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Consequences of Estrogen Receptor Beta Phosphorylation in the Aged Female Brain and Heart

Elena Pinceti
Loyola University Chicago, elenapinceti@gmail.com

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LOYOLA UNIVERSITY CHICAGO

CONSEQUENCES OF ESTROGEN RECEPTOR BETA PHOSPHORYLATION
IN THE AGED FEMALE BRAIN AND HEART

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

INTEGRATIVE CELL BIOLOGY PROGRAM

BY
ELENA PINCETI
CHICAGO, ILLINOIS
AUGUST 2016
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<td>AD</td>
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<td>AF-1</td>
<td>Activation Function-a</td>
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<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>AP</td>
<td>Alkaline Phosphatase</td>
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<td>BDNF</td>
<td>Brain-Derived Neutrophilic Factor</td>
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<td>CAM</td>
<td>Carbamidomethyl</td>
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<td>CD</td>
<td>Cardiovascular Disease</td>
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<td>CE</td>
<td>Collision Energy</td>
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<td>CHCA</td>
<td>α-cyano-4-hydroxycinnamic acid</td>
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<tr>
<td>CHD</td>
<td>Coronary Heart Disease</td>
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<tr>
<td>CID</td>
<td>Collision Induced Dissociation</td>
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<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
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<td>DBD</td>
<td>DNA-Binding Domain</td>
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<tr>
<td>DH</td>
<td>Dorsal Hippocampus</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DPN</td>
<td>Diarylpropionitrile</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>E1</td>
<td>Estrone</td>
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<td>EGF</td>
<td>Epydermal Growth Factor</td>
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ERK Extracellular signal Regulated Kinase
ERRα Estrogen Receptor Related α
ERα Estrogen Receptor α
ERβ Estrogen Receptor β
ET Estrogen Therapy
HR Hazard Ratio
ID Intrinsically disordered
IPTG Isopropyl β-D-1-thiogalactopyranoside
IQ intelligence quotient
JNK c-Jun-NH2-terminal Kinase
LBD Ligand-Binding Domain
LC Liquid Chromatography
LV Left Ventricle
MALDI-TOF Matrix Assisted Laser Desorption Ionization- Time Of Flight
MAPKs Mitogen Activated Protein Kinases
MCAO Middle Cerebral Artery Occlusion
MI Myocardial Infarction
mo months old
MRM Multiple Reaction Monitoring
mRNA Messager Ribonucleic Acid
MS Mass Spectrometry
mTOR mammalian Target Of Rapamycin
MW Molecular Weight
NCoR Nuclear Corepressor
NO  Nitric Oxide
NRF-1  Nuclear Respiratory Factor-1
OVX  Ovariectomy
PD  Parkinson's Disease
PPT  propyl pyrazole triol
PTM  Post Translational Modification
RNA  Ribonucleic acid
ROS  Reactive Oxygen Species
SADHART  Sertraline Antidepressant Heart attack Trial
SAPK  Stress-Activated Protein Kinase
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM  Standard Error of the Mean
SERM  Selective Estrogen-Receptor Modulators
SMRT  Silencing Mediator for Retinoid and Thyroid hormone receptor
SRC  Steroid Receptor Coactivator
SRM  Selected Reaction Monitoring
SSRI  Serotonin Reuptake Inhibitor
TAM  Tamoxifen
TBP  TATA Binding Protein
TGY  Threonine-Glycine-Threonine
VH  Ventral Hippocampus
WHI  Women's Health Initiative
WT  Wild Type
CHAPTER I

STATEMENT OF THE PROBLEM

Female life expectancy in the United States has steadily increased and now averages 81 years, meanwhile the age at which women reach menopause remains constant at 51 years. Therefore, women live over one third of their life in a post-menopausal, low estrogenic state. Both neurological and cardiovascular disorders increase following menopause, affecting women’s quality of life and leading to large health care costs.

Various epidemiological and basic science studies show that estrogen administration (Estrogen Therapy (ET)) can have neuroprotective effects and improve cognitive abilities in aging women, as well as reduce heart disease and overall mortality. The Women’s Health Initiative (WHI), a large clinical study conducted in menopausal women, had contradicting conclusions but established that age is an important factor in determining positive or negative outcome of ET. This led to the formulation of the “Timing Hypothesis”: exposure to estrogens is beneficial for younger women but has detrimental outcomes in post-menopausal women who have experienced an extensive hypo-estrogenic period. While its underlying mechanisms are unclear, it is well accepted that the female brain and heart respond differently to ET before and after menopause, indicating that cellular and molecular components in these tissues undergo some kind of modification during the perimenopausal transition.
The effects of estrogen are mediated by two high affinity nuclear receptors, Estrogen Receptor α (ERα) and Estrogen Receptor β (ERβ) and the G-protein coupled estrogen receptor1 (GPER). Evidence supports that ERβ plays an important role in neuroprotection and cardioprotection, but aspects of ERβ molecular signaling are still not fully understood. One possibility is that aging and estrogen deprivation alter ERβ post translational modifications, such as phosphorylation. Indeed the mitogen activated protein kinases (MAPKs) responsible for phosphorylation of ERβ have been shown to be modulated by aging and steroid hormones. The goal of my dissertation is to determine the consequences of ERβ phosphorylation and how prolonged estrogen deprivation prior to estrogen treatment alters MAPKs signaling in the brain and heart.
Estrogens are the primary female sex hormones; they are mostly produced by the ovaries, corpus luteum and placenta in premenopausal women [1]. A smaller amount of estrogens is also produced by non gonadal organs such as the brain, liver, heart and skin. The three major forms of estrogens in women are estrone (E1), 17β-estradiol (E2) and estriol (E3). The most potent and prevalent estrogen in premenopausal women is E2, which is produced within the ovary by thecal and granulosa cells. Aromatase is the enzyme necessary for the final step of E2 synthesis, and it is expressed in granulosa cells as well as in peripheral organs. During the menstrual cycle, E2 is highest right before ovulation, reaching 110-410 pg/ml. In perimenopause E2 levels fluctuate and then decrease to below 35 pg/ml in postmenopausal women [1, 2]. The average age at menopause is 48-52 in developed countries and reflects the exhaustion of functional follicles in the ovaries. The number of follicular oocytes is set before birth and declines with apoptotic events before and after puberty. When women reach 45-55 years of age the oocytes are exhausted leading to a cascade of events ending with neuroendocrine desynchronization, fluctuations in estrogens levels and ultimately menopause [2].
Following menopause, estrone is the prevalent circulating estrogen; estrone is synthetized from conversion of androstenedione in adipose tissue, neurons and astrocytes [3]. Analyses of post-menopausal women who underwent natural menopause determined that adipose tissue mass correlates with estrone levels, which had osteoprotective effects and increased bone mineral density as well as decreased colorectal cancer risk [4] [5]. However, high estrone levels in post menopausal women also have been found to correlate with increased ER-positive breast cancer [6]. Estrone has higher affinity for ERα than ERβ, however it was shown to induce coregulatory protein recruitment to ERβ more efficiently than ERα [7, 8]. The third physiological estrogen, estriol, is the weakest activator of ERs and is produced during pregnancy by the fetal liver and adrenal glands [9]. Interestingly, estriol has been found to decrease the symptoms of multiple sclerosis (MS), explaining the improvement of women with MS during pregnancy [10].

NEUROPROTECTION OF ESTROGEN

Learning and Memory

The study of the effects of estrogens on cognitive functions started in the 1950’s when a random controlled study on 75-year old women showed that 12 months of treatment with E2 improved their verbal intelligence quotient (IQ) while the IQ scores of the placebo treated women decreased over that same period of time [11]. Since then randomized observational and longitudinal studies have investigated the clinical effects of estrogens on cognitive functions, anxiety, mood, and memory using standard tests of neuropsychological function. Overall 71% of studies concluded a significant
beneficial effects of E2 [12]. However interpretation of these studies is complicated by several confounding variables such as age and dosage, tests used to evaluate cognition, and length of treatment. A more definitive understanding of the molecular effects of E2 is needed to help physicians better treat their patients.

Basic science studies have elucidated some of the mechanisms behind the neuroprotective effects of estrogens. In the 1990s, Catherine Woolley and Bruce McEwen found that E2 levels fluctuating across the estrous cycle correlate with synaptic changes in the hippocampus of female rats [13, 14]. Indeed, higher E2 levels coincided with increased synaptic density, increased dendritic spines and arborization. In an in vivo study using ERβ-null mice, Liu showed that ERβ is required for the E2-enhanced hippocampal synaptic plasticity and hippocampal dependent cognition [15]. These and other studies have therefore linked E2 to synaptic plasticity changes in neuronal circuitry and architecture [16]. Moreover, behavioral experiments using female rats show that changes in their learning strategy vary with natural hormonal fluctuations, such as during the estrous cycle, strongly implying that hippocampal mediated learning and cognition was intrinsically linked with circulating levels of estrogens [17]. In an extensive behavioral analysis, Kiss et al. evaluated the effects of E2 treatment on cognition and depressive-like behaviors in young (3 mo), adult (7 mo) and middle-aged (12 mo) female rats [18]. Their results showed that E2 treatment improved spatial reference memory in all age groups and was beneficial for depressive-like behaviors in young and middle-aged rats. Correlating with the behavioral data with neurochemical evaluations, hippocampal brain-derived neurotrophic factor
(BDNF) levels were increased by E2 treatment at all time points, while decreasing with age. BDNF mediates neuronal growth, survival, and plasticity in the nervous system. Several lines of evidence support an interaction between E2 and BDNF and their downstream neuroprotective, neurotrophic, and neuromodulatory effects [19]. Estrogens and BDNF have similar effects on the hippocampus and in the past years many studies have focused on the idea that BDNF is a mediator of estrogens’ effects. [20, 21]. By comparing the immunoreactivity of BDNF in the hippocampus during the estrous cycle, Sharfman showed that BDNF levels also cycle according to cycling estrogens [22].

Pathogenic conditions

Interestingly, studies suggest that E2 might prevent or delay the onset of Alzheimer’s disease (AD), the most common cause of dementia in women. In a 1998 meta-analysis Yaffe et al. concluded that treatment with estrogens decreased the risk of developing dementia in postmenopausal women by 29% and that several plausible biological mechanisms exist that could support estrogens’ use as a preventative and beneficial treatment of AD [23]. Case-controlled clinical studies have been conducted since then, and a 2001 meta-analysis concluded there is a 34% decreased risk of Alzheimer disease in women taking hormone treatment [24]. Research using animal models has also investigated a putative link between AD and E2 therapy. For instance, a recent study evaluated the use of a nonsteroidal selective androgen receptor agonist together with an ERβ agonist in a mouse model of AD, which resulted in improved cognition, reduced anxiety behaviors as well as an increase in Amyloid-β degrading enzymes [25]. The authors speculate that ERβ agonists
could be used as a potential therapy of patients with AD by interfering with the progression of the disease through degrading and clearing Amyloid β. In transgenic models of AD, different ERβ agonists led to prolonged survival, improved spatial recognition memory, cognition and lowered Amyloid-β accumulation [25, 26]. In the case of Parkinson’s disease (PD) and other neurodegenerative diseases, estrogens have been shown to protect the nigrostriatal dopaminergic pathway that is affected by these diseases [27, 28]. It is thought that both ERα and ERβ are necessary for this protection; however an animal model of PD showed that treatment with the ERβ agonist AC-186 prevented motor and cognitive deficits and mitigated neuronal loss in males but not in females [29, 30].

Other neuroprotective effects of estrogens have been attributed to ERβ. For example, several ERβ selective agonists have been shown to induce remyelination and repair in animal models of multiple sclerosis [31]. Inflammatory and neuropathic pains are also targets of ERβ regulation, as shown in vivo using the ERβ selective agent ERb-131 [32, 33]. Gliomas, aggressive brain tumors, are more common in males than females, indicating a possible beneficial effect of the higher levels of circulating estrogens in females; interestingly ERβ is highly expressed in male and female glioma cells, while ERα is not detected [34]. Indeed treatment with ERβ agonists DPN (Diarylpropionitrile) or liquiritigenin reduced proliferation in an in vitro glioma model and in vivo in mice that had a glioma xenograft [34].

Estrogens have also been shown to be neuroprotective in models of cerebral ischemia. Women overall have a lower incidence of stroke than men, but following menopause this difference decreases [35]. Studies have proven
that E2 exerts dramatic protection if administered either before or after the ischemic injury [36, 37]. For example, E2 treatment for seven days significantly reduced infarct size in a middle cerebral artery occlusion (MCAO) ischemic injury paradigm [37]. Moreover, a 2013 study showed periodic E2 treatment increased neuronal survival following cerebral ischemia via bilateral carotid occlusion and systemic hypotension. [38] The study also demonstrated that learning and memory following ischemic damage was improved when the rats where administered an ERβ specific agonist, which might have been mediated by the ERβ dependent increase in CREB phosphorylation.

**Mood and affective disorders**

The role of estrogen in anxiety and depressive disorders has been investigated both in clinical and basic science fields. Women experience increased depression, sleep disturbances, irritability, anxiety and panic disorders after menopause and ET has been show to improve mood, feelings of wellbeing and an overall increase in activity [39]. However, women in general report higher incidence of depression than men, yet animal models have shown both anxiogenic and anxiolytic actions of E2. These discrepant findings can be partly explained by differential actions mediated by the two estrogen receptor isoforms, ERα and ERβ. For instance, a 2005 paper by Lund et al., demonstrated that treatment with an ERβ specific agonist decreased anxiety-related behaviors in rats, whereas ERα specific agonists were anxiogenic [39]. Their findings suggest that the conflicting effects of estrogens might be due to inverse actions on anxiety behaviors by the two ERs. Indeed, studies have shown that ERα and ERβ have different signaling
pathways and downstream gene targets despite their equivalent binding affinity for E2 [40]. Overall, ERβ has beneficial effects on neurological diseases that have very different etiologies and in which different cell types are involved, highlighting its multifaceted functions.

CARDIOPROTECTION OF ESTROGEN

The sex difference in the incidence of cardiovascular disease is well documented, women are less likely to develop heart disease than men, but this advantage is lost after menopause presumably due to significant reduction in circulating E2 [41-43].

Both estrogen receptor isoforms, ERα and ERβ, are widely expressed in cardiovascular tissue, in cardiac myocytes, fibroblasts, vascular smooth muscle cells and endothelial cells and can influence gene expression as well as have rapid non genomic effects [44]. ERβ deficient mice develop hypertension, vascular dysfunction, and abnormal heart morphology as they age and therefore have been used to reveal the role for this receptor isoform in mediating E2 effects in the cardiovascular system. A study from Dr. E. Murphy found that ERβ mediates functional recovery following ischemia/reperfusion injury possibly by its regulation of fatty acid metabolism genes important in ischemic injury [45].

The development of several SERMs (selective estrogen-receptor modulators) also contributed to the understanding of the importance of ERβ in the cardiovascular system. ERβ-specific agonists reduced arterial pressure in ovariectomized spontaneously hypertensive rats, increased cardiac output and left ventricular stroke volume [46]. The same study also showed that ERβ activation also improved vascular reactivity and increased ERβ expression in
the aortic ring, both in endothelial cells in the intima and vascular smooth muscle cells [46]. Overall the study showed that ERβ specific agonists attenuate hypertension, vascular resistance and cardiac hypertrophy in spontaneously hypertensive rats. The authors also speculated that these positive effects might be mediated by enhanced vascular Nitric Oxide (NO) generation both via genomic and non-genomic mechanisms. Indeed ER increases expression of enzymes responsible for NO synthesis as well as activating them, possibly by recruiting and activating kinases [47].

ERβ is necessary for the observed gendered differences in the attenuation of the hypertrophic response to pressure overload and improved recovery from ischemia/reperfusion injury [48, 49]. Furthermore, in animal models of ischemia reperfusion injury, treatment with the ERβ specific agonist improves functional recovery and upregulates protective genes such as antiapoptotic proteins, heat shock proteins and cyclooxygenase 2 [50]. These and other studies led to the proposal by Kathryn Sandber and Hong Ji that ERβ could be the “Alpha-Dog” in estrogen receptor-mediated protection from hypertension [51].

An additional factor to consider when studying the effects of estrogen in post menopause is the comorbidity of depression and cardiovascular disease (CD) which ranges from 20 to 40% according to different studies. Middle-aged women are particularly susceptible to higher mortality following myocardial infarction (MI) if they have depressive symptoms (50% greater risk) [52-54]. The interesting link between heart disease and depression was analyzed in the SADHART trial (Sertraline AntiDepressant Heart Attack Trial), which was aimed at evaluating the effectiveness of the selective serotonin
reuptake inhibitor (SSRI) Sertraline in treating major depression in patients with acute myocardial infarction (MI) or unstable angina. The trial concluded that Sertaline is safe and efficacious in depressed patients with ischemic heart disease, but could not detect differences in mortality between placebo and Sertaline treated patients because it was underpowered. It is also difficult to determine whether depression is a risk factor for coronary heart disease (CHD) or a cause of it. Kenneth E Freeland and Robert M Carney in a recent report supported the idea that depression predicts worse outcome in patients with CHD and possibly improving the depressive symptoms could determine better cardiovascular prognosis [55]. Not only is comorbidity of depression and CD increasingly prevalent in middle-aged patients, but also menopausal women are particularly susceptible to have both these conditions. The elevated risk of both depression and CD in menopausal women is well accepted but the reason for this or the possible improvement from using HT needs further investigation [53].

Overall, several studies confirm estrogen’s positive effects on the cardiovascular system and the importance of ERβ in mediating these responses, but how age and timing of ET influences cardioprotection needs further investigation.
**Figure 1** Summary of studies depicting the specific cardioprotective and neuroprotective effects of ERβ.
THE TIMING HYPOTHESIS

The constant theme in neuroprotection and cardioprotection of estrogens in clinical studies is the importance of the time of initiation of ET (estrogen therapy alone) in women. The concepts referred to as “window of opportunity” or “timing hypothesis” emerged when analyzing several clinical studies and finding that the variety of results was due to the span of ages of the women enrolled in the studies. When analyzing data by grouping for the age of initiation of ET, it was clear that estrogens alone reduced risk of cognitive decline and dementia when administered to women in the early stage of menopause [56]. Postmenopausal women receiving ET in a 2012 Danish study also had significantly reduced risk of mortality and heart failure, without an increased risk of breast cancer or stroke [57]. The WHI ET follow-up showed that women 50-59 of age had statistically significant reduction in coronary heart disease (Hazard Ratio (HR) of 0.59), myocardial infarction (HR 0.54) and overall mortality (0.73) [58]. These are only a few examples of how cognitive and cardiovascular health of menopausal women can be improved if ET is started at the right time (i.e. in early menopause).

Despite the well-established evidence for cardioprotective effects of E2, the large scale clinical trial designed to assess the health benefits of E2 in postmenopausal women – the WHI- was suspended because of an increased risk of stroke and coronary heart disease among the HT participants [59].
Unfortunately, most physicians stopped prescribing ET to their patients after the WHI prematurely interrupted its study in 2002 and published their early results that showed adverse effects and increased mortality. A recent analysis by Dr. Phillip Sarrel published in the American Journal of Public health made the news when the author stated that avoiding ET by women in 2002-2011 caused the premature death of 50000 women [60]. He then emphasized the need to call attention to the beneficial effects of ET for women 50-59 which should prompt doctors to prescribe it to their patients in that age group. A better understanding of the contribution of age on the effects of estrogen on cognitive and cardiovascular health are needed to help guide physicians in providing the best care to their patients, and my project will shed light on the molecular mechanisms for this age-related shift in E2 efficacy.
ESTROGEN RECEPTOR BETA

Estrogen receptors are nuclear steroid receptors widely expressed in both reproductive and non-reproductive tissues and act as transcription factors; therefore they play a central role in many life processes including normal physiology [61]. In 1996 a group led by Jan-Ake Gustavsson successfully cloned the second estrogen receptor, expressed in rat prostate and ovary, named ERβ [62]. Initially described as a 485 amino acid long protein, further studies revealed an upstream AUG start site, making rERβ 530 amino acids long, with a calculated molecular weight (MW) of 59.152 KDa [63]. Subsequent studies identified several splice variants, the first and better characterized being ERβ2, first described in 1998 and widely expressed [64]. In the brain ERβ is expressed in several hypothalamic nuclei, hippocampus, cerebellum, olfactory bulb, amygdala, cortex, and spinal cord [65]. Peripherally it is expressed in the cardiovascular system (myocytes, endothelial cells, fibrocytes), ovary, prostate, skeletal system (osteoblasts, osteoclasts and osteocytes), gastrointestinal tract, pancreas, adipose tissue and liver [61].

Figure. 3. Domains of ERβ.
The structure of ERβ is that of a classical nuclear steroid receptor organized in domains each with a particular structure and specific functions (Fig.3). The N-terminal A and B domains are collectively defined as AF-1 (Activation Function-1, or N-terminal transactivation domain), a highly variable domain amongst the steroid receptor family. Many residues in the AF-1 domain are phosphorylated by various kinases; the AF-1 domain is also fundamental for binding to coregulatory proteins [63, 66]. The AF-1 domain is intrinsically disordered (ID) in solution, therefore its structure is difficult to analyze, but progress is being made in understanding its binding to coregulatory proteins that leads to modulation of its transcriptional regulation [67]. This domain is also thought to mediate the known ligand independent regulation of transcription by ERβ [68-70]. The C domain is the DNA-Binding Domain (DBD) involved in DNA recognition and binding. In the case of estrogen receptors the DBD recognizes Estrogen Responsive Elements (EREs), in the promoter of target genes. The DBD is partially conserved with ERα, which can also recognize and bind to ERE sequences. The ERE is an inverted palindromic DNA consensus sequence (5’ GGTCAnnnTGACC 3’) that the DBD has a high affinity for [71]. The D domain of ERβ is the hinge domain, a flexible domain allowing for the conformational changes of the receptor. The E domain is the Ligand Binding Domain (LBD) or AF-2, which is responsible for ligand binding of both agonists and antagonists, dimerization, interactions with coregulatory proteins and conformational changes caused by ligand binding [72]. The precise function of the small C-terminal F domain of ERβ has not been identified yet, although it has been proposed to influence ligand binding and coactivator binding [73].
According to the classic model of steroid receptor functioning, ERβ is normally present in the cytoplasm bound to chaperone proteins. Following ligand binding it dimerizes and translocates to the nucleus, where it binds to the ERE and, together with coregulatory proteins, activates gene transcription (Fig. 4). Steroid receptors are therefore highly dynamic, being shuttled to and from the nucleus and in proximity of the cell membrane or cellular organelles.

