ESTROGEN TREATMENT IN THE HIPPOCAMPUS: ANALYSIS OF GENE EXPRESSION AND CONGNITIVE FUNCTION IN THE AGING FEMALE MOUSE

By

KRISTINA K. AENLLE

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2009

© 2009 Kristina K. Aenlle

To Jeffrey, Dante and Dominic

ACKNOWLEDGMENTS

I would not have been able to accomplish all that I have with out the love and support from my friends and family. First, I want to thank my husband, Jeff, for the encouragement, love and sacrifice he has shown me. I must also thank my amazing beautiful boys, Dante and Dominic. Their smiling faces, laughs and love make each and every day more than I could ever dream of. Next, I want to ackowledge the love and support my family and I have seen especially from, Tom, Marianne, Liz, Steve, Beverly, Tony, Lisa, Scot, Michele, Jenny, Robert, Josephine and our Aunts, Uncles and cousins. I appreciate all of you have done for us.

During my time at the Univeristy of Florida, I meet many amazing people and made many wonderful friendships. Thank you to: Dustin, Kristi, Amber, Tolga, Missy, Shannon, Shankar and Stephanie for their support and friendships.

I would like to thank my colleagues and mentors at the University of Florida for their guidance and encouragment. In particular, I thank Dr. Thomas Foster for the honor of working with him and his support and guidance. Also, I thank Dr. Sue Semple-Rowland for her guidance and assistance. Finally , I thank the Foster lab, Ashok, Li, Asha, Travis, Karthik, Wei, Olga and Zane for making everyday so much fun.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

ESTROGEN TREATMENT IN THE HIPPOCAMPUS: ANALYSIS OF GENE EXPRESSION AND CONGNITIVE FUNCTION IN THE AGING FEMALE MOUSE

By

Kristina K. Aenlle

December 2009

Chair: Thomas C Foster
Major: Medical Sciences – Neuroscience

The use of estrogen replacement therapy to treat age-related cognitive decline remains controversial. While many basic, translational and clinical studies show protective effects of estrogen treatment, others show little if any cognitive reinforcement. The discrepancies in these studies, for the most part, are due to the lack of knowledge researchers have in estrogen signaling and how estrogen signaling changes with age. To gain a better understanding of estrogen's effect on the hippocampus during aging, Morris Water Maze and microarray analyses were used to compare hippocampal function and gene expression responses to estogen treatment during aging. The results show that estrogen treatment maintains hippocampal dependent spatial memory in middle-aged female mice. Moreover, estrogen treatment reversed age related gene expression and promoted the expression of neuroprotective genes in both young and middle-aged mice but the gene expression response in aged animals was attenuated. Furthermore, while estrogen treatment supported the expression of genes involved in synapse in young and middle-aged mice, gene expression in aged mice was shifted toward rapid signaling pathways. The differences in gene expression likely involved age-related decreases in estrogen receptors. Accordingly, microarray analyses of estrogen receptor alpha knockout and estrogen receptor beta knockout mice revealed significant alteration of hippocampal gene expression resulting from the alteration

of receptor ratio. Together, these results suggest that age-related alteration of estrogen receptors and membrane activity contribute to the reduced gene expression changes found in aged mice.

CHAPTER 1
INTRODUCTION

Estrogen and Cognition

Dementia is one of the leading concerns in the public health community. Affecting only 1% of the population at age 65, the numbers escalate to roughly 25% by the age of 85. Alzheimer's disease accounts for the majority (50-60%) of dementia diagnoses. By 2040 it is projected that 81 million people worldwide will suffer from Alzheimer's disease(Ferri et al., 2005) with the majority of cases being women (68%) (Brookmeyer et al., 1998). Current research suggests estrogen treatment may delay the onset of memory loss associated with normal aging and Alzheimer's disease (Henderson, 2004; Kawas et al., 1997). However, estrogen has diverse effects throughout the central nervous system and the challenge remains to determine which of these effects are important for memory. This challenge has grown more complicated due to the discovery of several estrogen receptors and the emerging evidence of rapid effects from estrogen.

