (GalNAc-T4)	glycosylase
gi|293354091	1073517	6.7	1.52	choline acetyltransferase	acetlytransferase

Table 17. Metabolic proteins co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

Miscellaneous proteins					
Accession No.	Molecular weight (Da)	PEAKS	% Coverage	ID	Function
gi|300796069	217237	21.4	0.46	thyroid adenoma associated	unknown
gi|841190	166864	6	0.8	Huntington gene product	unknown
gi|149017712	174407	8.2	1.31	tetratricopeptide repeat domain 3	unknown
gi|109464191	164236	10.5	1.36	tetratricopeptide repeat domain 37	unknown
gi|109461632	167630	7.4	0.78	similar to WD repeat domain 62 isoform 1	unknown
gi|157822497	49873	6.3	2	WD repeat-containing protein 37	unknown
gi|208973290	123209	5.5	0.63	WD repeat-containing protein 72	unknown
gi|157822973	73495	7.1	1.7	peptidylprolyl isomerase domain and WD repeat-containing protein 1	unknown
gi|281604148	391821	8.3	0.37	WD repeat and FYVE domain-containing protein 3 [Rattus norvegicus]	unknown
gi|62078549	72836	5.4	0.92	WD repeat-containing protein 70	unknown
gi|157822069	42251	6.2	2.92	cyclin-I	unknown
gi|166706873	119811	15.9	1.12	leucine zipper protein 1	unknown
gi|149045175	327503	7.1	0.64	desmoplakin	cell adhesion
gi|149046582	383239	16.3	0.52	reelin	cell adhesion
gi|12621132	480657	8.3	0.25	FAT tumor suppressor homolog 2 Multiple epidermal growth factor-like domains 1	cell adhesion
gi|109502781	132289	5.7	1	protocadherin 9 isoform 3	cell adhesion
gi|300796674	343526	6.4	0.33	TPR and ankyrin repeat-containing protein 1	ATP binding
gi|281306844	76767	25.5	2.32	FCH and double SH3 domains protein 1 isoform 1	CASK interacting
gi|21728404	14549	7.1	18.11	chibby homolog 1	leucine beta catenin interacting
gi|46396067	51653	6.7	1.53	Kynurenine--oxoglutarate transaminase 1	transaminase
gi|441473	27024	9.9	5.24	granzyme-like protein III	peptidase
gi|1698704	26314	6.2	3.77	mast cell protease 9	protease
gi|129887	221116	26	0.28	Aggrecan core protein	extracellular matrix
gi|149031833	87365	6.6	1.55	HCR (a-helix coiled-coil rod homolog)	differentiation
gi|157819065	104029	24.2	0.63	ADAM metallopeptidase type 1 motif 15	metalloproteinase
gi|157819247	47385	10	2.85	carboxypeptidase A4	metalloproteinase

Table 18. Multifunctional proteins co-immunoprecipitated with ERβ in the ventral hippocampus after DTBP cross linking

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VITA

The author, Natasha Mott is the daughter of Robert and Rhonda Bogatitus, born in Midlothian, Illinois on March 8, 1986. After moving to Cape Coral, Florida and graduating from Mariner High School in 2004 as salutatorian, she attended the University of Central Florida in Orlando, seeking a degree in Molecular and Microbiology. During her time in Orlando, Natasha explored many fields of biological research ranging from gestational hormones in the Cape ground squirrel *Xerus inauris* with Dr. Jane Waterman, prostate cancer in the lab of Dr. Karl Chai and the parasite *Plasmodium falciparum* under Dr. Debopam Chakrabarti. Natasha received her B.S. in August 2007, just before matriculating in the former Cell Biology, Anatomy and Neurobiology Ph.D program at Loyola University Chicago.

In January of 2008, Natasha entered the lab of Dr. Toni Pak, where she immediately found her passion for neuroscience and endocrinology. She began working on fibroblast growth factor signaling and autoregulation in GnRH neurons. Natasha then began work on her dissertation project which was aimed at identifying molecular mechanisms by which estrogen receptor beta (ERβ) could mediate ligand-independent transcription of target genes. Natasha became interested in the effects of ERβ in brain regions regulating cognition and affect during a physiologically relevant hypoestrogenic period, menopause.

Natasha has presented her work at two national and local meetings every year since 2008. She has won awards including the Endocrine Society Trainee Day Award in 2009, as well as second place in Loyola University Medical Center St. Albert's day Graduate Symposium in 2012. In 2011, she was also awarded the Federation for European Neuroscience Youth Travel Fund award to attend a student-centered nuclear receptor meeting in Spetses, Greece. Natasha has been funded by an institutional pre-doctoral National Research Service Award (NRSA) T32 on aging, and in 2011 Natasha was awarded an F31 NRSA also from the National Institute on Aging (NIA).

Natasha has also served on a number of committees as a graduate student. She represented CBNA on the Biomedical Graduate Student Council (2009-2010). She also served a 2-year term on The Endocrine Society's Trainee and Development Core Committee (2010-2012), where also served as chair of the Trainee Day Subcommitee. In addition to this work, Natasha was elected to the Board of Directors for a local non-profit called This is Me Inc., (2009-2011) where she developed and implemented science- and reading- based curricula for two after school programs.

In May of 2007, Natasha married her husband Kenneth Mott, and in February of 2012, Natasha had her first child Nova Renee. Natasha will pursue a postdoctoral position with Dr. Charles Roselli at Oregon Health Science University investigating the sexually dimorphic nucleus to identify the role of estrogens in neural circuits directing partner preference.