**Figure 4.** Classical model of ERβ’s action.
The ERα and ERβ isoforms can often be co-expressed in a tissue and form homo or heterodimers. It was shown in 1997 that ERα and β can form heterodimers on the DNA at ERE sequences and are capable of binding to coregulatory proteins and activating transcription [74]. However the heterodimer is potentially functionally distinct from the homodimer, giving ERs a wider possibility of gene regulation. The characteristics of the heterodimer were analyzed in vitro in a 2004 paper by Li et al. showing that heterodimer binding to E2 and activation of ERE were similar to the α-α homodimer [75]. However ERβ is known to act as a dominant inhibitor of ERα, and it was initially thought that the function of ERβ was to modulate ERα transcriptional activity [76]. This adds to the complexity of estrogens' signaling and determines the variety of effects estrogens can have in vivo in different tissues that display different expression of ERα and ERβ.

PHOSPHORYLATION OF ERβ

Post translational modifications (PTMs) such as phosphorylation, ubiquitination, acetylation and sumoylation have been identified for several steroid receptors, including ERs. These modifications can potentially alter each step of ERs function from ligand binding, dimerization, protein:protein interactions, DNA binding and ultimately ER function [66, 77]. For example, the first evidence that ERs can have ligand independent function was attributed to phosphorylation of the receptor after epidermal growth factor (EGF) treatment [78]. Furthermore EGF treatment to ovariectomized rats mimicked the effects of estrogens, as it leads to proliferation in the uterus [79]. We now know that extracellular factors such as EGF can initiate the
signaling cascade leading to kinase activation and ER phosphorylation and activation.

Although consensus sites for several kinases are present in ERβ, only a few have been described and confirmed as actual phosphorylation sites [66]. The experimentally confirmed phosphorylation sites for the human ERβ are S87 and S105 [80, 81]. Studies of the phosphorylation of the mouse isoform of ERβ have also shown phosphorylation at the homologous sites (S106 and S124) and other sites mostly in the N-terminal domain, which are less characterized [68].

Phosphorylation of ERβ has important functional implications. In a 2012 paper by Lam et al. phosphorylation of S105 inhibited breast cancer cell invasion and migration in vitro [81]. Phosphorylated S105 is also highly prevalent in human samples of breast cancer tissue, where ERβ is known to be antiproliferative [81, 82]. Phosphorylation of hERβ at S87 and S105 (mouse S106 S124) was shown to recruit the coregulatory protein steroid receptor coactivator-1 (SRC-1) in vitro and increase transcriptional activation at ERE in a ligand independent way [68]. The phosphorylation of ERβ has been shown to be mediated by two MAPKs, p38 and ERK, in in vitro kinase assays on the human and mouse ERβ form [68, 81].
Overall phosphorylation of steroid receptors is pivotal for their functioning, and the little that is understood about the actual phosphorylation of ERβ and how these influence their functioning is mostly from in vitro studies using breast cancer cell models. More needs to be done to characterize the molecular functional consequences of phosphorylation and describe ERβ phosphorylation in non-cancerous tissues, such as the brain and heart where ERβ is known to have important functions. Furthermore, to my knowledge there are no reports describing the effects of age and E2 on ERβ phosphorylation.

COREGULATORY PROTEINS

The recruitment of coregulatory proteins to steroid receptors is a fundamental step in their functioning. Coregulatory proteins can activate (coactivators) or repress (corepressors) gene transcription and are required for proper signaling of all steroid receptors. After binding to the steroid receptor they serve as scaffolds for the larger complex of proteins that form the pre-initiation complex on the promoter of the responsive gene. Most of the classic coregulatory proteins for ER belong to the SRC (Steroid Receptor Coregulator) family and were first identified in 1994 in the lab of Myles Brown as estrogen-receptor associated proteins necessary for ER functions [83]. Since then the functions of SRC proteins have been elucidated and their importance in cell physiology has grown. Coregulatory proteins were recently
defined by Dr. Bert O’Malley as “masters of system biology” to emphasize their importance [84]. They are not mere bridges between ERβ and the transcriptional machinery but integrate signaling cascades by binding to multiple transcription factors, and their dysregulation can lead to serious consequences such as development of cancer [85]. The coactivators SRC1 and SRC3 interact and positively regulate transcriptional regulation mediated by several steroid receptors, including ERβ. Corepressors, such as silencing mediator for retinoid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (NCoR), repress or silence steroid receptor mediated regulation of transcriptional activity [85].

To date, more than 350 steroid receptor coregulators have been reported. However the ER interactome comprises an impressive amount of proteins, not only classic coregulators but binding partners involved with cell functions ranging from apoptosis to cell structural proteins to metabolic proteins to RNA splicing. For example, over 743 proteins associate with ERβ in the ventral hippocampus and several of these interactions are age and estrogen dependent [86]. Other studies have shown that the interactome of ERs in breast cancers is made of hundreds of proteins [87, 88]. These newly discovered interacting partners are an insight to the potentials of ER signaling.

MITOGEN ACTIVATED PROTEIN KINASES

Age and E2 modulate cell signaling pathways critical for regulating basic cellular processes. Mitogen Activated Protein Kinases (MAPKs) are a family of Serine-Threonine protein kinases that link extracellular signals to intracellular responses [89, 90]. They are highly complex and interesting as they regulate cell activities including proliferation, differentiation, apoptosis
and cell survival. Nearly every physiological and pathological event studied involves MAPKs, which indicates not only their essentiality but also their complexity. The MAPK family includes p38 MAPK, extracellular signal-regulated kinase (ERK) and c-Jun NH2-terminal kinase (JNK), however as only p38 and ERK kinases have been shown to target ERβ for phosphorylation we will focus our attention to those [68, 81].

MAPK family members were first discovered and sequenced in the 1980s and 90s after their activation was observed following mitogens stimulation, hence their name [90]. These kinases are regulated themselves by phosphorylation at their Threonine-x-Tyrosine phosphorylation site motif. MAPKs activation is the last of a three step activation cascade from MAP3K to MAP2K to MAPK. Once phosphorylated (i.e. activated) they in turn phosphorylate substrate proteins at Serine or Threonine residues in specific recognition sequences [91]. While MAPKs phosphorylate S/T residues included in a conserved motif, this sequence is variable, allowing for substrate discrimination and a wider pool of targets [92]. The strongest requirement for phosphorylation common to p38 and ERK kinases is the presence of a Proline directly at the C- Terminus of the phosphorylation site. Another characteristic feature of MAPKs is the presence of docking sites in their substrates; the best characterized being D-sites and F-sites [93]. These mediate the interaction between sequence motifs in the target protein far from the phosphorylation sites and region of the kinase separate from the active site. Because of their heterogeneous preference for D- and F- sites, ERK and p38 interact with different target proteins, therefore having differential downstream effects. Furthermore docking sites assure specificity and selectivity of kinase
substrate interactions, reducing the number of potential substrates [93]. Interestingly 30% of human proteins have at least one MAPK phosphorylation site; however fewer have the docking sites necessary for the kinases to interact strongly and phosphorylate them.

The knowledge of the activators, substrates and functions of MAPKs are ever expanding, however when they were first discovered they were deemed stress-activated protein kinases (SAPKs) because of their responsiveness to toxins, physical stresses and inflammatory cytokines [92, 94]. Generally it can be said that ERK1/2 are preferentially activated in response to growth factors and extracellular stimuli while p38 is more responsive to stresses such as osmotic shock, ionizing radiation and cytokines [95]. MAPKs can be present in several cell compartments, such as the nucleus, cytoplasm, mitochondria and close to the cell membrane. Among their targets are other kinases and transcription factors allowing them to integrate signals and activate a variety of responses. While it can be difficult to dissect the effects of MAPKs activation from a specific stimulus, it is known that they can regulate mitosis, apoptosis, survival, differentiation, gene expression and more. MAPKs involvement in human disease has been proven and several kinases have been considered as targets of pharmaceutical modulation [96]. Of note, cancers such as melanoma and breast and ovarian tumors can be brought about by mutations in proteins either participating in the MAPKs pathways or direct targets [97]. Inflammatory related diseases such a COPD (chronic obstructive pulmonary disease), rheumatoid arthritis and systemic lupus also show MAPKs dysregulation.
Interestingly, neurodegenerative disease such as Alzheimer’s, Parkinson’s and ALS (Amyotrophic lateral sclerosis) are characterized by p38 and ERK alterations. For example, it has been shown that in a model of Alzheimer’s disease the oxidative stress induced by Amyloid beta accumulation leads to p38 activation and tau protein phosphorylation \[98\]. The cardiovascular system is also regulated by MAPKs and these have been considered as potential targets in cases of infarction, hypertrophy and heart failure \[99, 100\]. Pharmaceutical companies are investigating small molecule inhibitors or activators of MAPKs and several clinical studies are underway to investigate whether these could prevent, treat or decrease the symptoms of the diseases they are involved in.

In mammals, four splice variants of p38 kinase exist: p38α, p38β, p38γ and p38δ \[101\]. P38α was first isolated as a 38 KDa protein that was rapidly phosphorylated in a characteristic Thr-Gly-Tyr (TGY) motif following application of pro inflammatory stimuli \[102-105\]. The four splice variant proteins have a 60% identity in sequence, but can be differentially activated by upstream kinases and have different target proteins. P38α and p38β are widely expressed, while p38γ and p38δ are only expressed in certain cell types. Because of the sequence similarity of the different p38 isoforms, it is difficult to separately investigate the functioning of each. The perfect homology in the TGY motif results in the impossibility to raise antibodies for only one of the active isoforms. Therefore phosphop38 antibodies will bind to all p38 isoforms. Further complicating the study of each isoform, small molecule inhibitors also do not discriminate between isoforms.
The extracellular signal regulated kinase subfamily is comprised of the evolutionarily conserved ERK1 and ERK2 which have 83% amino acid identity [95]. Similarly to the p38 isoforms, ERK1 and ERK2 have perfect homology in their activating phosphorylation motif, resulting in antibodies that bind to both active forms.

To our interest p38 has been found to be regulated by aging and estrogen treatment in different cell and animal settings. In a 2011 study, Li et al. hypothesized that p38, a key regulator of pro-inflammatory cytokine biosynthesis, would be activated by the low grade inflammation that is associated with the aging process [106]. By measuring cytokines and markers of inflammation in the lungs of young (2mo) and old (20mo) C57BL/6J mice they showed a significant increase of inflammatory markers with age [106]. They also measured p38 activation in the lung and whole brain homogenate, showing the activation of p38 was doubled in the older mice, confirming their initial hypothesis [106]. Furthermore, Yousin Suh measured age specific changes in MAPKs expression and activity in the brain and liver of 2 mo and 26 mo female Fisher344 rats [107]. Again, p38 expression and activity was increased by 2.5 fold in the aged brain while no change in expression was measured in the liver [107]. In a separate study, the adrenal gland of 24-27 mo rats had increased levels of active p38 compared to younger animals [108]. In contrast to that, age dependent inhibition of p38 activity was detected in the cortex of 24 mo rats compared to 6 and 12 mo animals [109]. MAPKs can be activated by extracellular stimuli and E2 has been shown to activate p38 and ERK in a rapid, non-genomic way in isolated cardiomyocytes as well as in neuronal cells [110] [111].
P38 has also been shown to have a role in reproductive physiology, and it is more highly activated in the granulosa cells of older patients [112]. The same study also examined cellular localization of p38 and found that activated p38 was more highly present in the nucleus of younger cells, while it mostly resided in the cytoplasm in granulosa cells of older patients [113]. The authors concluded that oxidative stress might be the cause for the different subcellular localization of p38, since reactive oxidative species (ROS) are known to induce translocation of p38 to the cytoplasm and are abundant in aged cells. In the cardiovascular system, activated p38 has been implicated in a wide spectrum of pathologies, such as hypertrophy, infarction, and systolic and diastolic heart failure [100, 114-116]. The signaling pathways that p38 regulates could be linked to its role in heart pathology including apoptosis, pro-inflammatory gene regulation, myofilament modulation and remodeling of matrix proteins [115]. Several clinical trials involving p38 inhibitors have not yielded the positive results expected in regards to cardiovascular diseases, however there have been more encouraging results in rheumatoid arthritis treatment [100].

Overall it is well understood that MAPKs signaling plays an integral role in aging, and their aberrant regulation might be involved in age-related disorders. The molecular mechanisms by which p38 activation can be detrimental are not well understood, but we can infer from the literature that the age-dependent activation of MAPKs might be at least part of the cause for cardiovascular disease in post-menopausal women.
Figure 6. ERK and p38 signaling.
EXPERIMENTAL DESIGN

ANIMAL PARADIGM

The Timing Hypothesis originated from clinical data and clearly points to time after onset of menopause as an important factor in HT success. Therefore, we designed an animal paradigm that allows us to discern the relative contributions of age, length of E2 deprivation and subsequent E2 replacement over molecular and physiological responses (Fig. 7). The literature on animal models of menopause indicates that rats are the most appropriate non-primate animal model for menopause [117]. However, unlike primates, aging rats do not undergo a rapid decline in E2 levels so bilateral ovariectomy (OVX) is necessary to abolish endogenous E2, thereby ensuring an equivalent hormonal milieu. Our preliminary data show that a subcutaneous injection of 2.5 µg/kg E2 in aged (18mo) Fisher344 rats achieves circulating E2 levels of 50.56+/− 8.78 pg/ml, which is within the physiological range for post-menopausal patients receiving HT (17-75pg/ml; [118, 119]). Importantly, this paradigm will allow us to draw conclusions on the effects of duration of E2 deprivation prior to HT. Fisher344 rats (18mo; N = 10/group, total = 90) will be administered E2 or safflower oil (vehicle) once/day x 3 days at 1 week, 4 weeks, 8 weeks or 12 weeks after OVX, reflecting women who begin HT closer to menopause versus those who waited for longer periods of time.
Figure 7. Animal Paradigm.
HYPOTHESIS AND QUESTIONS

Estrogens are neuroprotective and cardioprotective for women, however when administered long after menopause, they fail to improve their health. This age related switch in estrogens’ effects after prolonged deprivation has not been explained. It is possible that the estrogen receptors (ER) that mediate the protective effects are modified following menopause and no longer function as in the past. Phosphorylation is the most common protein modification and it can fundamentally alter a protein’s activity. The ERβ isoform of the receptor is highly expressed in the brain and heart and has been shown to mediate protective neurological and cardiovascular effects. Little is known about ERβ phosphorylation, but the Mitogen Activated Protein Kinases can \textit{in vitro} phosphorylate ERβ in its N-terminal domain. This part of the protein mediates its interactions with coregulatory proteins that dictate its function as a transcription factor. The MAPKs responsible for this modification respond to cellular and extracellular stresses, which can increase with aging and disease and have been shown to be sensitive to estrogens. Therefore, I hypothesized that \textit{Mitogen Activated Protein Kinases are sensitive to age and prolonged estrogen deprivation leading to a differential phosphorylation of ERβ which results in altered transcriptional regulation.}

These are the questions I sought to answer:

1. Is phosphorylated ERβ present \textit{in vivo} in the brain and heart? (see Chapter III)
2. Does site specific phosphorylation of ERβ alter its activity as a transcription factor? (see Chapter III)
3. Does phosphorylation of ERβ alter its ability to bind to DNA? (see Chapter III)

4. Is the activity of the MAPKs ERK and p38 sensitive to prolonged estrogen deprivation and estrogen treatment? (see Chapter IV)

5. Is the expression of ERK and p38 dependent on prolonged estrogen deprivation and estrogen treatment? (see Chapter IV)

6. Which ER mediates the effects of estrogen on MAPKs activation? (see Chapter IV)

7. Can phosphorylated ERβ be detected and quantified in vivo? (see Chapter V)
CHAPTER III
PHOSPHORYLATION ALTERS ESTROGEN RECEPTOR B-MEDIATED TRANSCRIPTION IN NEURONS

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INTRODUCTION

Nuclear steroid receptors are master regulators of a broad range of physiological processes through their actions as ligand-activated transcription factors. Estrogen receptors (ERα and ERβ) are members of this receptor superfamily and their cognate endogenous ligand is 17β-estradiol (E2), which is the major circulating form of estrogen in premenopausal women. ERα and ERβ are widely expressed in a variety of tissues, although ERα is particularly abundant in breast, uterus and ovary, due to its primary role in mediating the reproductive-related effects of estrogens. By contrast, ERβ is highly expressed in many non-reproductive tissues such as nervous, cardiovascular, skeletal, gastric, and adipose tissues, and has been implicated in mediating estrogens effects on anxiety, mood, and memory, as well as numerous other physiological processes [15, 16, 40, 65, 120, 121]. Our basic understanding of ERα structure, function, and signaling pathways has significantly advanced in recent years, however similar aspects of ERβ function, especially in non-reproductive tissue/cell types, remain unclear.
The overall goal of these studies was to define the functional consequences of post-translational modifications to ERβ, namely site-specific phosphorylation, on its ability to transcriptionally activate gene promoter activity in neuronal cells.

Post-translational modifications (PTMs) such as phosphorylation, ubiquitination, acetylation, and sumoylation have been identified for several nuclear steroid receptors, including ERs [122-126]. These modifications have the potential to alter all aspects of ER function including ligand binding, dimerization, protein:protein interactions, DNA binding and, ultimately, alter ER-mediated transcription. Although several kinase consensus sites have been predicted for ERβ, only a few have been experimentally confirmed [66]. Specifically, the serine residues S87 and S105 located in the N-terminal domain of ERβ are highly conserved among the mouse, rat, and human, suggesting that ERβ phosphorylation at these sites could be a common regulatory mechanism across species [81]. The N-terminal domain of ERβ exhibits greater than 80% homology across species and the specific amino acid residues flanking S87 and S105 are highly conserved [127]. By contrast, the N-terminal domain of ERβ has relatively low homology with ERα, which could contribute to the divergent actions of the two receptors. These S87 and S105 sites are targets of MAPKs (Mitogen Activated Protein Kinases) and the specific MAPKs, P38 and ERK, have been shown to phosphorylate human and mouse ERβ in vitro [81, 128]. Functionally, phosphorylation of these sites increased recruitment of the coregulatory protein SRC-1 (steroid receptor coactivator-1), while coincidentally increasing transcriptional activation at an estrogen response element (ERE) [128].
Phosphorylation of ERβ has been studied primarily \textit{in vitro} using breast tumor cell models. The only reports of detection of phosphorylation of ERβ \textit{in vivo} come from immunohistochemistry analysis of human breast cancer tissue using a human-specific antibody generated against the phosphorylated S105 ERβ [81]. However, ERβ is highly expressed in non reproductive tissue such as the brain and heart, and its phosphorylation would have important functional consequences on those systems. Therefore, my first goal in these studies was to determine whether ERβ is phosphorylated \textit{in vivo} in the brain as well as in the heart of female rats. Using PhosTag\textsuperscript{TM} Acrylamide I was able to detect several phosphorylated species of ERβ in the dorsal hippocampus and left ventricle of the heart of aged (18 mo. Old) female rats. This, to my knowledge, is the first report of phosphorylated ERβ detection outside of the breast in any species.

Previous work by my laboratory showed that p38 kinase inhibition altered ERβ-dependent activation of ERE and AP-1 (activator protein-1) promoter activity in neurons [70]. However p38 kinase inhibitors are broad-spectrum inhibitors and can affect multiple signaling pathways in the cell, thereby making it unclear whether ERβ was a direct target of phosphorylation by p38 in those studies. Furthermore ERβ phosphorylation states were not determined. Therefore, in these studies I created phospho-mutants of ERβ to directly assess the consequences of ERβ phosphorylation on its transcriptional activity in neuronal cells. I hypothesized that phosphorylation of ERβ at specific sites, S87 and S105, would alter ERβ mediated gene regulation in neuronal cells, both directly at a canonical ERE site and also indirectly through protein:protein interactions at an AP-1 site. I also tested
whether phosphorylation of S87 and S105 altered the binding affinity of ERβ to a canonical ERE sequence. Collectively, my results demonstrate that phosphorylation of S87 and S105 altered both estrogen-independent and estrogen-dependent ERβ mediated transcriptional regulation at ERE and AP-1 sites in neuronal cells, but did not alter the DNA binding affinity to ERE sequences. Taken together, these data suggest that altered kinase activity in the brain, as occurs during aging, has the potential to alter the downstream expression of ERβ gene targets resulting in fundamental changes in brain function.

RESULTS

PHOSPHORYLATED SPECIES OF ERβ ARE PRESENT IN THE DORSAL HIPPOCAMPUS OF AGED FEMALE RATS.

The detection of phosphorylated ERβ in the brain and heart in vivo has not been previously demonstrated, likely due to its relatively low expression and the lack of commercially available phospho-specific antibodies targeting rERβ. A powerful tool that has recently emerged is PhosTag™, a phosphate-binding tag that slows the migration of phosphorylated protein during the electrophoretic run on polyacrylamide gels [129]. Use of PhosTag™ acrylamide results in the detection of several bands when analyzed by Western Blot using specific antibodies for the protein of interest, and each band represents the target protein with a different degree of phosphorylation. For instance, higher bands indicate that the target protein contains several phosphate groups, which result in a slower migration through the gel and is visualized as a higher band shift. To verify the specificity of the phosphorylated bands detected, protein samples can be treated with alkaline
phosphatase (AP), which will dephosphorylate the protein and result in the absence of detectable phosphorylated bands on the Western blot. The only band detected will be a lower band representing the non-phosphorylated protein of interest.

I tested whether phosphorylated ERβ is present in the dorsal hippocampus and left ventricle of aged female rats to provide rationale for assessing the functional consequences of phosphorylated ERβ in neuronal cells. Estradiol (E2) is known to have direct effects on the dorsal hippocampus, a brain region involved in cognition and memory formation and a region where ERβ is more highly expressed than ERα [130, 131]. Because E2 therapy is often prescribed to ameliorate the negative cognitive and cardiovascular issues that accompany menopause, I used a model of surgically-induced menopause followed by acute E2 treatment to test whether 1) phosphorylated ERβ is present in the brain and heart, and 2) E2 treatment alters ERβ phosphorylation levels in the brain. Aged Fisher 344 rats (18 mo. Old) underwent ovariectomy (OVX) followed by acute E2 or vehicle administration for 3 days (see Methods).

Fig. 8 shows a representative PhosTag™ SDS-PAGE blot probed for ERβ with the ERβ specific antibody. Phosphorylated ERβ was detected in the dorsal hippocampus of both the vehicle and E2-treated animals (Fig. 8A, arrow). Following AP treatment this same band disappeared, indicating that the band that shifted was specific for phosphorylated ERβ. AP treatment did not diminish the intensity of the lower band, which represents unphosphorylated ERβ, however the higher band is very dark indicating that a
large amount of ERβ in the aged female dorsal hippocampus was phosphorylated to some degree (Fig. 8A).

In Fig.8B phosphorylated ERβ is detected in the left ventricle of aged female rats. Conversely to the dorsal hippocampus, more than one band is absent following AP treatment. This could have different explanation. First, it is possible that an increasing amount of phosphates are bound to ERβ. This would result in the several bands detected: the higher being ERβ with several phosphates attached which would cause a slower electrophoretic run in the PhosTag™ gel. The other possible explanation is that splice variants of ERβ are present in the heart, and that those are also phosphorylated. ERβ2 is a widely expressed splice variant which has as an additional 18 amino acid insertion far from the antibody binding site, therefore it could recognize and bind it as well [132-134]. ERβ2 protein has not been detected in the heart but its mRNA has been detected in the cardiovascular system [134]. I hypothesize that the lower band in the AP treated samples is ERβ1, the band above ERβ2 and bands that are only present in absence of AP treatment a combination of phosphorylated species of ERβ1 and ERβ2.
Figure 8. Expression of phosphorylated ERβ in the dorsal hippocampus of aged female rats. Protein isolated from the dorsal hippocampus (50 μg) was resolved using Phos-Tag™ SDS-PAGE electrophoresis and probed for ERβ with the ERβ specific antibody (SC-150). Phos-Tag™ binds phosphate groups and increased protein phosphorylation retards gel migration. Alkaline phosphatase (AP) treatment removes phosphate groups allowing the protein to migrate further through gel. Aliquots of the same dorsal hippocampus protein sample were treated with different amounts of AP. Arrow indicates phosphorylated ERβ. Samples not treated with AP show upper band, indicating presence of phosphorylated ERβ that is abrogated in samples treated with either 30 or 60 units of AP.
ERβ PHOSPHORYLATION ALTERS LIGAND DEPENDENT AND LIGAND INDEPENDENT ACTIVATION OF ERE-MEDIATED TRANSCRIPTION IN NEURONAL CELLS.

To study the functional effects of phosphorylation of ERβ in neurons, I used site-directed mutagenesis to mutate S87 and S105 into alanine (A), a residue that cannot be phosphorylated, or glutamic acid (E), a residue that resembles a phosphorylated serine because of its similar negative charge and molecular bulk [135]. The expression vector constructs used in the transient transfection analysis are listed in Table 1. ERβ acts as a transcription factor in cis by directly binding to DNA at consensus ERE sequences and activating downstream gene transcription. To test whether phosphorylation of ERβ alters its ability to regulate ERE-mediated transcription, I transiently co-transfected wild type (WT)-ERβ or one of the mutants listed in Table 1 with the reporter construct ERE-tk-luc in a hippocampal-derived cell line (HT-22).
<table>
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<tr>
<th>Vector name</th>
<th>Characteristics</th>
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<tr>
<td>ERβ</td>
<td>Wild type ERβ</td>
<td>Wild type</td>
</tr>
<tr>
<td>87E</td>
<td>Phospho mimetic at S87</td>
<td>Phospho mutants</td>
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<tr>
<td>87A</td>
<td>Phospho null at S87</td>
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<tr>
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<td>105A</td>
<td>Phospho null at S105</td>
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<tr>
<td>AA</td>
<td>Phospho null at S87 and S105</td>
<td>Double mutants</td>
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<td>EE</td>
<td>Phospho mimetic at S87 and S105</td>
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*Table 1. List of ERβ expression vectors.*
First I analyzed the ERE-mediated promoter activity for each of the phospho-mutants with a single site mutated. A two-factor ANOVA analysis revealed that there was a statistically significant interaction between plasmid and treatment, demonstrating that the effect of E2 on ERE-mediated promoter activity depends on whether ERβ is phosphorylated at serine 87 and/or 105 (Fig. 9A; F (5,74)= 9.790, p<0.001). Consistent with previous studies, WT-ERβ tended to increase ERE-dependent transcription in the absence of ligand (an approximate 2 fold increase) and E2 treatment increased it to a much greater statistically significant extent (an approximate 5.5 fold increase, Fig. 2A) [136]. I then analyzed the ERE-mediated promoter activity for each of the phospho-mutant receptors. Mutation of ERβ at serine 87 had differential effects on ERE-mediated promoter activity depending on whether it was phospho-null or phospho-mimetic. First, mutation to alanine (87A, phospho-null) increased ERE-mediated promoter activity to a similar extent as WT-ERβ in the absence of E2, whereas E2 treatment significantly increased ERE-mediated activity to a greater extent. These results suggest that phosphorylation at this site hinders the E2-dependent activation of the receptor. Interestingly, the opposite effect was observed for mutations at the serine 105 site. In that case, the phospho-null mutation (105A) was not different from WT-ERβ. By stark contrast, the phospho-mimetic (105E) increased not only the E2-independent (by approximately 4 fold), but also the E2-dependent, activation of ERβ.
Figure 9. Effects of ERβ phosphorylation on ERβ-mediated promoter activity at an ERE site. Hippocampal-derived (HT-22) cell lines were transiently co-transfected with an ERE-tk-Luciferase reporter construct and the wild type ERβ or (A) single phospho-mutant ERβ expression vector (S87A, S87E, S105A, S105E) or (B) double phospho-mutant ERβ expression vector (S87A+S105A; S87E+S105E; S87A+S105E, S87E+S105A) (B). Cells were treated with 100 nM E2 or vehicle (0.01% ethanol) for 15 hours. Data are expressed as the mean percent change compared to empty vector control ± SEM. Different letters denote statistically significant differences as calculated with two-way ANOVA and Tukey post-hoc analysis (p<0.05).
The previous experiments demonstrated the effects of a single amino acid manipulation on ERE- and AP-1-mediated transcription. In those experiments, the phosphorylation status of the opposing site was unknown and entirely dependent on the endogenous kinase activity in the cell. Therefore, in this next series of experiments I tested whether simultaneous phosphorylation (i.e. S87E + S105E (EE)) or complete absence of phosphorylation (i.e. S87A and S105A (AA)) at both serine residues could further alter ERβ regulation. In addition, I also tested the effects on ERE-mediated transcription when one site was phosphorylated, but not the other (i.e. S87A + S105E (AE); S87E + S105A (EA)). Each of the double mutant vectors (see table 1) were transiently co-transfected with an ERE-tk-luc reporter construct in HT-22 cells as described before.

A two-factor ANOVA analysis revealed a statistically significant interaction between plasmid and treatment, once again indicating that E2 regulation of promoter activity depends on the phosphorylation status of both ERβ S87 and S105 residues (Fig. 9, F( 5,78)= 5.271, p< 0.001). Figure 9B demonstrates the effects of the double mutants on ERE-mediated promoter activity. All of the double mutants increased ERE-mediated transcription in the presence of E2, although the increase was greater when the two serine residues had an opposite phosphorylation status (AE, EA, Fig. 9B). Moreover, the AE mutation increased ERE-mediated activity by more than 2 fold in the absence of ligand.

Overall, these data demonstrate that phosphorylation of ERβ at both S87 and S105 has important functional consequences on its ability to transcriptionally activate ERE-mediated promoters in neurons.
ERβ PHOSPHORYLATION ALTERS LIGAND DEPENDENT AND LIGAND INDEPENDENT ACTIVATION OF AP-1 DEPENDENT TRANSCRIPTION IN NEURONAL CELLS.

Estrogen receptor β can also act as a transcription factor *in trans* by tethering other transcription factors, thereby regulating a larger subset of genes whose promoters might lack a consensus ERE. For instance, ERβ-mediated regulation at AP-1 sites requires ERβ interaction with transcription factors of the Fos and Jun families. Similar to my observed results on ERE-mediated promoter activity, a two-factor ANOVA analysis revealed that there was a statistically significant interaction between plasmid and treatment (Fig. 10A; F (5, 59)= 6.046, p< 0.001). First, WT-ERβ had a constitutive (ligand independent) inhibition of AP-1-mediated promoter activity (an approximate 50% decrease, Fig. 10A), which showed a trend towards a decrease following E2 treatment. These results are consistent with my previous reports of ERβ repression of AP-1-mediated promoter activity in neuronal cells [70, 136].

Surprisingly, however, mutation of S87 or S105 to any form (phospho-null or phospho-mimetic) completely abolished the ligand independent inhibition of AP-1-mediated transcriptional regulation, yet the E2-induced reduction was preserved. These results suggest that S87 and S105 are critical residues mediating the ligand independent actions of ERβ at an AP-1 site.
Figure 10. Effects of ERβ phosphorylation on ERβ-mediated promoter activity at an AP-1 site. Hippocampal-derived (HT-22) cell lines were transiently co-transfected with an AP-1-tk-Luciferase reporter construct and the wild type ERβ or (A) single phospho-mutant ERβ expression vector (S87A, S87E, S105A, S105E) or (B) double phospho-mutant ERβ expression vector (S87A+S105A; S87E+S105E; S87A+S105E, S87E+S105A) (B). Cells were treated with 100 nM E2 or vehicle (0.01% ethanol) for 15 hours. Data are expressed as the mean percent change compared to empty vector control ± SEM. Different letters denote statistically significant differences as calculated with two-way ANOVA and Tukey post-hoc analysis (p<0.05).
I next tested whether simultaneous phosphorylation (i.e. S87E + S105E (EE)) or complete absence of phosphorylation (i.e. S87A and S105A (AA)) at both serine residues could further alter ERβ regulation at an AP-1 site. The regulation of AP-1-mediated transcription by double ERβ mutants is shown in Figure 10B. Surprisingly, the ligand independent inhibition of AP-1 was restored when both S87 and S105 were concurrently mutated, while the single mutation of those same sites abolished the constitutive inhibition of AP-1 regulation by WT-ERβ (Fig. 10B). Moreover, E2 significantly inhibited the constitutive repression to a greater extent when S105 was mutated to a phospho-mimetic I regardless of the phosphorylation status of S87 (Fig. 10B; F (5, 70)= 3.741, p< 0.005).

Overall, I show that ERβ regulation of AP-1 dependent transcription is fundamentally altered by single, but not double, mutation of S87 and S105.

PHOSPHORYLATION OF ERβ ALTERS TAMOXIFEN EFFECTS ON ERE AND AP-1 TRANSCRIPTION IN NEURONAL CELLS.

Next, I tested whether phosphorylation of ERβ alters the effects of tamoxifen (TAM), a known selective ER modulator (SERM). TAM can be both agonistic and antagonistic, depending on cell type, ER subtype, and promoter response element [137-141]. I have previously shown than TAM abrogates the constitutive activation of ERE in HT-22 neuronal cells, and this was also true for some of the phospho-mutants tested (Fig. 11) [136]. Similar to the previously described experimental results, a two-factor ANOVA revealed a significant interaction between the two factors, plasmid type and TAM treatment (F (5, 71) = 12.978, p< 0.001). Specifically, TAM treatment completely eliminated the constitutive ligand-independent activation (i.e.
vehicle-treated) of ERE-mediated transcription (Fig. 11A). Further, phosphorylation of S105 (105E) increased constitutive activity to the greatest extent (>400%), and also increased TAM dependent activation of ERE-mediated transcription compared to wild type ERβ (Fig. 11A). These results suggest that phosphorylation of S105 would alter transcription of ERE regulated genes following TAM treatment.
Figure 11. Effects of tamoxifen treatment on ERβ or ERβ mutant-mediated promoter activity at an ERE or AP-1 site. Hippocampal-derived (HT-22) cell lines were transiently co-transfected with an (A) ERE-tk-Luciferase or (B) AP-1-tk-Luciferase reporter construct and the wild type ERβ or phospho-mutant ERβ expression vector (S87A, S87E, S105A, S105E). 24 hours following transfection, cells were treated with 100 nM 4-OH-tamoxifen (TAM) or vehicle (0.01% ethanol) for 15 hours. Transfection efficiency was normalized using a second renilla luciferase reporter construct (rLUC) in all experiments. Data are expressed as the mean percent change in fLUC/rLUC compared to empty vector control ± SEM taken from 4 independent experiments with 6 replicates/experiment. Different letters denote statistically significant differences as calculated with two-way ANOVA and Tukey post-hoc analysis (p<0.05).
Our previous studies showed that TAM abolished the observed ligand independent inhibition of AP-1 activity by ERβ. The current studies confirm that TAM does abolish the ligand independent inhibition of AP-1 activity for the WT-ERβ (Fig. 11B). However, mutation of S87 or S105 alone abolished the ligand independent repression of AP-1-mediated transcription (see Figs. 10, 11B vehicle), and treatment with TAM did not have any additional effects.

Phosphorylation of ERβ does not alter binding to an ERE sequence

Phosphorylation of nuclear receptors has previously been shown to alter their binding affinity for consensus response elements, such as an ERE, in the promoter regions of their target genes. Moreover, I have previously shown that WT-ERβ can bind to consensus ERE sequences both in the absence and presence of E2, but the effects of phosphorylation of S87 or S105 have never been investigated [70, 136]. Therefore, I tested whether phosphorylation of ERβ at S87 or S105 altered its ability to bind to consensus ERE sequences using electromobility shift assays. After autoradiography, I observed that all receptor proteins produced an equally strong shift in the position of the $^{32}$P-labeled ERE oligonucleotide, demonstrating that phosphorylation of S87 and S105 did not affect ERβ:DNA binding to that consensus sequence (Fig. 12).
Figure 12 Effects of S87 and S105 phospho-mutation on DNA binding to a consensus ERE sequence. Vitellogenin ERE consensus sequence was 32P-end labeled and incubated with in vitro translated wild type or phospho-mutant ERβ protein receptor lysates. Receptor lysates were incubated with 100 nM E2 or vehicle for 16 hr. prior to 30 min. incubation with 32P-labeled ERE oligonucleotide and resolved on a 6% retardation gel. Gels were dried and exposed to a phosphorImager screen for 4 hours prior to imaging. Specific binding was confirmed by incubating with a scramble radiolabeled DNA sequence (lane 2) or by addition of a 1000X unlabeled ERE oligonucleotide prior to addition of radiolabeled vitellogenin ERE (lane 3). (Neg: negative control, no lysate). Relative densitometry of gel bands taken from six independent experiments were quantified using IMAGE J (NIH, Bethesda, MD, USA). The data are reported as the mean ± SEM density of pixels. Two-way ANOVA analysis indicated no statistically significant differences.
Unlabeled excess (500 or 1000x) of the same $^{32}$P-ERE oligonucleotide competed effectively for ERβ binding and no shift was observed when the receptors were incubated with a $^{32}$P-labeled scrambled DNA sequence. Densitometry was used to quantify the band width and there were no statistically significant differences in the average densities of each band between the WT-ERβ and the phospho-mutants, indicating that phosphorylation at S87 or S105 does not affect ERβ binding affinity to consensus ERE sequences (Fig. 12). These experiments were repeated at minimum of 10 times to account for lane-to-lane variability between gels, and the average band density from all gels combined was calculated to determine if phospho-mutation and/or ligand affected ERE binding. I did not observe any statistically significant differences in average band density between vehicle or E2 treatments for either WT-ERβ or the ERβ phospho-mutants, which is consistent with my previous reports for WT-ERβ [70, 136].

**DISCUSSION**

The overall objective of these studies was to determine the consequences of ERβ phosphorylation on its functional capacity to act as a transcription factor at known promoter enhancer sites in neurons. Further, I hypothesized that phosphorylated ERβ would be detectable in the brain of aged animals, due to the potential alterations in kinase activity that can occur with aging [107, 142-145]. My data demonstrated the novel finding that phosphorylated ERβ is not only present in the brain of aged females, it is likely the major form of ERβ expressed in the dorsal hippocampus and phosphorylation tended to increase following E2 treatment. These data provide strong evidence for the physiological relevance of my functional in
outside data, which together demonstrated that phosphorylation of ERβ at specific serine residues altered its ability to activate and/or repress promoter activity in neurons. Collectively, these data suggest that age-related changes in hormonal milieu and cellular kinase activity could impact the expression of ERβ-regulated genes, such as those mediating stress, anxiety, and cognitive function.
Table 2. Schematic summary of reporter assay results. Arrows indicate direction of change in relation to wild type ERβ. In grey are highlighted the instances of differences between each mutant compared to wild type ERβ.

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Post-translational modifications of nuclear steroid receptors have been widely investigated and these modifications are known to regulate their signaling abilities [66, 122, 125, 126]. Structurally, ERβ is similar to other members of the nuclear steroid receptor superfamily. The N-terminal A and B domains are collectively defined as the AF-1 (Activation Function-1, or N-terminal transactivation) domain, which is a highly variable domain amongst the steroid receptor family and is fundamental for binding coregulatory proteins that assist in transcriptional activation or repression [70, 136]. Phosphorylation of mouse ERβ at the N-terminal serine 106 and 124 has previously been shown to mediate ligand independent recruitment of the coregulatory protein SRC-1 (steroid receptor coactivator 1) and alter its subsequent transcriptional activity at an ERE in COS-1 cells [128]. In those studies, overexpression of SRC-1 significantly increased ligand independent activation at an ERE site. This effect was dependent on the MAPK phosphorylation of S106 and S124, as the double phospho-null mutation (S106A/S124A) completely abolished the SRC-1-induced activation at an ERE. However, that study did not evaluate the effects of S106A or S124A in the presence of ligand (E2), or the transcriptional activity at an ERE resulting from a S106/S124 phospho-mimetic (i.e. mutation to glutamic acid). In my studies the single mutation of S87A significantly increased E2-induced transcription at an ERE, but there was no effect in the absence of E2. Moreover, there was no difference in ERE-mediated transcription when S87 and S105 were both phospho-null (87A/105A) compared to WT-ERβ. The discrepancies between my results and the previously reported could be due to the overexpression of SRC-1 in those studies. In addition, it is possible that
there is a differential endogenous expression of SRC-1 in neurons or that ERs interact with different set of proteins depending on cell-type, resulting in altered signaling.

A similar study evaluated the effects of phosphorylated human ERβ at S105 in cancer cell lines [81]. In that study, phosphorylation of hERβ at S105 inhibited breast cancer cell invasion and migration in vitro, suggesting that phosphorylation of ERβ at S105 mediates the anti-proliferative action of ERβ in the breast. [81, 128]. Moreover, phosphorylated S105 ERβ has been detected both in benign and invasive breast cancer tissue samples using a human-specific phosphoS105 antibody; however no studies to date have detected the presence of phosphorylated ERβ in the brain. My results using neuronal cells showed that the phospho-mimetic S105E significantly increased both ligand independent and ligand dependent transcriptional activation at an ERE compared to WT-ERβ, whereas conversely, the opposite phospho-null mutation of S105A had no effect. The LBD of ERβ lies between residues 223-457, suggesting that phosphorylation of S87 or S105 is unlikely to alter ligand binding. However, cross talk between the N-terminal and C-terminal domain has been demonstrated, suggesting that altered ligand binding is a possible mechanism for phosphorylation-mediated changes in transcriptional activity [9].

It is well accepted that ERβ has lower transactivation ability at an ERE site compared to ERα and my data suggest that this discrepancy could be partly explained by the phosphorylation status of ERβ in the N-terminal AF-1 domain [137]. For instance, phosphorylation of the N-terminal domain could alter the ability of ERβ to bind consensus ERE sites, thereby allowing
preferential binding of ERα to those same sites. This concept is supported by
the observation that various types of post translational modifications have
been shown to alter DNA binding in other contexts. For example, acetylation
of ERα at two lysine residues in the DBD enhanced its ability to bind to an
ERE [146]. By contrast, sumoylation of estrogen-related receptor alpha
(ERRα) at two sites in the N-terminal domain did not affect its DNA binding
ability, yet still altered its transcriptional activity [147]. My results add to
existing evidence supporting the idea that post translational modifications in
nuclear receptors at regions distant to the DBD alters their ability to modulate
transcription, without affecting DNA binding.

Perhaps the most interesting results from this study were the effects of
ERβ phosphorylation at an AP-1 site. I have previously shown that ERβ exerts
strong transcriptional repression at an AP-1 site in neuronal cells in the
absence of ligand and the results presented here indicate that this might
depend on phosphorylation of the N-terminal domain [70, 136]. The single
mutation of either S87 or S105 completely abolished ligand independent
inhibition of AP-1 mediated transcription compared to WT-ERβ. However, AP-
1 mediated transcription was equally repressed with WT-ERβ as it was with
double mutations of AA, EE, or AE. Notably, only the 87E/105A mutation
showed a significant difference from the other mutants, suggesting that S87
phosphorylation status is the critical serine residue for mediating ERβ-induced
repression at an AP-1 site. The precise dynamics of p38 and ERK
phosphorylation of ERβ are unknown and the folding of singly phosphorylated
ERβ could render the other site inaccessible to the kinase. Nevertheless, my
data suggests that a single phosphorylation change in either S87 or S105
abrogates the ligand independent inhibition of AP-1, whereas no effect is observed due to concurrent phosphorylation of both sites. This could be explained by altered ERβ protein-protein interactions with Jun/Fos proteins or with other co-regulatory proteins recruited to the AP-1 complex, such as p160, based on evidence that phosphorylation alters coregulatory protein recruitment to ERβ [128]. The next step will be to determine which of these sites are phosphorylated in vivo and the precise molecular environment that facilitates those phosphorylation changes.

Recently, Vivar and colleagues identified 3 classes of ERβ target genes and discovered that the majority of genes (453) fell into the Class I category, which were genes regulated by unliganded ERβ [148]. Further, the Class I genes showed a high enrichment for AP-1 binding sites, demonstrating that ligand independent repression of AP-1-mediated promoter activity is a common mechanism of ERβ signaling. Similarly, Zhao et al. showed that over 60% of ERβ interacting regions in MCF-7 breast cancer cells contain an AP-1 site [149]. Together these studies underscore the significance of my findings that ERβ ligand independent activity at an AP-1 site is abolished when the N-terminal domain is phosphorylated. Aging in women results in dramatic declines in circulating estrogens raising the possibility that the ligand-independent actions of ERβ could play a prominent role in regulating gene transcription in the aging brain.

My studies were limited to the investigation of just one type of post-translational modification (phosphorylation) at specific serine residues. However, there is evidence that other post-translational modifications of ERβ could also be present, such as acetylation and sumoylation, and these
modifications can create cross talk between different residues of the receptor. For example, phosphorylation at S305 on ERα inhibits subsequent acetylation at K303 leading to enhanced transcriptional regulation [150]. Moreover, phosphorylation of S94 and S106 on mERβ leads to recruitment of ubiquinating enzymes to the AF-1 domain resulting in enhanced degradation of the receptor [151]. Future studies are required to determine the precise mechanisms and consequences resulting from post-translational modifications acting in concert to modulate overall ERβ function. My in vitro studies were conducted using rat ERβ expression vectors in a mouse-derived neuronal cell line. Given the high degree of homology between rat and mouse ERβ, I expect that similar results would be obtained using rat neuronal cell lines or primary neurons derived from either species [127]. However, it is important to consider these results in this context as both brain-region and species-specific effects of ERβ have been observed [152, 153].

Tamoxifen (TAM) has been described as a partial antagonist due to its cell-type specific effects [139, 141, 154-157]. Relevant to these studies, TAM action in the brain has been shown to block the neuroprotective effects of E2, confirming that the beneficial effects of E2 are mediated by classical estrogen receptors [120]. In addition, TAM can activate numerous second messenger signaling pathways, including MAPK, thereby potentially phosphorylating and altering ERβ transcriptional activity [158]. My results demonstrated that TAM worked as an antagonist of both the WT and phosphorylated forms of ERβ by preventing the ligand independent increase in ERE-mediated transcription. Consistent with my previous studies, TAM also blocked the ligand independent repression of WT-ERβ at an AP-1 site. The ligand binding
domain (LBD) of ERα and ERβ is more conserved than other domains and X-Ray crystallography studies have shown that both receptors bind several types of selective estrogen receptor modulators (SERMs), including TAM [159]. The structure of TAM complexed with the ERα or ERβ LBD have been resolved and TAM:LBD binding resulted in conformational changes that inhibited subsequent binding of coactivator proteins, pointing to a clear mechanism for TAM antagonism [160, 161].

In summary, my results show that phosphorylation of ERβ alters its function in neuronal cells both in a ligand dependent and independent manner, and that ERβ is phosphorylated in vivo in the hippocampus. My work highlights the importance of further understanding the effects of post-translational modifications on nuclear steroid receptors, as these modifications fundamentally alter their function as transcription factors and could result in clinically important physiological changes.
INTRODUCTION

Clinical and basic science studies have shown that estrogens are neuroprotective and cardioprotective. Treatment with estrogens (ET) can reduce the incidence of Alzheimer disease, improve survival following ischemic stroke, improve learning and memory, and reduce anxiety and mood disorders [23-25, 36-38, 155]. Moreover, women are less likely to develop heart disease than men, but this advantage is lost after menopause presumably due to a significant reduction in circulating estrogens [41-43]. Clinical correlates have indicated that the decline of circulating estrogens coincident with menopause leads to an increased risk of coronary heart disease, atherosclerosis, hypertension, stroke, neurodegenerative disease, cognitive decline, and mood disorders. However, the first large scale clinical trial designed to assess the health benefits of ET in postmenopausal women (The Women’s Health Initiative (WHI)) was prematurely suspended because of an unexpected increased risk of stroke and coronary heart disease among the participants [59].
Subsequent analysis of the clinical data revealed that the age of the participants significantly affected the outcome. Women who began ET at the time of menopause or within 10 years had significant reductions in heart disease and overall mortality [162]. This observation was coined the “Timing Hypothesis”, and postulated that ET administration is beneficial for early postmenopausal women, but detrimental in late postmenopausal women. Importantly, the underlying molecular mechanisms for these observations remain undetermined.

The actions of estrogens are mediated primarily by estrogen receptor (ER) α and ERβ. Both receptors can mediate the neuroprotective and cardioprotective effects of estrogens [16, 25, 45, 46, 51, 155, 163]. Estrogens bind ERs to regulate gene transcription through classical genomic pathways, or by modulating cell signaling pathways such as the MAPKs (mitogen activated protein kinases) ERK (extracellular signal-regulated kinase) and p38 [113, 164-170]. Moreover, aging alone modulates similar cell signaling pathways independent of estrogens [107, 110, 113, 143, 144, 169, 171, 172]. One possibility for these age-related changes is that MAPKs are sensitive to proinflammatory and oxidative stimuli, which are increased with age [173]. These converging data suggest that MAPK signaling could be a molecular mechanism underlying the discrepant effects of ET in postmenopausal women.

MAPK family members are activated by phosphorylation at their threonine-x-tyrosine phosphorylation site motif. MAPKs activation is the last of a three step activation cascade from MAP3K to MAP2K to MAPK. Once phosphorylated (i.e. activated) they in turn phosphorylate substrate proteins at
serine or threonine residues in specific recognition sequences [91]. The knowledge of the activators, substrates and functions of MAPKs are ever expanding, however when they were first discovered they were deemed stress-activated protein kinases (SAPKs) because of their responsiveness to toxins, physical stresses and inflammatory cytokines [92, 94]. Generally, ERK1/2 are preferentially activated in response to growth factors and extracellular stimuli, while p38 are more responsive to stresses such as osmotic shock, ionizing radiation and cytokines [95]. MAPKs can be present in several cell compartments, such as the nucleus, the cytoplasm and close to the cell membrane, thereby allowing them to integrate signals to coordinate a variety of physiological responses including mitosis, apoptosis, survival, cellular differentiation, and gene expression.

Activated MAPKs target a wide pool of proteins due to sequence variation in the conserved phosphorylation motif and in the availability of docking sites on their protein targets. The strongest requirement is the presence of a proline directly at the C-Terminus of the target protein phosphorylation site and this feature is a shared requirement for both p38 and ERK kinases. Another characteristic feature differentiating the downstream effects of MAPKs is the presence of docking sites in their substrates; the best characterized being D-sites and F-sites. ERK and p38 phosphorylate different target proteins, leading to differential downstream effects because of their heterogeneous recognition motif and respective preference for D- and F-sites in the targets [93]. Interestingly, 30% of human proteins have at least one MAPK phosphorylation site, yet few have the required docking sites to facilitate phosphorylation by MAPK family members.
The overall goal of this study was to quantify the total expression and activation of MAPKs (ERK and p38) in the brain and heart of aged female rats subjected to a paradigm designed to model the tenets of the Timing Hypothesis (see Fig.13). I hypothesized that the combination of age and low circulating estrogens alters the expression and activation of p38 and ERK kinases in the brain and heart. To test this hypothesis, I used a rat model of surgically-induced menopause and quantified changes in kinase activity following varying lengths of E2 deprivation. My data revealed age- and E2-dependent effects on kinase activity suggesting a potential mechanism explaining the variable effects of E2 following menopause.
Figure 13. Diagram of the animal paradigm. Female Fisher 344 rats were ovariectomized at day 0 (= 18 months old) and subjected to increasingly longer periods of hormone deprivation (1, 4, 8, and 12 weeks). Following the assigned length of deprivation, animals were treated with either vehicle (safflower oil) or 17β-estradiol (E2; 2.5 μg/kg) by subcutaneous injection once daily for 3 consecutive days (n=10/treatment group/deprivation time). Animals were euthanized 24 hours following the last treatment.
RESULTS

LENGTH OF E2 DEPRIVATION AND SUBSEQUENT E2 TREATMENT ALTERS ERK AND P38 KINASE ACTIVITY IN THE BRAIN AND HEART OF AGED FEMALE RATS.

I first tested whether the effects of E2 treatment on ERK and p38 kinase activity would depend on the length of time aged animals were deprived of circulating E2 (i.e. time post-ovx). I measured the ratio of phosphorylated and total ERK and p38 proteins as a measure of kinase activity in 3 distinct brain regions (hypothalamus, dorsal hippocampus, ventral hippocampus) and in the left ventricle of the heart. The hypothalamus regulates homeostasis, thermoregulation and stress response which are often dysregulated in post-menopausal women [174]. The hippocampus is functionally divided in two regions, dorsal and ventral [175] and both regions express ERs. The dorsal hippocampus (DH) mediates cognitive functions, while the ventral hippocampus (VH) mediates processes associated with emotional memory and stress [175]. In addition, I focused on the heart because the likeliness of developing heart disease increases in women post-menopause, and sex differences in cardiovascular disease are well characterized. The left ventricle (LV) is the main pumping chamber of the heart and it is most commonly subjected to diseases that accompany aging, such as hypertrophy. Estrogen receptors are highly expressed in the LV and the cardioprotective effects of E2 treatment are evident in the LV [163, 176, 177].

Brain – Hypothalamus. A two-factor ANOVA analysis revealed a significant interaction between length of E2 deprivation and subsequent E2
treatment (all F-values reported in Table 3), demonstrating that the effects of E2 are dependent on when it is administered following prolonged periods of E2 deprivation. Total ERK protein increased in E2-treated animals at the 12-week post-OVX time point, yet total protein in both groups remained stable at all other time points (Fig. 14A). Conversely, E2 treatment had a dramatic effect on the amount of phosphorylated ERK, which is an indicator for the active form of the protein. E2 treatment significantly decreased the amount of phosphorylated ERK to levels that were barely above detection at the 8- and 12-week post-OVX time points (Fig. 14B). The ratio of phosphorylated ERK to total ERK protein was significantly lower at all time points in E2-treated animals (Fig. 14C), representing a large magnitude shift from baseline compared to vehicle-treated controls (Fig. 14D).
Figure 14. Effects of age and E2 treatment on ERK protein expression and activation in the hypothalamus. Total ERK protein (A), phosphorylated ERK (B), calculated ratio of phospho:total-ERK (C), and percent change from vehicle following E2 treatment (D). Data are expressed as mean fold change ± SEM compared to vehicle-treated animals at one week post-OVX (A-C). An * indicates statistically significant difference from 1-week time point; # indicates significant difference within the same time point.
By stark contrast to ERK activity in the hypothalamus, total p38 protein was unchanged over time and with treatment (Fig. 15A); however phosphorylated p38 increased 3-fold at the 12-week time point in both vehicle and E2-treated groups (Fig. 15B). The ratio of phospho:total p38 was not altered by E2 treatment (Fig. 15C), however it is notable that it was increased by 170% in the E2-treated animals at 1-week post-OVX compared to vehicle treated animals.
Figure 15. Effects of age and E2 treatment on p38 protein expression and activation in the hypothalamus. Total p38 protein (A), phosphorylated p38 (B), calculated ratio of phospho:total-p38 (C), and percent change from vehicle following E2 treatment (D). Data are expressed as mean fold change ± SEM compared to vehicle-treated animals at one week post-OVX (A-C). An * indicates statistically significant difference from 1-week time point; # indicates significant difference within the same time point.
**Brain - Dorsal hippocampus.** I then measured ERK and p38 protein and phosphoprotein in the dorsal hippocampus (Figs.16, 17). Similar to the results observed in the hypothalamus, a two-factor ANOVA revealed a significant interaction between length of E2 deprivation (i.e. time post-OVX) and subsequent E2 treatment (Table 3). E2 treatment significantly decreased phosphorylated ERK at the 1-week time point, yet there were no changes in total ERK protein in either group (Fig. 16A, B). The ratio of phospho:total ERK was significantly lower than vehicle-treated animals at 1-week post-OVX (Fig. 16C) and the overall levels of phosphoERK were consistently lower in E2-treated animals across the treatment paradigm (Fig. 16D).
Figure 16. Effects of age and E2 treatment on ERK protein expression and activation in the dorsal hippocampus. Total ERK protein (A), phosphorylated ERK (B), calculated ratio of phospho:total-ERK (C), and percent change from vehicle following E2 treatment (D). Data are expressed as mean fold change ± SEM compared to vehicle-treated animals at one week post-OVX (A-C). An * indicates statistically significant difference from 1-week time point; # indicates significant difference within the same time point.
Next, I measured p38 expression and activation in the dorsal hippocampus (Fig. 17). A two-factor ANOVA analysis revealed a significant main effect of time post-OVX, yet no significant interaction suggesting the two variables were not dependent on each other (Table 3). Similar to ERK, E2 treatment significantly inhibited phospho-p38 levels 1 week post-OVX (Fig. 17B), yet the ratio of phospho:total p38 was not significantly different at any time point (Fig. 17C, D).
Figure 17. Effects of age and E2 treatment on p38 protein expression and activation in the dorsal hippocampus. Total p38 protein (A), phosphorylated p38 (B), calculated ratio of phospho:total-p38 (C), and percent change from vehicle following E2 treatment (D). Data are expressed as mean fold change ± SEM compared to vehicle-treated animals at one week post-OVX (A-C). An * indicates statistically significant difference from 1-week time point; # indicates significant difference within the same time point.
**Brain – Ventral hippocampus.** A two-factor ANOVA revealed a significant interaction between length of E2 deprivation and subsequent E2 treatment, similar to the results from the other brain regions (hypothalamus and dorsal hippocampus) (Table 3). Specifically, total ERK protein was significantly decreased at 4 weeks post-OVX in both vehicle and E2-treated animals, and these levels increased back to the levels observed at 1-week post-OVX by the 8 and 12-week time points (Fig. 18A). E2 treatment decreased phosphoERK by 70%, at 1-week post-OVX, but both vehicle and E2-treated animals had significantly lower levels of phosphoERK at the 4 and 8 weeks post-OVX time points (Fig. 18B). Interestingly, the ratio of phospho:total ERK was not different between treatment groups or with longer periods of E2 deprivation (Fig. 18C) and this was likely due to parallel changes that occurred in total available ERK protein. The strong inhibitory action of E2 on ERK activation was only present at the early E2 deprivation time point (1 week post-OVX), while at later time points (4 and 8 weeks post-OVX) E2 treatment increased ERK activation (Fig. 18D).
Figure 18. Effects of age and E2 treatment on ERK protein expression and activation in the ventral hippocampus. Total ERK protein (A), phosphorylated ERK (B), calculated ratio of phospho:total-ERK (C), and percent change from vehicle following E2 treatment (D). Data are expressed as mean fold change ± SEM compared to vehicle-treated animals at one week post-OVX (A-C). An * indicates statistically significant difference from 1-week time point; # indicates significant difference within the same time point.
There were few observed differences between treatment groups or with length of E2 deprivation in total or phospho-p38 levels (Fig. 19A, B). A two-factor ANOVA revealed no significant interaction between the two factors, but there was a significant main effect of time post-OVX (Table 3). E2 treatment significantly decreased the amount of total p38 protein at 12-weeks post-OVX (Fig. 19A) without any corresponding change in the levels of phospho-p38 (Fig. 19B). The ratio of phospho:total p38 was also significantly decreased after 8 weeks of E2 deprivation, but there was no difference between treatment groups (Fig. 19C). Overall, E2-treated animals had lower phosphorylated levels of p38 compared to vehicle treated animals until the 12-week post-OVX time point. At 12 weeks post-OVX there was a dramatic reversal with E2-treated animals having 88% higher levels of phosphorylated p38 compared to vehicle-treated animals (Fig. 19D).
Figure 19. Effects of age and E2 treatment on p38 protein expression and activation in the ventral hippocampus. Total p38 protein (A), phosphorylated p38 (B), calculated ratio of phospho:total-p38 (C), and percent change from vehicle following E2 treatment (D). Data are expressed as mean fold change ± SEM compared to vehicle-treated animals at one week post-OVX (A-C). An * indicates statistically significant difference from 1-week time point; # indicates significant difference within the same time point.
Heart – Left ventricle. Total ERK protein levels increased 12 weeks post OVX in the E2-treated animals compared to the vehicle-treated animals (Fig. 20A). In contrast to results obtained in the brain, length of E2 deprivation (i.e. age alone) had the most striking effect on ERK activation, while E2 treatment had a modest, yet statistically significant effect. Moreover, a two-factor ANOVA revealed a significant interaction between length of E2 deprivation and subsequent E2 treatment (Table 3), demonstrating that the effects of E2 replacement on ERK activity are altered following prolonged periods of time post-OVX in both the heart and brain. Specifically, phospho-ERK significantly decreased in both vehicle- and E2-treated groups at 4 and 8 weeks, yet there was a dramatic rebound in phospho-ERK in the E2-treated animals after 12 weeks of E2 deprivation (Fig. 20B). However, after comparing the ratio of active to total ERK it is clear that the length of E2 deprivation was the most important factor regulating ERK activity (Fig. 20C), as both treatment groups showed significant declines (80% decrease) at 4 and 8 weeks that returned to baseline by 12 weeks (Fig. 20D). Notably, E2 treatment led to even further declines in active ERK (to nearly undetectable levels) at 4 and 8 weeks post-OVX (Fig. 20D).
Figure 20. Effects of age and E2 treatment on ERK protein expression and activation in the left ventricle. Total ERK protein (A), phosphorylated ERK (B), calculated ratio of phospho:total-ERK (C), and percent change from vehicle following E2 treatment (D). Data are expressed as mean fold change ± SEM compared to vehicle-treated animals at one week post-OVX (A-C). An * indicates statistically significant difference from 1-week time point; # indicates significant difference within the same time point.
Total p38 protein levels were not significantly altered in the heart in my paradigm (Fig. 21A). However, a two-factor ANOVA analysis revealed a significant interaction between the two factors, E2 treatment and length of time post-OVX, on the levels of phospho-p38 in the heart. Consistent with the observations for ERK, p38 activation decreased with longer periods of deprivation post-OVX regardless of treatment in the heart (Fig. 21C). There was a strong main effect of time post-OVX, as the phospho:total p38 declined by more than 40% by the 4-week time point (Fig. 21C). These lower levels were stable up to 12 weeks post-OVX, in E2 and vehicle treated animals, in contrast with ERK whose activation levels were restored 12 weeks post-OVX. Although E2-treatment significantly decreased phospho-p38 at 8 weeks post-OVX, this effect did not have a major impact on the amount of active p38 (Fig. 21B, C, D).
Figure 21. Effects of age and E2 treatment on p38 protein expression and activation in the left ventricle. Total p38 protein (A), phosphorylated p38 (B), calculated ratio of phospho:total-p38 (C), and percent change from vehicle following E2 treatment (D). Data are expressed as mean fold change ± SEM compared to vehicle-treated animals at one week post-OVX (A-C). An * indicates statistically significant difference from 1-week time point; # indicates significant difference within the same time point.
LENGTH OF E2 DEPRIVATION AND SUBSEQUENT E2 TREATMENT ALTERS ERK AND P38 MRNA EXPRESSION

To determine if steady-state mRNA expression paralleled the observed changes in protein levels, I measured ERK (Fig. 22) and p38 (Fig. 23) mRNA in each brain region and in the left ventricle of animals subjected to my E2-deprivation paradigm. A two-factor ANOVA revealed statistically significant differences between treatment groups in the hypothalamus (Fig. 22A) and the heart (Fig. 22D), but not in either region of the hippocampus (Fig. 22B, C). Treatment with E2 increased ERK mRNA 1-week post-OVX, (Fig. 22A, dashed line), however ERK mRNA levels increased progressively over time and this age-related increase was prevented by E2 treatment (Fig. 22A). Similarly, prolonged periods of E2 deprivation increased ERK mRNA in the heart and this was significantly decreased following E2-treatment at the 12 weeks post-OVX time point (Fig. 22D).
Figure 22. Effects of age and E2 treatment on ERK mRNA expression. ERK mRNA was measured using RT-qPCR in the hypothalamus (A), dorsal hippocampus (B), ventral hippocampus (C), and left ventricle (D). Data are expressed as mean fold change ± SEM compared to vehicle-treated animals at one week post-OVX. An * indicates statistically significant difference from 1-week time point; # indicates significant difference within the same time point.
Expression of p38 mRNA levels were also statistically different between groups subjected to my E2-deprivation paradigm (Fig. 23). In the hypothalamus (Fig. 23A) p38 expression was significantly increased 4 and 8 weeks post-OVX, with no significant effect of E2 treatment. In the dorsal hippocampus a significant main effect of time, but not treatment was detected and pairwise comparisons did not reveal statistically significant changes between groups (Fig. 23B). A significant interaction of treatment and time post-OVX was detected in the ventral hippocampus (Table 3), where E2 treatment significantly reduced p38 mRNA at the 12-week time point (Fig. 23C). There were no differences between groups in p38 mRNA expression in the heart (Fig. 23D).
Figure 23. Effects of age and E2 treatment on p38 mRNA expression. P38 mRNA was measured using RT-qPCR in the hypothalamus (A), dorsal hippocampus (B), ventral hippocampus (C), and left ventricle (D). Data are expressed as mean fold change ± SEM compared to vehicle-treated animals at one week post-OVX. * indicates statistically significant difference from 1-week time point; # indicates significant difference within the same time point.
ERK AND P38 ACTIVATION ARE DIFFERENTIALLY REGULATED BY ERα AND ERβ

The differential effects of ERβ and ERα on MAPKs signaling have been previously reported in different experimental systems, and I detected both forms of the receptor in my samples [178, 179]. Therefore, in order to determine the contribution of each estrogen receptor, I treated a set of 18 mo Fisher 344 with the ERβ or ERα specific agonists DPN and PPT, 1 week post OVX to compare with the vehicle or E2 treated animals at the same time point. In Fig. 24, the ratio of phosphorylated ERK and total ERK was measured via Western blot in samples from the hypothalamus (A), dorsal hippocampus (B), ventral hippocampus (C) and heart (D). In the hypothalamus, E2 treatment 1 week post OVX determined a significant inhibition of ERK activation, which was replicated in animals treated with the ERα agonist PPT. The ERβ specific agonist DPN did not significantly alter the activation of ERK, indicating that in the hypothalamus ERα is the main mediator of the inhibition of ERK activity. In the dorsal hippocampus E2 was found to inhibit ERK activation 1 week post OVX, and in panel B of Fig. 24 ERβ and ERα specific agonists show a similar effect. All treatments significantly inhibited ERK activation compared to vehicle treated animals. No significant changes in ERK activation were detected in the ventral hippocampus when animals were treated with E2, DPN or PPT. Although a trend to a decrease was observed in the E2 treated animals. In the heart, E2 treatment does not alter ERK activation 1 week post OVX and neither do DPN PPT (see Fig. 24 D).
Figure 24. ERK activation is differentially regulated by ER isoform specific agonists. Ratio of phospho:totalERK in the hypothalamus (A), dorsal hippocampus (B), ventral hippocampus (C), heart (D) of 18 months old rats treated with V, E2, DPN (ERβ agonist) or PPT (ERα agonist) 1 week post OVX. Total and phosphorylated ERK were measured via Western blot; ratio of the two is represented here. Fold change compared to vehicle-treated animals. An * indicates statistically significant difference between groups using one-way ANOVA p<0.05 (n=4/6 animals per group)
The ratio of phosphorylated p38 and total p38 was calculated following Western blot analysis in the hypothalamus (A), dorsal hippocampus (B), ventral hippocampus (C) and heart (D) as shown in Fig. 25. In the hypothalamus PPT treatment alone significantly increased p38 activation 5 fold, while in E2 and DPN treated animals levels were similar to the vehicle group. The fact that E2 alone does not increase p38 activation while PPT does might be reflective of E2 binding to heterodimers. The other regions did not show any statistically significant effect of DPN or PPT on p38 activation.
Figure 25. p38 activation is differentially regulated by ER isoform specific agonists. Ratio of phospho:total-p38 in the hypothalamus (A), dorsal hippocampus (B), ventral hippocampus (C), heart (D) of 18 months old rats treated with V, E2, DPN (ERβ agonist) or PPT (ERα agonist) 1 week post OVX. Total and phosphorylated p38 were measured via Western blot; ratio of the two is represented here. Fold change compared to vehicle-treated animals. An * indicates statistically significant difference between groups using one-way ANOVA p<0.05 (n=4/6 animals per group)
Consistent with what has been observed throughout this study, ERK activity was more sensitive to my animal paradigm than p38. Interestingly, I found that the ER isoforms differentially alter kinase activity. Overall I found that in the hypothalamus ERα plays a more important role on MAPKs regulation. I have shown that E2 treatment leads to inhibition of ERK likely by activating ERα. Furthermore, stimulation of ERα but not ERβ activates p38. However, in the dorsal hippocampus both receptor specific agonists had the same effects as E2.
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DISCUSSION

MAPKs are central components of second messenger signaling pathways and are ubiquitously expressed in all cell types, therefore the functional implications for these findings are widespread. Here, I report the novel findings that E2 treatment differentially affects ERK and p38 activation, as well as total protein and mRNA expression, in the brain and the heart dependent on age and length of time following E2 deprivation (i.e. OVX/menopause). Importantly, length of E2 deprivation was a critical factor in every parameter analyzed. For example, ERK and p38 were significantly less active 4 or more weeks following OVX regardless of treatment in the heart, possibly reflecting an age-related change. Age has been shown to both increase and decrease MAPKs activation, depending on tissue analyzed, sex, age and species of the animal model, as well as experimental design [106, 107, 180, 181]. For instance, Li et al. hypothesized that p38, a key regulator of pro-inflammatory cytokine biosynthesis, would be activated by the low grade inflammation that is associated with the aging process. They observed increased levels of inflammatory markers accompanied by doubling in p38 activation in the lung and whole brain homogenate of old (20 mo.) compared to young (2 mo.) C57BL/6J mice [106]. Similarly, p38 expression and activity was increased by 2.5 fold in the brain of 26 mo. compared to 2 mo. Fisher 344 rats [107]. These findings align with my results in the hypothalamus where p38 had a 2.5 fold increase 12 weeks post-OVX, regardless of treatment. However, these results were brain region specific, as I did not observe age-related changes for phospho-p38 in other brain regions analyzed.

Perhaps most intriguing was the observation that E2-induced changes
in activated ERK and p38 were prolonged, given that the last dose of E2 was administered 24 hours before euthanasia. The prevailing view is that the non-genomic actions of E2 occur within minutes and the effects on MAPK phosphorylation are transitory. Indeed, this has been observed repeatedly in vitro, however accumulating evidence suggests that the in vivo regulation of MAPKs is more complex [110, 114, 169, 182-187]. One possibility is that E2, acting through classical genomic pathways, altered cellular components required for maintaining balance between active and inactive MAPKs. For example, crosstalk between phosphorylation and ubiquitination pathways can exert long-term changes in cellular processes through multiple feedback loops that ultimately impact apoptosis and cell proliferation [188]. Moreover, I along with others have shown that E2 can regulate microRNAs in the brain and the heart, and some of these miRNAs could target components of MAPK signaling pathways [179, 189-192] providing a putative mechanism for sustained activation of these kinases. These results were further corroborated in a 2013 study which showed E2 could regulate long-term activation of MAPKs by altering expression of microRNAs that silence upstream inhibitors of ERK activation [192]. The findings herein combined with other published studies suggest that E2 can exert prolonged changes in MAPKs activation that cannot be explained by the acute non-genomic actions of E2 alone.

The significant interactions observed between the two factors of time and E2 treatment for ERK, but not p38, in nearly every brain region and in the heart could have important functional ramifications for understanding the physiological consequences of ET in postmenopausal women. Specifically, the timing of E2 treatment would not be expected to impact p38 signaling in
these tissues, despite the fact p38 activity was independently altered by E2 and age. Conversely, activated ERK was mainly decreased following E2 treatment and the timing of E2 treatment dictated the magnitude of decline. Activation of ERK induces downstream signaling pathways that mediate both neuro- and cardioprotection. For instance, the formation of dendritic spines was increased by E2 treatment through ERK-mediated mTOR (mammalian target of rapamycin) and this E2-induced synaptic plasticity is a key mechanism underlying memory consolidation and storage [193]. Similarly, activated ERK mediates cardioprotective pathways in the heart and phospho-ERK is increased by E2 in young adult rat cardiomyocytes [110, 170, 194-196]. The E2-induced decrease of activated ERK in the brain and heart following longer periods post-OVX reveal a putative mechanism for memory decline and reduced cardioprotection following ET in late postmenopausal women.

Increased estrogen receptor-mediated gene transcription is the most likely explanation for my observed increases in total ERK and p38 protein levels. Indeed, I found that the mRNA levels of both kinases were significantly altered by E2 treatment and length of deprivation in the brain and heart. Sequence analysis of the ERK and p38 gene promoters revealed an abundance of binding sites for several transcription factors such as NF-kB and GATA families as well as multiple estrogen response elements (ERE) [197]. The canonical actions of ERs are through direct ERE binding, however ERs can also regulate transcription by tethering to other transcription factors, such as members of the Jun and Fos families acting at AP-1 sites (activator protein-1) [198]. Notably, there are 28 and 34 identified AP-1 sites within 2000
bp upstream of the p38 and ERK translation start sites, respectively. These data demonstrate that there are multiple mechanisms for E2 to regulate ERK and p38 transcription directly at the level of the gene promoter.

In the last decade, sophisticated genomic and proteomic tools have allowed for the quantitative molecular analysis of complex biological samples. These experiments revealed low correlations between mRNA and protein levels in many samples [199]. Similarly, changes in ERK and p38 protein levels did not tightly correlate with their altered mRNA levels in my paradigm. This suggests that age and/or E2 treatment can regulate compensatory factors that affect protein translation or stability. For example I observed a 3 to 5-fold increase of ERK mRNA following prolonged E2 deprivation in the hypothalamus, yet total ERK protein was unchanged. This could be partly explained by the semi-quantitative nature of the techniques (i.e. Western blot). Alternatively, this observation could be due to increased mRNA turnover or translational inhibition. Several mechanisms of post transcriptional regulation of mRNA have been described and microRNAs are interesting example of regulatory molecules that can repress target mRNA translation [200]. My recent work demonstrated that microRNA expression is differentially regulated by prolonged E2 deprivation and subsequent E2 treatment using this same animal treatment paradigm in aged rats [179, 190]. In line with the current findings, several of those E2-regulated microRNAs have the potential to inhibit p38 and ERK mRNA translation, resulting in decreased protein levels. I also identified a subset of microRNAs that are differentially regulated by E2 in young (3 mo.) vs. old (18 mo.) rats using a microRNA microarray platform [190]. Bioinformatics pathway analyses revealed that the MAPK pathway was
predicted as the most represented cellular pathway targeted by the microRNAs I identified as E2 regulated [190]. Regulation of microRNAs is just one possible explanation for the discrepancies seen between mRNA and protein levels in complex biological samples.

I also sought to determine which ERs might be mediating the effects on MAPKs activity in my animal paradigm. My group has recently demonstrated that in animals subject to my experimental paradigm both ERα and ERβ are expressed in the brain regions in analysis and in the heart throughout all time points and treatments [178, 201]. Furthermore I have analyzed the expression of ERβ2, a splice variant of ERβ, which is a constitutively active form of the receptor, and found that it was regulated by estrogen deprivation following OVX as well as E2 [178]. Here I have determined that 1 week post OVX E2 significantly inhibits ERK activation, likely via ERα whose activation also significantly inhibited ERK activation. Similarly p38 activation was also activated by E2 treatment and ERα agonist PPT in the hypothalamus, while ERβ agonist DPN had no effect. In the dorsal hippocampus however both ERα and ERβ agonists inhibited ERK activation as did E2. Isoform specific effects of ERs are known, however this is the first in vivo analysis of MAPKs regulation in the brain and heart.

Despite the understanding of the importance of the time of initiation of ET in women for neuroprotection and cardioprotection gained from clinical studies, clear mechanistic insight is still lacking. I designed my study to model a main tenet of the timing hypothesis, as based on clinical observations [59, 202, 203]. One limitation is that rodent reproductive senescence is not comparable to the menopausal transition in women [204, 205]. However, the
surgically-induced menopause model used in rodents is the most accurate method to determine the length of time following total ovarian hormone depletion, and surgically-induced menopause is clinically relevant for some women. I have also demonstrated previously that 18 months of age is a physiologically relevant comparison to human in this strain of rat (Fisher 344), although Sprague Dawley rats demonstrate much earlier reproductive senescence [178]. The concepts referred as “window of opportunity” or “timing hypothesis” emerged from clinical studies that found that age of ET initiation determined the successful outcome of the study. Indeed estrogens reduced risk of cognitive decline and dementia when administered to women in early stage of menopause [56]. Postmenopausal women receiving ET in a 2012 Danish study also had significantly reduced risk of mortality and heart failure, without an increased risk of breast cancer or stroke [57]. The WHI ET follow-up showed that women 50-59 of age had statistically significant reduction in coronary heart disease (Hazard Ratio HR of 0.59), myocardial infarction (HR 0.54) and overall mortality (0.73) [58]. These are only a few examples of how cognitive and cardiovascular health of menopausal women can be improved if ET is started at the right time (i.e. in early menopause). My data highlights MAPKs as a possible focus of further analysis, as these kinases are critical regulators of cell signaling pathways.
CHAPTER V

QUANTITATIVE MASS SPECTROMETRY FOR IN VIVO ANALYSIS OF PHOSPHORYLATED ERβ

INTRODUCTION

Phosphorylation of ERβ significantly alters its function as a transcription factor as shown in Chapter III [206]. ERβ can be phosphorylated in its N-terminal domain at two sites, S87 and S105. This was shown first by mutating mouse ERβ in the homologous Serines into Alanines and performing in vitro phosphorylation with radioactive p32ATP [128]. The Mutant ERβ failed to incorporate p32ATP, indicating that S87 and S105 are phosphorylated at those sites. Separate groups have found that ERβ can be phosphorylated in vitro by the MAPKs ERK1, ERK2, p38 but not Src or PKA [81, 128]. Later, phosphorylation of purified human ERβ at S105 was verified following in vitro phosphorylation using mass spectrometry (MS) [81]. I have extended these findings by demonstrating detection of phosphorylated ERβ, in vivo, in the brain and heart of aged rats using PhosTag™ immunoblotting (Chapter III). While this technique allowed for detection of phosphorylated forms of ERβ, the site or sites at which the phosphates are attached cannot be determined with this technique. Importantly, site specific modifications altered ERβ function, as shown in Chapter III, therefore the identification of distinct phosphorylated residues in vivo is a critical next step.
Phosphorylation of ERβ in vivo was also detected using antibodies directed against the phosphoS105ERβ peptide through immunohistochemical staining of human breast tissue samples [81]. While this is a positive indication of the presence of phosphoS105ERβ in human breast, antibody based assays are often misleading when studying ERβ. Indeed a thorough analysis of nine antibodies available revealed that while some do recognize ERβ used in certain techniques, most lack specificity or only work correctly in a specific application [207]. The lack of reliable tools for investigating ERβ in vivo is a recurring theme in the field and most experts attribute this to the partially unfolded state of the N-terminal domain of ERβ [208-211]. The dynamic structural behavior of ERβ gives it the ability to rapidly interact with different coregulatory proteins, localize to different intracellular organelles and transition on and off the DNA quickly, overall determining its functional heterogeneity that makes it an important target of investigation.

The activity of the MAPKs p38 and ERK, the kinases that have been shown to phosphorylate ERβ, is sensitive to aging and estrogen treatment. Indeed the findings described in Chapter IV demonstrated that the length of estrogen deprivation prior to estrogen treatment altered MAPKs activation in the brain and heart of aged rats. Therefore, it is likely that levels of phosphorylated ERβ are different in the tissues of rats that underwent the estrogen deprivation paradigm. ERβ mediates important neuroprotective and cardioprotective effects of estrogens and its phosphorylation has functional consequences as shown in Chapter III. Therefore it is pivotal to detect and compare levels of phosphorylated ERβ in brain and hearts.
The experimental approach I chose was to use Multiple Reaction Monitoring (MRM) (also referred to as Selected Reaction Monitoring or SRM); a mass spectrometry based method that allows for detection and quantification of low abundance peptides in complex samples. Briefly, during an MRM experiment a predefined peptide of interest is detected using a Triple Quadrupole mass spectrometer. By mixing a known amount of a peptide identical to the target peptide but incorporating heavy isotopes, the amount of unknown peptide can be readily quantified. Further, using heavy labelled peptides that are phosphorylated at the site of interest allows for the quantification of the phosphorylated form of the peptide.

There are multiple steps that must be performed in order to create the MRM including 1) analysis of the protein sequence of interest to obtain a theoretical digestion profile, 2) empirical testing of digestion profile with a purified protein, 3) optimization of instrumentation parameters using synthetically developed peptide fragments, 4) development of the MRM using increased sample complexity, 5) isolation of *in vivo* experimental protein samples and mass spectrometry analysis. Fig. 26 is a schematic representation of the experiments described in this Chapter and details the preliminary steps necessary for MRM method design.
Figure 26. Outline of the experiments described in this Chapter.
MRM experiments are usually performed using Liquid Chromatography (LC) coupled to Triple Quadrupole mass spectrometers. These types of instruments are designed to follow the workflow diagram depicted in Fig. 27.

First, samples are injected in the chromatography column; typically a C18 column which is made of an 18 carbon chain bound on a silica substrate. Peptides adsorb to the column and by gradually increasing the amount of polar solvent in the mobile phase they elute separately according to their biochemical characteristics. This step reduces the complexity of the sample prior to injection in the mass spectrometer. Second, ionization of the peptides is achieved by applying a high voltage to the peptides to create an aerosol with the electrospray ionization (ESI) probe. ESI does not fragment the peptides and can create multiply charged ions (z>1). Third, the ionized peptides move into the first quadrupole (Q1) and are analyzed by mass. Fourth, in the second quadrupole (Q2) the ions are collided with high pressure gas (argon) inducing dissociation of the ions in a process called collision induced dissociation (CID). Each ionized peptide that entered Q1 (precursor ion) will then be fragmented in Q2 into product ions that will be detected by Q3, which also analyzes each fragment by mass. The m/z values of precursor ions and product ions that will be generated after CID can be predicted based on the sequence for the peptide of interest. The collision induces breakage of the amide bond and loss of amino acids at the N- and C- termini of the peptide, and the resulting ions are called y- and b- ions. In MRM, the precursor ion of interest is selected in Q1 and the product ions of interest are monitored in Q3. MRM is an example of a targeted mass spectrometry approach, as this method only detects a selected peptide of interest. The
AQUA (absolute quantification) method was developed to directly quantify peptides using MRM [212]. Briefly, synthetic peptides of the same sequence as the peptide of interest are synthetized using a heavy labelled amino acid, shifting their mass by a known amount. These heavy peptides are used as internal standards and spiked with the unknown sample containing the light peptide of interest. The heavy peptides have the same retention time, ionization and fragmentation characteristics, but have a shift in mass which allows for comparison of intensity of endogenous (light) and heavy peptides.

**Figure 27.** Schematic representation of the Triple Quadrupole mass spectrometer used for MRM.
RESULTS

1) Analyze ERβ Sequence

The first step in determining whether MRM is a feasible approach to detect and quantify ERβ phosphorylation is to analyze its sequence. Indeed it is necessary to enzymatically cleave proteins in smaller peptides that can be detected by Triple Quadrupole mass spectrometers for MRM experiments. Protein digestion is a common procedure in proteomics and several proteolytic enzymes are available commercially. Each protease has very specific cleavage sites; empirical digestion of the protein of interest can be tested using bioinformatics tools such as Expasy Peptide Cutter [213]. The size of the peptide of interest is also an important consideration for MRM experiments. A maximum length of 25 amino acids is indicated for a few main reasons. First, mass of larger peptides is over the limit of detection of Triple Quadrupole mass spectrometers. Furthermore long peptides are typically unsuitable for synthesis. Finally amino acid side chains can potentially be modified during ionization and dissociation in the mass spectrometer, and longer sequences have more potential for modifications which would complicate the analysis. Another crucial factor when deciding which enzyme to use for digestion is the uniqueness of the sequence of the peptide of interest. Therefore, to avoid false positive results, it is essential to scan the NCBI database to confirm that the target peptide fragment is unique to the protein of interest. Finally, protease efficiency is high, however some cleavage sites are more optimal than others, and bioinformaticians have developed algorithms to predict the likeliness of a “missed cleavage” to occur [214].
I performed the steps described above using the rERβ sequence available from Uniprot (accession number Q62986). The goal was to identify a protease that would digest ERβ containing the desired S87 and S105 sites in peptide fragments that met the optimal criteria for MRM. I first used Expasy peptide cutter to obtain ERβ digestion profiles for eight common proteases. Figure 28 shows the results of the theoretical digestion. The right column lists the cleavage sites for each proteases and I focused on proteases that cleaved the protein at residues close to S87 and S105. I calculated the size of the peptides containing S87 and S105 and evaluated my options. Arg-C, Asp-N, low-specificity-Chymotrypsin and Proteinase K were not feasible enzymes as they would not yield ERβ peptides I could use for MRM. Cleavage of ERβ with Lys-C or Lys-N yielded S87 and S105 containing fragments of 100 or 21 amino acid length each. On average MRM experiments use peptide of 10 amino acids, however it is possible to use peptides up to 25 amino acid long. Chymotrypsin digestion of ERβ generated a 43 amino acid peptide with S87 and an 8 amino acid peptide with S105. Trypsin digestion resulted in a 33 amino acid peptide encompassing S87 and a 7 amino acid peptide encompassing S105. This preliminary analysis revealed that none of the available enzymes would digest ERβ so that S87 would be contained in a fragment of the length required for MRM. This depends on the protein in the region surrounding S87, and makes the study of phosphorylation of S87 with the MRM approach unfeasible. However, as shown in chapter III, phosphorylation of S87 leads to more modest changes in ERβ function compared to S105, suggesting that phosphorylation at that site is less important \textit{in vivo}. Furthermore, phosphorylated S105-ERβ was detected in
breast tissue samples and inhibited breast cancer cells invasiveness and migration [81]. Together, these data suggested that S105 was the best target for additional proteomics analysis and both high-specificity Chymostrypsin and Trypsin are feasible proteolytic enzymes (Fig. 28). Trypsin is the universal enzyme used in proteomics because it is readily available, economical, and several validated protocols are published. Furthermore, large data sets using mass spectrometry are available online allowing for preliminary identification about whether a protein of interest has been detected using similar techniques and most of the data collected used Trypsin digestion. Analysis of the available data sets showed that human and rat sequences are identical in the N-terminal region containing S105. Also, in vitro phosphorylated human ERβ at S105 was detected following tryptic digestion [81]. In that study, an Orbitrap mass spectrometer was used to detect the phosphorylated form of ERβ, but this was a positive indication that empirical tryptic digestion of ERβ would result in that peptide fragment (i.e. it is not a missed cleavage), and that the peptide can be ionized and detected using a mass spectrometer.
**Fig. 28** Theoretical digestion of rERβ with 8 available enzymes. (PeptideCutter, Expasy.org)
Next, analysis of the predicted ERβ tryptic digestion was done using PeptideMass, an Expasy bioinformatics tool, to analyze the mass of the peptide of interest. Common to most digestion protocols is the treatment of the samples with Dithiothreitol (DTT), a reducing agent, following by Iodacetamide, an alkylation reagent. The first step is necessary to convert cysteine side chains from disulfide bonds into free cysteine side chains. Iodacetamide treatment then alkylates the free sulfhydryl side chains adding a carbamidomethyl group (CAM), ensuring that no free sulfhydryl groups reform. These steps are necessary to allow for Trypsin’s full access to all cleavage sites and results in a 57.021 Da increase in mass to all cysteine containing peptides. Fig. 29, shows that following tryptic digestion of ERβ S105 results in a 7 amino acid peptide: SPWCEAR. The mass of the peptide is 905.3934 Da following CAM modification on the cysteine, which is ideal for detection using a Triple Quadrupole. This same peptide was detected, with the CAM modification, in the Lam et al. study, indicating successful digestion and modification [81].

While commercially available Trypsin is highly efficient, missed cleavage can occur. As explained earlier, missed cleavage probability can be predicted by algorithms created after analysis of all peptides that contain a missed cleavage in a large data repository (PeptideAtlas). Therefore, I tested the probability of missed cleavage upstream and downstream of the SPWCEAR sequence using the missed cleavage predictor program developed by King’s University [214], and the results are shown in Fig. 30. The right column indicates a score from 0-1, with the missed cleavages resulting in a higher score. Both N- and C- terminal cleavage sites have a
relatively low score, a positive indication that digestion with trypsin will successfully yield the target peptide containing S105.

**Figure 29.** Analysis of ERβ tryptic peptides (PeptideMass, Expasy.org).
**Figure 30.** Analysis of Tryptic digestion of ERβ for potential missed cleavage sites. (Missed Cleavage Predictor described in [214]).
2) **Empirical ERβ digestion**

2a) Obtain recombinant ERβ

While theoretical digestion analyses are the basis of all targeted mass spectrometry experiments, empirical digestion has to be performed before moving on with the synthesis of the heavy labelled peptides that will be used for the MRM. In collaboration with Mengjie Zhang, Research Specialist in Dr. Pieter de Tombe’s laboratory, I used an *in vitro* bacterial expression system to obtain large amounts of purified ERβ [215]. First, the rERβ sequence was cloned into the pEXP5-CT/TOPO expression vector, so that a 6histidine tag is co-translated at the C-terminus of ERβ. In this vector, the T7 RNA polymerase necessary for translation of ERβ-His is under regulation of a Lac promoter. Therefore, induction with the lactose metabolite IPTG (Isopropyl β-D-1-thiogalactopyranoside) was required to obtain ERβ-His6. BL21 DE3 competent cells were transformed with the ERβ-His6 plasmid and plated overnight. Five colonies were picked and grown in liquid culture before being split into 2 aliquots. One of the aliquots (B) was induced with IPTG, the other (A), was not induced and served as the control. Following overnight incubation, cells were lysed and protein isolated. Screening of the colonies that most efficiently produced ERβ-His6 after induction was done by analyzing Coomassie stained gels containing the protein. As seen in Fig. 31, IPTG induced samples showed a strong band at the expected 50KDa mass (see arrow), which was not present in the in control (IPTG-free = A) samples. Clone 5 had the highest intensity band, indicating a stronger induction of translation.
of the ERβ-His6 protein, and was chosen for large-scale protein production.

Figure 31. Screening of 5 clones following protein induction with IPTG. Protein visualized with Coomassie staining. Arrows indicate induced protein of the expected molecular weight of ERβ-His6.
Next, protein was isolated from 4 liters of bacterial culture and purified using a nickel affinity column that binds to the His6-tag of the ERβ-His6 protein. After binding to the column and a wash cycle, elution buffer containing Imidazole (which competes for binding to the nickel column) was added and eluted protein fractions were collected. The example in Fig. 32 shows protein fractions 12-21; as the arrow indicates, fraction 19 contained the target ERβ-His6 protein. Western blot analysis of the protein confirmed that both ERβ and the His-tag6 were correctly translated (Fig. 33).

**Figure 32.** Screening of eluted fractions 12-21 after IMAC. Protein visualized with Coomassie staining. Arrow indicates induced protein of the expected molecular weight of ERβ-His.
Figure 33. Representative Western blot staining of protein from the eluted fractions containing putative ERβ based on Coomassie stain. Membrane was incubated with primary antibody targeting N-terminal ERβ confirming expression at expected molecular weight.
2b) Recombinant ERβ digestion

Confirmation of the theoretical and previously published results were then accomplished by tryptic digestion of the recombinant ERβ-His6 protein (obtained by pooling and concentrating IMAC fractions) [81]. First, ERβ-His6 protein was run on a SDS-PAGE gel and the gel band containing ERβ was excised, as well as a portion of the gel void of protein to be used as a negative control (see arrows in Fig. 34). Second, a standard in gel Trypsin digestion protocol was used to clean, reduce and alkylate the protein prior to an overnight incubation with Trypsin (see Methods for details). Digested peptides were then washed out from the gel and concentrated to a smaller volume using a SpeedVac centrifuge. The peptides were then mixed with a matrix (α-Cyano-4-hydroxycinnamic acid, CHCA) which facilities ionization in MALDI-TOF mass spectrometry (Matrix Assisted Laser Desorption ionization-Time of Flight). Briefly, the samples mixed with the matrix are irradiated with a laser, causing the matrix together with the peptides to ionize and vaporize. Using an Axima-CFR Plus MALDI-TOF MS system in “reflecton mode”, the charged ions are reflected using an electrical field and are accelerated in a vacuum. The time of flight to the detector is dependent on the mass of the peptides. Therefore, using calibrants of known mass and measuring their TOF, it is possible to calculate the mass of the sample peptides. I used a 7-point calibration curve and recalibrated the instrument between each sample. Repetitively, I was able to detect several expected tryptic peptides or ERβ, including the peptide of interest containing S105. Fig. 35 is an example of a spectrum acquired as described above. The y-axis shows the normalized % intensity of the ions and the x-axis shows the mass to charge ratio (m/z).
Importantly, only charged ions can be analyzed and detected by the mass spectrometer, hence the need of the matrix and laser bombardment. The red and blue traces represent two separate duplicates of the in-gel digestion products of ERβ-His6 protein. The numbers indicate m/z of ions that were automatically detected by having high intensity. Indeed several of those, and others at lower m/z not showed in this example, match the mass of tryptic products of ERβ. Importantly a peak of 905.4 m/z is present in this example and others; this matches the mass of a singly charged SPWCEAR peptide containing S105. Ions are usually only singly charged in MALDI because it is a milder form of ionization compared to others such as ESI, which will be introduced in the next section.

The empirical digestion of ERβ-His6 protein with Trypsin successfully led to the peptide of interest containing S105. This process confirmed the theoretical results and the empirical results with the human form of ERβ [81]. These steps were necessary to evaluate whether the MRM approach would be feasible and the synthesis of target peptide would be necessary.
Figure 34. ERβ-His6 protein was run on a SDS-PAGE gel in duplicate and stained with Coomassie stain. Gel bands containing the protein of interest and a negative control were excised (See arrows).
Figure 35. Representative MALDI-TOF spectrum of gel digestion products of ERβ-His6.
3) Use target ERβ peptides to develop the MRM

The target SPWCEAR peptides required for development and validation of AQUA-MRM were synthesized by ThermoFisher Scientific with heavy Arginine which is 10 Da heavier due to the presence of C13 and N15 isotopes. Phosphorylated peptides were also synthetized in the light and heavy version (Fig. 36).

<table>
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<tr>
<th>Posno</th>
<th>Well_ID</th>
<th>Barcode ID</th>
<th>Peptide Name</th>
<th>Solvent</th>
<th>Nterm</th>
<th>Peptide Sequence</th>
<th>CTerm</th>
<th>MW</th>
<th>Amount [mg]</th>
<th>Volume[µL]</th>
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<tr>
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<td>A1</td>
<td>191257113</td>
<td>KS35084.1 ERB-light</td>
<td>50% ACN / 0.1% TFA</td>
<td>[H]</td>
<td>SPWCEAR</td>
<td>[OH]</td>
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<td>400</td>
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<td>50% ACN / 0.1% TFA</td>
<td>[H]</td>
<td>[SPWCEAR][S]</td>
<td>[OH]</td>
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<td>3.1</td>
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</tr>
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<td>SPWCEAR(R)</td>
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**Figure 36.** Characteristics of the four SPWCEAR peptides purchased.
To develop a reliable MRM method, each peptide has to be tested for both its ionization and fragmentation characteristic. After electrospray the peptides can be multiply charged and the most prevalent ion form needs to be selected in Q1. Then the ion has to go through CID and the full spectrum of the product ions scanned. From all the product ions that are generated after dissociation, the most prevalent will be selected in Q3 in the MRM method.

The predicted y- and b- product ions of each peptide, can be obtained using bioinformatics tool such as MS-product, a program developed by the University of California. Fig. 37 shows the precursors and products m/z for the light and heavy SPWCEAR peptides. Note that the heavy peptide is 10m/z heavier that the light form in both the precursor and product ions that contain the heavy arginine.
**SPWCEAR**

User AA Formula 1: C2 H3 N1 O1
Elemental Composition: C36 H54 N11 O11 S1

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<tr>
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<th>MH⁺&lt;sup&gt;2&lt;/sup&gt;(av)</th>
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[←] Main Sequence Ions

b       y       y<sup>+2</sup>
---      1 S 7    ---      ---
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371.1714 3 W 5 664.2872 332.6472
474.1806 4 C 4 478.2078 339.6076
603.2232 5 E 3 375.1987 188.1030
674.2603 6 A 2 246.1561 123.5817
---      7 R 1 175.1190  88.0631

**SPWCEAR(+10)**

User AA Formula 1: C2 H3 N1 O1

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</table>

[←] Main Sequence Ions

b       y       y<sup>+2</sup>
---      1 S 7    ---      ---
185.0921 2 P 6 771.3399 386.1736
371.1714 3 W 5 674.2872 337.6472
474.1806 4 C 4 488.2078 344.6076
603.2232 5 E 3 385.1987 193.1030
674.2603 6 A 2 256.1561 128.5817
---      7 R(+10) 1 185.1190 93.0631

*Figure 37.* Predicted precursor and product ions for the light and heavy unphosphorylated SPWCEAR peptide.
A full scan analysis of each peptide, with no dissociation induced, shows which charged species are generated for each peptide. The spectrum in Fig. 38 was collected from a directly infused short unphosphorylated light peptide. As indicated by the arrows, both the 1+ and 2+ species are generated. Because of the double charge the m/z of the 2+ species is equal to 1/2 of the ion’s mass. The most prevalent species was the 2+ ion, and was selected in Q1 for the MRM experiment. For all four peptides the doubly charged ion was the most prevalent following electrospray ionization, therefore the 2+ ions was selected for fragmentation.

The subsequent step used Q3 to detect the full spectrum of product ions derived from dissociation of the 2+ precursor ions. Representative spectra of the light and heavy unphosphorylated peptides are shown in Fig. 39. As indicated by the arrows, several of the predicted product ions were detected.
Figure 38. Spectrum for the SPWCEAR peptide. Arrows indicate the 1+ and 2+ species.
Figure 39. Spectra detected in Q3 showing product ions of the 424.66 (above) and 429.63 (below) precursor ions, the 2+ ion of the light unphosphorylated light (above) and heavy (below) peptides. Arrows indicate the m/z values that match the predicted products (for a list see Fig. 37).
Collision energy (CE) applied in Q2 to induce ion dissociation must be optimized in order to determine that all of the precursor ions are dissociated into product ions. Optimal CE can be theoretically calculated, but then must be empirically verified. My results showed that the phosphorylated peptides required slightly higher energy for a complete dissociation, due to their larger size because of the 79Da addition of the phosphate, however efficient dissociation was obtained with similar CE.

In AQUA-MRM experiments, confident detection and quantification is best achieved through the use of four transitions (precursor and product ions pairs). In addition, the MRM method must be designed to target the CAM modified peptides to mimic the true MRM experiment, in which the peptides go through the trypsin digestion protocol. The four transitions that were highly reproducible and were chosen for the final MRM design are listed in Fig. 40.
<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Peptide Modified Sequence</th>
<th>Ion Formula</th>
<th>Precursor Mz</th>
<th>Precursor Charge</th>
<th>Product Mz</th>
<th>Fragment Ion</th>
<th>Product Charge</th>
<th>Cleavage Aa</th>
</tr>
</thead>
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<tr>
<td>ERBETA</td>
<td>SPWC [+57.1] EAR</td>
<td>C38H56N12O12S</td>
<td>453.504846</td>
<td>2</td>
<td>721.30622 y5</td>
<td>1</td>
<td>W</td>
<td></td>
</tr>
<tr>
<td>ERBETA</td>
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<td>453.504846</td>
<td>2</td>
<td>535.229507 y4</td>
<td>1</td>
<td>C</td>
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<tr>
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<td>453.504846</td>
<td>2</td>
<td>375.19865 y3</td>
<td>1</td>
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<td>453.504846</td>
<td>2</td>
<td>246.150606 y2</td>
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<td>ERBETA</td>
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<td>458.469178</td>
<td>2</td>
<td>731.316880 y5</td>
<td>1</td>
<td>W</td>
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<td>458.469178</td>
<td>2</td>
<td>256.164335 y2</td>
<td>1</td>
<td>A</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 40.** List of transitions chosen for each target peptide.
4) Develop LC-MRM method

The initial development of the MRM established the ionization and fragmentation characteristics of the pure peptides directly injected in the mass spectrometer by-passing the LC stage. However the final experiment is based on the ability of the endogenous (light) and exogenously added heavy peptides to be detected in a complex tissue protein sample following liquid chromatography. Therefore the LC step of the experiment must be optimized to determine the elution time of the peptide (i.e. retention time). During a LC-MRM method, the instrument cycles through the list of defined transitions for the duration of the program. Using the Skyline software, created by the MacCoss lab at the University of Washington, the transitions acquired across time can be easily viewed. To first test the LC-MRM method, I mixed equal amounts of each peptide to detect each retention time and tested whether the transitions observed with the pure peptides were reproducible.

Fig. 41 shows a screenshot of the Skyline software analysis of the LC-MRM of the sample mix. On the left panel, each transition is highlighted and assigned a different color. The chromatogram shows a zoomed in window of the retention times at which the peptides eluted. The green and light orange traces represent the unphosphorylated light and heavy peptides which co-elute. This result confirmed that the incorporation of the heavy arginine does not affect retention time. The red traces represent the phosphorylated peptides which also co-eluted as expected. Each peptide should only elute once, when the amount of organic solvent in the mobile phase is enough for the peptide to come off the C18 column and elute.
Figure 41. Chromatograph of the 4 pure peptides after LC-MRM program. On the x-axis is retention time, the y-axis is the signal intensity. For color legend see panel of the left. (Skyline software, MacCoss lab)

Analysis of the intensity of each transition is also possible, and the prevalence of each product ion should be consistent across replicates. Fig. 42 shows the four transitions monitored for the light unphosphorylated peptide in separate runs of the peptide alone (on the left) and mixed with the other peptides. As expected the pattern of each transition is conserved, with the 245.15 product (in orange) being the more intense in both samples.
Figure 42. The four transitions monitored for the light unphosphorylated peptide are shown in a run of the peptide alone (on the left) and mixed will all other peptides. Retention time and prevalence of each transition are conserved.
After optimization of the LC-MRM and establishment of retention time for each peptide, a standard curve will be prepared using the heavy labeled peptides. LC-MRM of serial fold dilutions of the unphosphorylated and phosphorylated heavy peptide will be used for the standard curve. Using the Xcalibur software, the intensity of the area under the curve of each dilution will be plotted and fit to a linear curve. The standard curve will be used for quantification of light (endogenous) peptide in the experimental tissue samples.

**Perform MRM with experimental samples**

The LC-MRM method developed above will be used to measure the ratio of ERβ that is phosphorylated at S105 in the brain and heart of animals that underwent the estrogen deprivation paradigm. A known amount of the heavy peptide will be spiked with the tissue sample and, following LC-MRM, the ratio of the intensity of the endogenous peptide and the added heavy peptide will be calculated for both the phosphorylated and unphosphorylated peptides.

**Comparison of trypsin digestion of the unphosphorylated and phosphorylated peptide fragment.**

The S105 of interest is directly downstream of the lysine residue recognized by trypsin in the cleavage site. It is possible that trypsin might not recognize and, therefore cleave, as efficiently when S105 is phosphorylated. To evaluate this possibility, I designed a longer peptide with 7 additional amino acids upstream of S105 (Fig.43). The heavy LYAEQPQKSPWCEAR peptide was synthetized with and without phosphorylation of S105 and then tested using the same digestion parameters as the shorter peptide. The
results demonstrated that the heavy phosphorylated peptide was cleaved after successful digestion with trypsin (Fig. 44, left panel) and the retention time and most prevalent product ions were similar to the short peptide (Fig. 44, right panel).

Figure 43. The blue box highlights the longer peptide synthesize to study whether phosphorylation at S105 affects recognition and cleavage by Trypsin.
Figure 44. The long phosphorylated ERβ peptide (left) is successfully cleaved and yields transitions that match the short heavy phosphorylated peptide (right).
DISCUSSION

In this chapter I have delineated the development of a LC-MRM experiment that can be used to detect and quantify phosphorylated ERβ. Optimization of the LC-MRM method is necessary before moving on to assaying the tissue samples, however I am confident that this experiment is the best approach to answer the fundamental next questions in this study. I have already demonstrated that phosphorylated ERβ is functionally distinct and alters downstream gene expression compared to the unphosphorylated form of the receptor (Chap. 3). Moreover, these functional differences are sensitive to the presence or absence of E2 in the cell, raising the possibility that ERβ differentially alters gene expression in postmenopausal women. Adding to this complexity, the activation of kinases that putatively phosphorylate ERβ is differentially altered by age and E2 in the brain and the heart (Chap. 4), thereby providing a mechanism for differential phosphorylation of ERβ with advanced age. The next step is to identify phosphorylated ERβ in the brain and heart of aged rats, and quantify changes in phosphorylation based on age and circulating levels of E2. These data will allow us to predict whether this functionally important modification is changed following longer deprivation of estrogen, and potentially is a basis for explaining the discrepant effects of E2 replacement therapy in postmenopausal women.
CHAPTER VI
FINAL DISCUSSION
SUMMARY OF KEY FINDINGS

The aim of this dissertation was to uncover potential molecular mechanisms of the age related switch of estrogen treatment post menopause. The project stemmed from the clinical observations brought about from the Women’s Health Initiative study and the Timing Hypothesis. Important findings of the WHI were that the neurological and cardiovascular systems specifically benefit from E2 administration closely in time to the menopausal transition.

The underlying hypothesis of this dissertation was that age and estrogen deprivation lead to altered kinase activation which could result in altered phosphorylation of ERβ in the brain and heart.

The first goal was to test whether phosphorylation on ERβ would alter its activity as a transcription factor in neurons, where estrogens can be beneficial. Indeed the data presented in Chapter III show that phosphorylation of ERβ increases its activation of transcription at ERE sites, while abolishing the ligand independent inhibition of AP-1 sites (see Fig. 45). These represent two very different modalities of ERβ activity as a transcription factor; the first requiring direct binding to the DNA at ERE sequences, the second by interacting with other transcription factors at the AP-1 sequences on the DNA.
Figure 45. Phosphorylation of ERβ at S105 increases ERE dependent transcription (above) and abolishes ligand independent inhibition of AP-1 sites (below).
The possibility that phosphorylation of ERβ alters its ability to bind to
the DNA at ERE sequences was also tested in Chapter III. The results
showed that ERE binding is not altered when ERβ is phosphorylated in the N-
terminal domain which is not known to participate in this interaction. However,
the N-terminal domain (specifically S87 and S105) is important in mediating
interactions with coregulatory proteins and participates in homo and
heterodimerization.

The Timing Hypothesis relates to the whole organism physiology,
therefore aging and estrogen deprivation need to be investigated in vivo. The
animal paradigm used in this study was designed to investigate the findings of
the WHI study and the Timing Hypothesis. Briefly, aged female rats were
ovariectomized to remove endogenous sources of estrogens. E2 or vehicle
was administered after different lengths of time following the surgery. This
design was chosen due to the clinical studies which showed that the
beneficial effects of E2 depend on how long after menopause women started
ET. The initial hypothesis that MAPKs are differentially activated depending
on age and estrogen deprivation could be tested in this model. Indeed,
measuring the activation of p38 and ERK, members of the MAPK family,
revealed a clear interaction between length of estrogen deprivation and
estrogen treatment. Namely, the length of time between ovariectomy and
beginning of treatment resulted in altered effects of E2 on the activation of
MAPKs. Among the striking results described in Chapter IV, are the clear
brain region-specific and kinase-specific effects detected. Also, the factors of
length of estrogen deprivation and E2 treatment showed significant
interactions, indicating that different effects of E2 treatment were detected depending on the time at which it was administered.

Additionally, in Chapter V this dissertation provides the description of a novel method for detecting and quantifying phosphorylated ERβ \textit{in vivo}. The mass spectrometry technique described has been successful for the detection of lowly expressed proteins but has never been attempted to study ERβ. Indeed, because of the difficulties experienced in the use of antibody based assays, the MRM method described offers a reliable alternative.

Taken together, the data presented in this dissertation demonstrate that alternative regulation of MAPKs signaling in the brain and heart could provide a novel mechanism explaining the variable effects of E2 following menopause. Furthermore, phosphorylated ERβ was detected in the heart and brain and the consequences of phosphorylation of ERβ on its regulation of transcription were determined.
PHOSPHORYLATION OF ERβ AND LIGAND INDEPENDENT ACTIONS

The findings presented in Chapter III add to the renowned complexity of ERβ signaling. ERβ has been found to function completely differently from its cognate steroid receptor ERα. ERα binds to DNA and regulates transcription only in the presence of a ligand, as has been shown by ChIP-seq experiments in breast cancer cells [216]. Conversely, ERβ can bind to EREs in absence of ligand, with microarray data showing that ERβ regulates transcription of three classes of genes [70, 148, 217]. The first and larger class of genes was regulated by unliganded ERβ. The second class of genes was regulated only in the presence of ligand, and genes in the smaller third class were regulated by ERβ both in presence or absence of ligand. Interestingly, several of the Class I genes in the Vivar et al. study were enriched for AP-1 sites, demonstrating that unliganded ERβ targets genes under AP-1 regulation. This is important to consider in light of the results shown in Chapter III that the ligand independent inhibition of AP-1, which is characteristic of ERβ, was abolished when either phosphorylation site (S87 or S105) were mutated. This points to the possibility that the N-terminal domain is involved in ERβ interaction with a coregulatory protein or directly with transcription factor Jun/Fos that binds to the AP-1 DNA sequence. Mutation of either S87 or S105 abolished the ligand independent inhibition of AP-1 sites, indicating that the genes that Vivar et al. identified as Class I would not be regulated by phosphorylated ERβ. This could be a potential way of directing ERβ towards other sets of genes that do not have AP-1 sites in their promoters. Indeed we found that phosphorylation of ERβ at S105 increased ERE dependent transcription both in presence or absence of ligand. It is
possible that when unphosphorylated, ERβ regulates both Class I and Class II genes but after phosphorylation it is limited to Class II, ERE genes.

As estrogen levels are low after menopause, ERβ is constantly functioning unliganded causing regulation of only Class I genes. Therefore it is possible that following menopause, in absence of estrogen, phosphorylation of ERβ would determine the set of genes it regulates. This modification would have critical consequences on ERβ signaling at a time when estrogen is low.

PHOSPHORYLATION OF ERβ AND THE DYNAMIC STRUCTURE OF THE N- TERMINAL DOMAIN

The dynamic structure of ERβ can also be the cause of the profound consequences of mutations of S87 and S105. The ligand binding domain and DNA binding domains of ERs have been described via X-ray crystallography in complex with ligands and ERE sequences respectively [218-224]. However, the three dimensional structure of the N-terminal domain of ERβ, along with many other nuclear receptors, has not yet been resolved. Indeed it has been shown that the AF-1 domains of nuclear receptors possess an intrinsically disordered (ID) conformation [225, 226]. The lack of particular structures has been attributed as the cause for the ability of nuclear receptors to interact with a variety of partners by easily changing structure to adapt and bind to a partner when the cellular milieu promotes that interaction. Both ERα and ERβ have an intrinsically disordered nature, however analysis of predicted secondary structural elements in the ER N-terminal domain shows that ERα has more α-helix and β-sheet elements than ERβ. The N-terminal domain of ERα contains 67% random coil conformation, whereas more than 80% of ERβ is unstructured. The possibility that an unstructured part of a transcription
factor becomes structured following interaction with a component of the transcriptional machinery or a coregulatory has been shown for nuclear receptors such as the glucocorticoid receptor and progesterone receptor [227-229]. This is true for ERα as well, which by interacting with TBP (TATA binding protein) loses its disordered characteristics and becomes folded as shown by surface plasmon resonance and circular dichroism spectroscopy [225]. The same study was not able to replicate the experiments performed with the N-terminal domain of ERα with ERβ. This was attributed to the impossibility of ERβ to interact with TBP or the need for other proteins that form the transcriptional complex.

It is also possible that phosphorylation alters the intrinsically disordered nature of the N-terminal domain and determines the binding partners of ERβ. Much of what we know about the structure of the N-terminal domain of ERβ comes from sequence analysis prediction. The “Network Protein Sequence Analysis” for example, is a web service that was developed by a biocomputing team from analysis of sequence and structure information available in large databases [230]. Analysis of the N-terminal domain of ERβ with a prediction model shows that the biggest majority of it is unstructured and made of random coil (see Fig. 46) [231]. Strikingly the amino acids close to S87 form a short α-helix right next to an extended strand region, followed by a random coil and another short α-helix following S105. Intuitively, it is plausible to think that even a small change in this sequence at the interface of unstructured regions and short helices might result in large effects on the structure of the region. Furthermore, a modification such as phosphorylation that brings the addition of a negative charge could also result in a
conformational switch in the area. Changing S87 or S105 into Glutamic acid (E) would mimic the effect of the phosphate, as experimentally done in Chapter III, and could help obtain a prediction of the structural conformation of the N-terminal domain of ERβ following phosphorylation. Indeed when S87 was changed into Glutamic acid, it destabilized the short α-helix close to the extended strand region and turned it into a random coil. Changing S105 into S105E had the opposite effect. Even if S105 is further away from the short helix than S87, S105E induces a conversion of the random coil into a helix (Fig. 46). This extends the α-helix from 5 to 8 amino acids; a longer α-helix could limit the dynamic movement of the region and direct ERβ towards interacting with one binding partner rather than another. As shown in Chapter III, phosphorylation of ERβ at S105 increased the ligand independent and ligand dependent activation of ERE dependent transcription which could result from the stabilization of the α-helix structure and the recruitment of the transcriptional machinery.

It has been proposed that the unstructured and dynamic status of the N-terminal domain of ERβ is at the base of its heterogeneous function [210, 226]. Phosphorylation in that region could result in different structural conformation leading ERβ to interact with a certain binding partner and therefore towards a specific function.
Figure 46. Network Protein Sequence Analysis of the N-terminal domain of ERβ shows the conformational changes potentially induced by phosphorylation [230].
PHOSPHORYLATION OF ERβ AND THE INTERACTING PROTEINS

The interactome of ERβ is vast, depending on the tissue under analysis, and can be altered by hormone treatment in vitro [86, 87]. Indeed ERβ interacting proteins in the ventral hippocampus were altered by aging and E2 as shown by a study from our group [86]. It is possible that the observed changes in ERβ interacting proteins in E2 treated versus vehicle treated animals might be due to ERβ phosphorylation. As described above, phosphorylation could deeply alter the structure of the N-terminal domain which is known to mediate interactions with several proteins such as coregulators or TATA-binding proteins [128, 225, 232]. Furthermore, as described in Chapter IV, E2 treatment in the ventral hippocampus inhibits both ERK and p38 activity. This could cause decreased levels of phosphorylated ERβ and altered recruitment of interacting proteins, which serves as a potential explanation for the changes detected in the previous study. Many of the interactions of ERβ are transient and make determining the effect of phosphorylation on these interactions difficult. An alternative approach to determine how phosphorylation of ERβ alters its interactome is using proteomics techniques such as APEX and BioID [233, 234]. These techniques are based on biotinylation of proteins proximal to the protein of interest which is tagged with a biotinylating enzyme. All biotinylated proteins have therefore interacted with the protein of interest and can be isolated using affinity chromatography. Mass spectrometry analysis is then used to reveal protein identity. While this is an in vitro approach to study if phosphorylated ERβ interacts with different proteins, it would potentially explain some of the
observed differences in its function.

MAPKS IN THE AGING FEMALE BRAIN AND HEART

Aging can be defined as “a progressive, generalized impairment of function, resulting in an increased vulnerability to environmental challenge and a growing risk of disease and death” [235]. Aging is a continuous process affecting the whole organism which is changed at all levels, from the molecular and cellular level to tissue and system levels. In women, reproductive aging is the decline in fertility that ends at menopause, when levels of circulating estrogens are low. The decline of circulating estrogens is accompanied by a decline in health and rise in neurological and cardiovascular disease [56, 58]. It is plausible to think that the concurrent aging and estrogen decline together lead to a continuous change in the brain and heart. Indeed the effects of ET are different depending on how long following menopause they are administered, indicating that something in the cellular milieu has changed. It was shown that there is a critical window at which estrogen can be protective, but the molecular basis for this change is unknown. It is also not understood whether there is one critical window for all systems or if estrogens might be beneficial for a larger span of time in one tissue or another. It was proposed that estrogen action has a “healthy cell bias”, meaning that if cells are healthy at time of estrogen exposure their response to estrogen will be beneficial [236]. In contrast, if estrogen is administered to a cell that is not healthy, estrogen will exacerbate the cellular stress.
The results shown in Chapter IV support the idea that the effects of estrogen treatment depend on the time of administration. Furthermore there is a clear difference between the results obtained in the heart and in the brain. In the brain E2 inhibited ERK activity; in the hypothalamus this was true at all times post OVX, in the hippocampus E2 was only effective 1 week post OVX. In the heart, however, E2 did not alter ERK or p38 at any time point. This differential regulation of MAPKs signaling could be indicating that the critical window of time is different for the brain and the heart, and indeed these two organs age differently. The brain is very sensitive to aging which is the biggest risk factor for neurodegenerative disease. The aging brain is characterized by underlying oxidative stress, neuroinflammation, glial activation, impaired protein processing and dysfunctional mitochondria [237]. The cardiovascular
system is also affected by aging which leads to atherosclerosis, thickening and loss of elasticity of the arterial walls, hypertrophy of the heart, and fibrosis [238].

A POTENTIAL ROLE FOR PROTEIN PHOSPHATASES

Protein phosphorylation is a reversible and transient mark placed by kinases and removed by phosphatases. These enzymes are part of a network that regulates cell signaling pathways and downstream effector proteins. In the last decade researches have started to focus their studies on the phosphatases that are responsible in removing the phosphorylation mark placed by kinases as a potential tool for drug development [239]. Indeed kinases are the target of several common medications for diseases such as diabetes, obesity, cancer etc. yet fewer phosphatase modulators are currently available [239]. Phosphatases can be broadly categorized based on their substrate recognition as protein Serine/Threonine phosphatases (PSTPs), Tyrosine phosphatases (PTPs) or dual-specificity phosphatases. Interestingly, phosphatases have been shown to be differentially expressed and activated by aging and hormonal status similar to many kinases [240-244]. A 2015 study showed that in primates aging significantly inhibited protein phosphatase 2A (PP2A) and consequently increased α-synuclein phosphorylation and oligomerization [243]. Similarly, STEP (Striatal-enriched Tyrosine phosphatase) is less active in the aged rats in several brain regions [242]. The activity of STEP has also been linked to age-associated neurologic disorders possibly because it is regulated by oxidative stress. Furthermore, the hormonal regulation of phosphatases is complex as previously discussed
for kinases. It was shown that both PSTPs and PTPs can be regulated by estrogen in healthy and cancer cells [244-248].

The specific phosphatases that target ERβ’s S87 and S105 have not been investigated, but *in vitro* experiments could specifically identify which enzymes are responsible for removing the PTM placed by the MAPKs. It is plausible that these phosphatases are regulated by age and estrogen similarly to the results shown in Chapter IV for the MAPKs p38 and ERK. While it was not a focus of this dissertation, Tyrosine 36 has been recently identified as a phosphorylation site of ERβ [249]. Using *in vitro* and *in vivo* approaches T36 phosphorylation was identified as a switch in determining the antitumor activity of ERβ in breast cancer. Indeed, phosphorylation of T36 was necessary for recruitment of coregulatory proteins at promoters of antitumor genes. Furthermore, the phosphatase EYA2 was shown to remove the phosphate mark placed by the kinase c-ABL. This is the first example of a well-defined signaling circuitry that regulates ERβ’s antitumor activity based on a specific phosphorylation mark.

Identifying the phosphatases responsible for removal of phosphorylation at S87 and S105 and measuring its changes with age and estrogen are promising avenues that would complement and strengthen the data presented in Chapter IV.
MITOCHONDRIAL DYSFUNCTIONS WITH AGE AND ESTROGEN EFFECTS

Known targets of estrogen regulation are mitochondria [250-255]. Interestingly mitochondrial dysfunction is common with age, and the “Mitochondrial theory of aging” proposes that the large amount of reactive oxygen species (ROS) produced in the mitochondria makes them a prime target for oxidative damage, which in turns decreases mitochondrial function and activates signaling pathways such as MAPKs [235, 256]. The three factors of aging, estrogen signaling and mitochondria are clearly intertwined and likely play an important role in the insurgence of the neurological and cardiovascular diseases associated with aging in women.

Estrogen can regulate mitochondrial function both in a genomic way, by regulating transcription of mitochondrial proteins, and in a non-genomic way by regulating signaling pathways such as MAPKs that converge on mitochondria. Interestingly, two studies have looked at the mitochondrial proteome of the heart or the brain following estrogen treatment or estrogen deprivation via ovariectomy [257, 258]. The majority of the proteins that were altered by estrogen were involved with regulation of oxidative stress, apoptosis, and electron transport complex proteins. Furthermore, the respiratory rate and activity of respiratory chain enzymes were increased in brain mitochondria 24 hours following E2 treatment, indicating the possibility for either a genomic regulation of transcription of genes in those functions or a non-genomic activation of signaling pathways that led to regulation of mitochondrial respiration [259].
Many of the beneficial effects of estrogen on mitochondria are thought to be due to ER dependent transcription of NRF-1, nuclear respiratory factor-1 [260]. NRF-1 is a transcription factor that regulates transcription of mitochondrial genes in the nuclear DNA. Interestingly, the NRF-1 promoter has ERE and AP-1 sites in its promoter and both ERα and ERβ can induce its transcription [260, 261]. In breast cancer cells, it was found that unliganded ERβ represses the NRF-1 promoter via the AP-1 site, but E2 or Tamoxifen treatment relieves this repression [261]. An in vivo study E2 treatment in ovariectomized rats determined an increase in NRF-1 and several mitochondrial protein levels and enzymatic activity in mitochondria isolated from cerebral blood vessels [262]. The authors conclude that vasoprotection by estrogen treatment might be due to the measured improved mitochondrial function, improved respiration and reduced production of ROS.

The non genomic effects of estrogens on mitochondria often include MAPKs signaling regulation. In in vitro experiments of human lens epithelial cells, E2 treatment reduced ROS produced by the mitochondria by activating ERK signaling which in turn stabilized the mitochondrial membrane potential [263]. Similarly, in cultured hippocampal neurons, brief E2 treatment reduced toxicity of elevated Glutamate which usually results in elevated Ca^{2+} [264]. Instead, brief E2 treatment increases the storage of Ca^{2+} in mitochondria and an elevation of the antiapoptotic gene Bcl2 [264]. In an in vitro model of AD, treatment with E2 inhibited the amyloid induced translocation of the proapoptotic protein Bax to the mitochondria and increased the antiapoptotic Bcl2 levels [265].
This estrogen regulation of mitochondrial function via MAPKs has been identified in vivo as well. Exercise-induced myocardial hypertrophy is a physiological response to exercise that does not lead to fibrosis and is reversible. Interestingly females develop larger physiological hypertrophy which is proposed to contribute to the more favorable remodeling in response to pathological stimuli seen in females [266]. It was shown that exercise in females induces activation of ERK and p38 which phosphorylate transcription factors that activate expression of mitochondrial proteins, leading to increased mitochondrial mass to better cope with the increased size of myocytes. This process was not present in ERβ knockout mice, indicating that ERβ is necessary for the activation of MAPKs and improved response to exercise [266].

As shown above, often the non-genomic effects of estrogen on mitochondrial function require activation of MAPKs which does not correlate with the results from our deprivation paradigm. In the hypothalamus, for example, E2 treatment strongly inhibited ERK at all time points. Does that mean that mitochondrial function is repressed in these samples? It is difficult to predict, because of the complexity of ERK signaling. However, if mitochondrial function was indeed reduced this could mean that the window for estrogen’s protective effects is closed in the hypothalamus. Interestingly in this brain region, longer deprivation caused stronger inhibition of ERK, possibly leading to an even worse phenotype. This could be an example of the “healthy cell bias”: longer deprivation from estrogen and aging could lead to unhealthier cells which more drastically respond to E2 treatment. In the heart both ERK and p38 were decreased by prolonged estrogen deprivation
and E2 treatment did not rescue this phenotype. This could again determine a 
failed protective response by E2, potentially due to altered cellular milieu. 
Because many of the protective effects of estrogen on mitochondrial functions 
are mediated by genomic regulation, it would be important to determine 
whether the NRF-1 transcription factor or the mitochondrial proteins known to 
be transcribed following estrogen treatment are increased in the E2 treated 
animals.

In conclusion, the role of mitochondria in the beneficial effects of 
estrogens is known and could be a mechanism by which aging and estrogen 
together lead to time dependent effects.
FUTURE DIRECTIONS

The studies presented here contribute to the understanding of estrogen receptor signaling and kinase regulation in the context of menopause. Importantly, these data support the ideas that post translational modifications alter ERβ’s functions and that the length of estrogen deprivation alone can affect the intracellular milieu of the aged brain and heart.

There are many new possible lines of investigation brought about from these findings. First, the *in vitro* evaluation of the effects of ERβ phosphorylation on its activity as a transcription factor was a necessary starting point. While the abolished ligand independent activity of ERβ by mutation of either phosphorylation site was striking, the data presented do not provide an explanation for this change. The idea proposed above, that phosphorylation alters ERβ’s network of interactions, is compelling also due to the dynamic structure of ERβ near its phosphorylation sites. Using an *in vitro* approach such as BioID or APEX, the consequences of ERβ phosphorylation on its interaction with other proteins could be investigated [233, 234].

Furthermore, the phosphorylation status of ERβ *in vivo* needs to be determined. The proteomics approach described in Chapter V will conclusively determine how phosphorylation of ERβ changes *in vivo*. This is crucial because of the results of Chapter III; namely ERβ’s regulation of transcription is altered by phosphorylation. Furthermore, in light of the results of Chapter IV, it is likely that phosphorylation of ERβ will be decreased in the brain and heart following E2 treatment, because ERK and p38 kinase were overall depressed.
The other potential for investigation that is opened by these studies is the possibility that the altered MAPKs signaling after prolonged estrogen deprivation might result in mitochondrial dysfunctions. Indeed, I would hypothesize that following prolonged estrogen deprivation mitochondrial function in the brain and the heart would be reduced. Measuring mitochondrial function in the deprivation paradigm used here could be a meaningful assessment of cellular health. Furthermore, in vitro studies have shown that activation of MAPKs signaling is necessary for estrogen’s positive effects on mitochondrial function. According to the data shown in Chapter IV, however, ERK and p38 were in general not activated by E2, suggesting that either MAPKs signaling is differentially regulated in vivo or potentially that the window of opportunity is closed in these tissues. Finally, the beneficial effects of E2 on mitochondrial function can be determined by increased NRF-1 levels which could be measured in the brain and heart in our deprivation paradigm. If the beneficial effects of E2 are mediated via the genomic pathway, NRF-1 will be increased, resulting in higher levels of mitochondrial proteins necessary for efficient mitochondrial respiration.
CHAPTER VIII
BROAD CLINICAL IMPLICATIONS
CLINICAL POTENTIAL OF ERβ

The Timing Hypothesis that resulted from the Women’s Health Initiative study is at the base of this dissertation work. Because of the age dependent effects of estrogen treatment in post-menopausal women physicians are often uncertain on whether it is a good option for their patients. Furthermore, the activation of ERα in the uterus or breast with estrogen treatment could lead to proliferation and cancer. This is often a concern of physicians and patients who decide to avoid ET. Therefore, ERβ selective agonists have been evaluated in the past 10 years, because ERβ does not induce reproductive tissue proliferation and is active in non-reproductive organs such as the brain and heart. For its neuroprotective and cardioprotective roles, ERβ is a potential target of regulation for age and menopause related diseases.
Figure 48. Potentials for ERβ specific treatment in post-menopausal women
NEED FOR BETTER UNDERSTANDING OF ERβ SIGNALING

A potential pitfall of using ERβ specific ligands in the clinic is that many aspects of ERβ signaling are still unknown. The characteristic ligand independent activity of ERβ in activating ERE dependent transcription and inhibiting AP-1 dependent transcription remains mostly unexplained. Indeed ERβ can interact with coregulatory proteins or transcription factors, bind to DNA and regulate transcription in absence of ligand. Furthermore while classically steroid receptors only translocate to the nucleus following ligand binding, ERβ can be found in the nucleus or other subcellular locations in absence of ligand. It is thought that the ligand independent functions of ERβ are particularly important after menopause when estrogens are low. Taking advantage of the functional and structural differences between ERα and ERβ several receptor specific ligands (SERMs) have been developed. ERβ specific botanical compounds known as phytoestrogens had been used for centuries in Chinese medicine. Different formulations of phytoestrogens have been tested in preclinical and clinical studies and have been deemed viable options for postmenopausal symptoms such as hot flashes, decreased memory function and menopause associated neurological decline [267-270]. Several phytoestrogens can bind to both ER isoforms, but selectively act on ERβ to induce recruitment of coregulatory proteins and activate transcription [271]. This selectivity ensures that ERα in reproductive tissues such as the breast and uterus is not activated and does not lead to proliferation and possibly cancer [271]. An approach used to limit ERα activation in postmenopausal women with breast cancer is to use Aromatase inhibitors [272]. Inhibition of aromatase leads to decreased circulating estrogens and reduced ERα
activation. Because ERβ can act in absence of ligand, use of Aromatase inhibitors could reduce endogenous estrogens thereby highlighting ligand independent actions of ERβ. More agreement on the beneficial effects of ERβ’s activation post menopause is leading to development of pharmaceuticals that take advantage of the growing understanding of ERβ’s to improve post-menopausal women’s life.

Clinical and basic science studies have evaluated the use of SERMs on ERβ; however it is possible that following phosphorylation of ERβ treatment with SERMs does not lead to the same effects. Indeed in Chapter III I show that phosphorylation of ERβ alters its E2 dependent and independent regulation of transcription in neurons. It is a possibility that treatment with other ligands could have alternate effects. The results in Chapter IV show that MAPKs are differentially activated following estrogen deprivation and E2 treatment which could correlate with altered levels of ERβ phosphorylation *in vivo*. While it is difficult to speculate on the physiological consequences of altered levels of phosphorylated ERβ in the brain and heart, *in vitro* studies point out that a big effects would be the abolished ligand independent inhibition of AP-1 dependent transcription. This would result in increased transcription of the genes that are usually repressed by ERβ in absence of ligand when ERβ phosphorylation is high. The other important effect of ERβ phosphorylation was the increased activation of ERE dependent transcription. Taken together, increased phosphorylation of ERβ *in vivo* due to increased MAPKs activity would lead to a shift in its action from the inhibition of AP-1 to the activation of ERE.
A further complicating factor is that while only ERβ1 transcriptional regulation was tested in Chapter III, several splice variants exist. Interestingly all splice variants still include the N-terminal domain, therefore could be phosphorylated as ERβ1 is. Furthermore the human ERβ splice variants are truncated in the C-terminal domain, therefore lack the ligand binding domain. These ERβ splice variants can regulate transcription in absence of ligand, indicating that the N-terminal domain is necessary for its constitutive activity [70]. A recent study by our group detected ERβ2 splice variant, which has an insertion of 18 amino acids in the ligand binding domain, in the brain regions analyzed in Chapter IV [273]. Therefore it is possible that both ERβ1 and ERβ2 are differentially phosphorylated correlating with the altered MAPKs activation measured. Indeed more understanding on the dynamics of ERβ splicing and phosphorylation in the context of aging and estrogen deprivation might uncover a potential target for regulation. Drugs targeting splicing and phosphorylation are available, therefore understanding which splice variant and phosphorylation status are optimal for ERβ to have most benefits would be highly important for physicians.

CLOSING REMARKS

My findings shed light on the molecular characteristics of phosphorylated ERβ as well as highlighting the potential contribution of MAPKs signaling in the brain and heart in response to estrogen deprivation and E2. As predicted, I found that the cellular milieu of the aged brain and heart is altered by prolonged estrogen deprivation. While MAPKs signaling could affect brain and heart physiology through many critical cellular pathways, we found that when phosphorylated ERβ differentially activates
transcription. Therefore, if indeed MAPKs and consequently ERβ are differentially modulated by age and estrogen deprivation this could provide a molecular basis for altered response to ET. These findings highlight the need for further investigations on the effects of prolonged estrogen deprivation in post-menopausal women and provide a molecular explanation for the Timing Hypothesis.

While the understanding of the molecular underpinnings of the Timing Hypothesis is still unclear, the clinical evidence that points toward the benefits of ET in post-menopausal women is strong. Studies enrolling large numbers of women and with the appropriate experimental design show that ET is neuroprotective and cardioprotective following menopause. Furthermore, recent studies evaluating ERβ selective agonists are very encouraging and could remove the disadvantage of activating ERα in reproductive tissues. The societal and economical burden of post-menopausal issues ranging from hot flashes to dementia or atherosclerosis should serve as an incentive for doctors to prescribe ET to women, but at the right time (i.e. in perimenopause). In conclusion, I would recommend the use of ET to perimenopausal women, and I predict that soon ERβ specific agonists will have strong evidence in support of their use in the clinic.
CHAPTER VIII
GENERAL METHODS
Ethics Statement

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

Animals and deprivation paradigm

Female Fischer 344 rats were obtained from the National Institute of Aging (NIA) colony (Taconic) at 18 months (N = 80) of age. The animals were allowed to acclimate to the housing facility for 7 days after arrival. Animals were housed two per cage and were allowed free access to standard rat chow and tap water. One week after arrival, animals were deeply anesthetized with vaporized isoflurane and bilaterally ovariectomized (OVX). Briefly, the ovary and distal end of the uterine horn were pulled from the body cavity through a 1 cm incision made through the skin and body wall. The uterine horn was clamped with a hemostat and ligated proximal to the clamp. The entire ovary and distal uterine horn were then removed. Animals were singly housed and provided with acetaminophen analgesic (122.7 mg/kg) in their water for 3 days postoperative.
During this time, animals were weighed once/day and their water intake was measured. Following 3 days of analgesia the animals were pair-housed with their previous cage mate for the duration of the experiment. Following OVX animals recovered for 1, 4, 8, or 12 weeks (N=20/age group, Fig. 7). After the designated recovery time the animals were given a subcutaneous injection of either safflower oil (vehicle) (N=10/age group) or 2.5 μg/kg 17β-estradiol (E2, N=10/age group) dissolved in safflower oil once/day for 3 days. This dose of E2 elevated levels in OVX female rats (56.5 ± 6.3 pg/ml, which is within physiological range of women who received hormone replacement therapy during post-menopause (17-75 pg/ml)[178]. Animals were euthanized 24 hours after the last injection, trunk blood was collected, brain and heart rapidly removed and flash frozen.

Tissue Collection

The hypothalamus, dorsal and ventral hippocampus were microdissected using a Palkovit’s brain punch tool (Stoelting, Inc., Wood Dale, IL) according to “The Rat Brain in Stereotaxic coordinates” [274]. The left ventricle was also rapidly removed from heart that had been flash frozen and homogenized using silica beads and a Mini Beadbeater-8 (Biospec Products, Bartlesville OK).

CHAPTER III METHODS

PhosTag™ Electrophoresis

50 ug of dorsal hippocampus or heart (left ventricle) protein was run on precast PhosTag™ Acrylamide 12.5% Acrylamide gels, (Wako Pure Chemical Industries, Osaka, Japan). The gel was then transferred on a PVDF membrane (Promega, Madison WI), blocked for 1 hour with 5% BSA, then
incubated with the Estrogen receptor β antibody H150 (epitope: 1-150 fragment of hERβ, N-terminal domain) (Santa Cruz, sc-8974, Dallas TX) at a 1:250 dilution in 5% BSA TBST overnight. Blots were washed twice with TBST for 10 minutes prior to application of 1:5000 goat α-rabbit-HRP (Santa Cruz, sc-2004, Dallas TX) in 5% BSA TBST. Blots were washed twice with TBST for 10 minutes and imaged on the Bio-rad Chemidoc XRS+ imager (Bio-rad, Hercules, CA) using ECL Chemiluminescent substrate (Pierce Scientific, Rockford IL). Densitometry was performed using ImageLab software. Antibody specificity was confirmed with parallel Western Blots and PhosTagTM blots using ERα H-184 antibody (Santa Cruz, sc-7207, 1:1000 dilution; data not shown).

Alkaline Phosphatase treatment
Specificity of phosphorylated proteins were confirmed by treating 50 µg of dorsal hippocampus protein (vehicle-treated animals) with 0, 30 or 60 units of alkaline phosphatase (Roche, Basel, Switzerland) for 2 hours at 37°C to dephosphorylate all phosphorylated proteins.

Cell culture
The mouse hippocampal-derived cell line HT-22 (generously provided by Dr. David Schubert, Scripps Institute, San Diego, CA) was maintained in DMEM (Corning, Tewksbury, MA) containing 4.5% glucose and L-glutamine supplemented with 1x nonessential amino acids (Corning, Tewksbury, MA) and 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA). Cells were used at 70-80% confluency for all experiments.

Hormone treatments
Cells at 70-80% confluency were rinsed with 1x PBS and then media replaced with phenol red-free DMEM plus 10% charcoal-stripped FBS (Atlanta Biologicals, Norcross, GA) at least 36 hours prior to hormone treatments in order to remove all exogenous hormone sources. 17β-estradiol (Sigma, St. Louis, MO) and 4-OH Tamoxifen (Sigma, St. Louis, MO) were diluted in molecular grade ethanol (EtOH) (Sigma, St. Louis, MO) and used at a final concentration of 100 nM as described previously [70, 136].

Expression vectors and reporter constructs

Plasmid expression vector (pcDNA 3.0; Invitrogen, Carlsbad, CA) containing inserts for rER-β1 was provided by Dr. Tom Brown (Pfizer Corp., Cambridge, MA) and has been extensively characterized [8]. The ERE-tk-luciferase reporter construct (generously donated by Dr. Paul Budworth, Case Western Reserve University, Cleveland, OH) contains two repeats of the consensus vitellogenin ERE sequence upstream of the minimal thymidine kinase promoter-firefly luciferase (2xERE-tk-luc) in pGL2-basic plasmid (Promega, Madison, WI). The AP-1-tk-luciferase reporter construct (generously provided by Dr. Colin Clay, Colorado State University, Fort Collins, CO) contains three repeats of the AP-1 sequence into pGL2-basic plasmid. The renilla luciferase pGL4 reporter construct (Promega, Madison, WI) was used as an internal control for transfection efficiency.

Site directed mutagenesis

The pcDNA3.0 plasmid expression vector (Invitrogen, Carlsbad, CA) containing a cDNA insert coding rat ERβ1 was mutated using the Quick Change II XL site-directed mutagenesis kit (Agilent, Santa Clara, CA) to create the phospho-mutants (see Table 1). Primers were designed using the
QuickChange primer design available from the Agilent website and point mutations were inserted following manufacturer’s instructions. Vectors were validated by DNA sequencing (ACGT, Inc, Wheeling, IL) to confirm successful site directed mutagenesis.

Transient Transfections
HT-22 cells were plated at a density of 20000 cells /well in 96-well plates for 48 h before transfection. Transfections were carried out using Fugene6 (Roche, Basel, Switzerland) or Fugene9 (Roche, Basel, Switzerland) according to manufacturer’s instructions. Twenty-four hours after transfection, cells were washed with 1x PBS and incubated with dextran charcoal-stripped media containing hormone treatment or vehicle (EtOH) for 15 h and then lysed for luciferase assays. Transfection efficiency and expression was verified prior to luciferase experiments using GFP-tagged constructs (data not shown).

Luciferase assays
Following lysis, control reporter (Renilla Luciferase) and reporter (Firefly Luciferase) activities were measured using the Dual-Luciferase Reporter Assay system (DLR; Promega, Madison, WI). Relative light units (RLU) were detected using a Biotek Synergy HT plate reader (Biotek, Winooski, VT) with automatic dual injector system and represented as a ratio of Firefly/Renilla Luciferase RLU. All experiments were conducted with 6 replicates for each condition in each 96 well plate and each assay was repeated in 4 or more independent experiments.

Statistics
Two way ANOVA was performed to determine statistical significance and interaction between the groups followed by Tukey post hoc test for comparisons between mutants and wild type vector. Significance was set at P value < 0.05. All transfection data are represented as the mean percent change in fLUC/rLUC compared to vehicle-treated cells transfected with empty vector ± SEM.

CHAPTER IV METHODS

RNA Isolation
Trizol reagent (Invitrogen, Carlsbad CA) was used to isolate total RNA from the hypothalamus, ventral hippocampus, dorsal hippocampus, and left ventricle of the heart. All RNA samples were quantified using Nanodrop spectrophotometry and analyzed for quality by visualization of the RNA on 1.5% agarose gel.

Quantitative reverse transcription PCR (RT-qPCR)
Following RNA isolation, 1.0 µg total RNA was reverse transcribed using the SuperMix VILO cDNA synthesis kit for RT-qPCR (Invitrogen, Carlsbad, CA). Roche FastStart SYBR Green Master Mix was added to intron-spanning ERK and p38 primers: ERK forward: 5’CTCGGATTCCGCCATGAGAA3’, reverse: 5’GGTCGCAAGGTGGTGTGATA3’; p38 forward: 5’CAGGAAACGGGACGAACAGA3’, reverse: 5’CCACAGAACTGCATGTCCT3’. Then, 2 µL cDNA templates were added to duplicate reactions performed in 96 well plates. The following program was used for RT-qPCR: 1) 95°C for 10 minutes, 2) 95°C for 30 seconds, 3) 59°C for 30 seconds, 4) 72°C for 30 seconds, and melting curve analysis. All samples were normalized to the hypoxanthine guanine phosphoribosyl transferase 1 (HPRT) housekeeping gene (primers: forward:
5’AGCAGTACAGCCCCAAAATGG3’, reverse: 5’TGCGCTCATCTTAGGCTTTGT3’), as it is not altered by E2 treatment [178, 179]. Quantification of the target gene expression was achieved using the ∆∆CT method [275].

Protein Isolation
Total protein was extracted from the hypothalamus, dorsal hippocampus and ventral hippocampus using T-Per reagent (ThermoFisher Scientific, Waltham MA) supplemented with Pierce Protease and Phosphatase Inhibitor Tablet, EDTA Free (ThermoFisher Scientific, Waltham MA). Similarly, total protein was extracted from the left ventricle of the heart using RIPA buffer supplemented with Pierce Protease and Phosphatase Inhibitor Tablet, EDTA Free (ThermoFisher Scientific, Waltham MA). Protein concentrations were measured using the Pierce BCA Protein Assay kit according to manufacturer’s directions (ThermoFisher Scientific, Waltham MA).

Western Blot
10 μg of isolated protein was electrophoresed on a 10% acrylamide gel. The gel was then transferred on a PVDF membrane (Promega, Madison WI), blocked for 1 hour with 5% Bovine Serum Albumin (BSA, ThermoFisher Scientific, Waltham MA) in Tris Buffered Saline with 0.1% Tween (TBST), then incubated with primary antibodies in 5% BSA TBST overnight (Table 4 – detailed antibody information). Blots were washed twice with TBST for 10 minutes prior to application of a secondary antibody in 5% BSA TBST for 2 hours, then blots were washed twice with TBST for 10 minutes and imaged using a Bio- rad Chemidoc XRS+ imager following application of the Pierce Enhanced Chemiluminescence (ECL) Western Blot substrate (ThermoFisher
Scientific, Waltham MA). PonceauS staining (MP Biomedicals, Santa Ana CA) was used to detect total protein. Quantification of bands was achieved by measuring the intensity of the bands following normalization to total protein with ImageLab software. Biological replicates were 6-8 per treatment/time point and samples were repeated in 2-3 technical replicates.

<table>
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<td>1:5000</td>
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</tbody>
</table>
CHAPTER V METHODS

Expression vectors and reporter constructs

Rat ERβ sequence was cloned in the plasmid expression vector pEXP-CT/TOPO (Invitrogen, Carlsbad, CA) using EcoRI (NEB, Ipswich, MA) restriction enzyme digestion. The insertion was verified by sequencing which confirmed that 6His were inserted after the last amino acid of ERβ.

Recombinant His-Tagged Protein Expression and Purification

BL21 DE3 (generously donated by the Lab of Dr. de Tombe) competent cells were transformed with ERβ-6His plasmid DNA, and grown on LB-Amp plate overnight. Five colonies were picked for screening and grown in duplicates until culture turbidity reached OD600=0.4. Then IPTG was added to one duplicate (B, induced cells) of each colony to induce expression of ERβ-His6 under control of the Lac promoter. After additional incubation at 37°C for 5 hours, cells were centrifuged and resuspended in Lysis Buffer. After sonication (10" pulses/4 times), supernatant was resuspended in Laemmli buffer, heated for 5" at 95°C and electrophoresed on a 4-15% acrylamide gel (Bio-rad, Hercules, CA). The gel was then stained with 1% Coomassie R-250, 10% Acetic Acid, 40% Methanol solution for 30’, destained for30” and imaged with Bio-rad Chemidoc XRS+ imager (Bio-rad, Hercules, CA). Based on the result, the highest expressing culture was inoculated in 10 ml of LB-Amp. After overnight growth the large scale protein production started by inoculating 8 ml of the culture in 2 L of LB-Amp. The culture was then induced with IPTG and incubated at 37 °C. Cell pellet was then sonicated (30” pulses/4 times) and centrifuged at 10000rpm, 20’, 4°C. Supernatant was used for protein purification performed by Menjie Zhang (Dr. de Tombe lab) with an
automated FPLC system (AKTÄ FPLC, GE Healthcare). The sample was loaded on a HisTrap nickel affinity column (GE Healthcare). The column was then washed until ultraviolet absorbance at 280 nm reached equilibrium. ERβ-His6 was then eluted using a buffer containing imidazole (which competes for binding the column) and the flow through was collected in separate fractions. Fractions were then electrophoresed and stained with Coomassie R-250 to verify which fractions contained ERβ-His6. The positive fractions were combined and concentrated using Pierce Protein Concentrator tubes (Pierce Scientific, Rockford IL). Protein concentration was measured using the Pierce BCA Protein Assay kit according to manufacturer’s directions (ThermoFisher Scientific, Waltham MA). To verify correct expression, ERβ-His6 protein was then electrophoresed on an Acrylamide 4-15% gels (Bio-rad, Hercules, CA). The gel was then transferred on a PVDF membrane (Promega, Madison WI), blocked for 1 hour with 5% BSA, then incubated with the Estrogen receptor β antibody H150 (epitope: 1-150 fragment of hERβ, N-terminal domain) (Santa Cruz, sc-8974, Dallas TX) at a 1:500 dilution in 5% BSA TBST overnight. Blot was washed twice with TBST for 10 minutes prior to application of 1:5000 goat α-rabbit-HRP (Santa Cruz, sc-2004, Dallas TX) in 5% BSA TBST. Blot was washed twice with TBST for 10 minutes and imaged on the Bio-rad Chemidoc XRS+ imager (Bio-rad, Hercules, CA) using ECL Chemiluminescent substrate (Pierce Scientific, Rockford IL). After confirmation of ERβ-His6 expression, recombinant His-tagged protein was aliquoted and stored at -80 °C.
Tryptic digestion and MALDI-TOF analysis

5 to 20 ug of Erbeta-His6 protein was electrophoresed on a 4-15% Acrylamide gel (Bio-rad, Hercules, CA). The gel was then stained with Coomassie G250 (1% Coomassie G-250, 10% acetic acid, 40% Methanol) for 1 hour and destained overnight. The band corresponding to the ERβ-His6 protein was excised using a sterile scalpel; a negative control band with no protein was also excised. Gel bands were individually put in a 1.5 ml tube and washed with 50% Acetonitrile 50 % 100mM Ammonium Bicarbonate for 15’ at 37°C while shaking at 800 rpm. Then the “wash cycle” was performed. First bands were washed with a 100% 100mM Ammonium Bicarbonate for 15’ at 37°C while shaking at 800 rpm. Then they were washed with a 50% Acetonitrile 50% 100mM Ammonium Bicarbonate solution for 15’ at 37°C while shaking at 800 rpm. Then they were washed with 100% Acetonitrile for 15’ at 37°C while shaking at 800 rpm. The gel bands were reduced using 10 mM DTT for 30’ at 37°C while shaking at 550 rpm. The “wash cycle” was then repeated. Then gel bands were alkylated with 55mM Iodacetamide in 100 mM Ammonium Bicarbonate solution for 45’ at 37°C while shaking at 550 rpm. The “wash cycle” was then repeated. The gel bands were then treated with 1 ug of Trypsin on ice 1 hour, then overnight at 37°C while shaking at 550 rpm. The supernatant containing most digested peptides was saved. The gel bands were then washed according to the “wash cycle” to collect all possible remaining peptides from the gel. The washes were collected and pooled. Peptides were concentrated using a Speedvac. Peptides were then resuspended in 0.1% Formic Acid.
The standard curve calibrants for MALDI-TOF and the peptides were then mixed with the α-Cyano-4-hydroxycinnamic acid matrix (CHCA) matrix (ThermoFisher Scientific, Waltham MA). The Axima-CFR Plus MALDI-TOF in reflecton mode was calibrated using a mix of the calibrants spotted on the target plate. Calibrants mix solution was pipetted next to each sample and calibration was repeated between each measurement.

LC-MRM development

A Thermo Scientific TSQ Vantage MS system was used for these experiments. The target peptides (SPWCEAR) were synthetized by ThermoFisher Scientific (Waltham MA) and used for the development of the MRM method. Peptides were resuspended in 50% Acetonitrile 0.1% Formic Acid to a 10picomole/ul concentration, aliquoted and stored at -80 °C. Peptides were diluted at 1picomol/ul and full scan was collected after direct injection of 5ul. Parent ions were then detected and identified. The 2+ parent ion was then selected for fragmentation and a full MS/MS scan was collected. Four transitions (precursor-product ions combinations) were picked for each peptide to be used for the MRM method. The MRM method was prepared using the Xcalibur software (ThermoFisher Scientific, Waltham MA). Peptides were combined in equal amounts and injected through in a Biobasic C18 HPLC column (ThermoFisher Scientific, Waltham MA) in a 66 minutes long LC-MRM method. The MS/MS spectra collected were analyzed using the Skyline software, created by the MacCoss lab at the University of Washington.
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VITA

The author Elena Pinceti was born in Genova, Italy, to Paolo and Laura. She is the oldest of three; Anna and Andrea are her siblings. In 2005 she spent a year as a High School foreign exchange student with the family of Thomas, Terri and Eliza Slanker and graduated from Plainfield Central High School. She attended the Universita’ degli Studi di Genova (Italy) and earned a Bachelor of Science in Biology cum laude, in Cell and Molecular Physiology. She worked in the lab of Dr. Ilaria Demori and defended her undergraduate thesis titled: “Nonalcoholic fatty acid disease: an in vitro system”.

In August 2010, Elena came to the Integrative Cell Biology program at Loyola University Chicago. In the summer of 2011 Elena joined the lab of Dr. Toni Pak where she studied phosphorylation of ERβ in the aging female heart and brain. While at Loyola, Elena has given oral and poster presentations at national and international meetings and published first author and contributing author articles. She obtained a Schmitt Fellowship award as well as a fellowship to attend the 8th International Meeting Steroids and Nervous System in Torino, Italy.