The use of estrogen to treat menopausal symptoms began in the 1940's (for review of the history of estrogen use (Stefanick, 2005); however, as research began to show that estrogen may protect against age related cognitive decline and may delay the onset of Alzheimer's disease, the use of estrogen rapidly increased. Furthermore, as life expectancy has increased, the age at which women enter menopause (cessation of menstrual cycle) has remained relatively constant; therefore, women are spending potentially one third of their lifespan in an estrogen-depleted state, leaving women more vulnerable to cognitive impairments. In the late 1980's Sherwin demonstrated that premenopausal women who had their uterus and/or ovaries removed maintained their performance on a test of verbal memory when receiving estrogen replacement. The participants receiving placebo experienced a significant decrease in their verbal memory

scores**.** Halbreich et al. (1995) followed up Sherwin's study and found that menopause was associated with a potential acceleration of age-related cognitive deficits. These initial studies illustrated that estrogen is not only involved in reproductive systems but may protect against cognitive decline found during aging. However, the mechanism in which estrogen can ameliorate age-related cognitive impairments is unknown and the data supporting estrogen's positive influence on cognition is not without controversy.

The divergence between the copious amount of basic science, epidemiological and clinical studies that supported the potential benefit of hormone therapy in preventing age-associated cognitive impairment and the results of studies suggesting adverse effects was recently emphasized by the published results of the Women's Health Initiative Memory Study (WHIMS) (Espeland et al., 2004; Shumaker et al., 2004). WHIMS compared the effect of estrogen alone and estrogen with progesterone on the incidence of probable dementia and global cognitive function in older women (Espeland et al., 2004; Shumaker et al., 2004). The WHIMS had a vast impact on the perception of estrogen therapy due to its large number of participants (2808 women) and the media's excitement over the WHIMS findings that indicated that not only did estrogen treatment with or with out progesterone fail to improve cognitive function, but may augment cognitive decline in postmenopausal women. Beyond WHIMS, other studies have also suggested no effect of estrogen treatment on memory impairments (Henderson et al., 2000; Wang et al., 2000). The question remains as to why rodent models, non-human primate models and many clinical trials show improved cognitive performance after estrogen treatment and others do not.

Following the conclusion of WHIMS many scientists speculated as to why the study failed to confirm previous findings of the beneficial effects of estrogen treatment. Sherwin (2005)

reviewed WHIMS and previous randomized controlled trials (RCTs) and found that the initial setup of many of the RCTs, including WHIMS, failed at many points. First, WHIMS failed to use a full assortment of cognitive tests. WHIMS used only 3MS (Modified Mini-Mental State Examination); therefore, any improvement on verbal memory, which previous RCTs found to benefit from estrogen treatment, was not assessed. The 3MS is a test of cognitive decline that is not suited to distinguish between specific cognitive domains. It is believed that estrogen can influence only certain types of memory; such as verbal and spatial memory. Second, many of the previous RCTs used estrogen alone and a combination of estrogen and progesterone treatment, referred to as hormone replacement therapy (HRT). More specifically many of these studies, including WHIMS, used a form of progesterone called medroxyprogesterone acetate (MPA). Research indicates that MPA counteracts estrogen's ability to protect neurons against glutamate induced neurotoxicity (Nilsen and Brinton, 2002). The WHIMS study found that estrogen + MPA treatment increased the risk of probable dementia and estrogen treatment alone did not improve cognitive function. Furthermore, previous studies have shown that progesterone may have opposing effects on biology (Woolley and McEwen, 1993) and memory compared to estrogen (El-Bakri et al., 2004). For example, aged ovariectomized (removal of the ovaries, OVX) rats given progesterone exhibited more working memory errors, compromised learning of working memory and reference memory errors compared with aged OVX rats that did not receive progesterone (Bimonte-Nelson et al., 2004). This same study showed OVX improved working memory in aged animals compared to shams, suggesting the removal of ovaries may eliminate the negative impact of progesterone. Considering that menopause and estropause is associated with increased levels of progesterone and decreased levels of estrogen, it is believed that an increase of progesterone may contribute to cognitive deficits in aged animals.

More important for understanding estrogen's effects on cognition, and a more compelling explanation for the failure of WHIMS, may be the age at which treatment was initiated. Evidence is mounting which suggests that beneficial effects from estrogen treatment may be reduced or nonexistent if treatment is initiated several years after estrogen declines during menopause (for review see, Sherwin, 2006). The age of the women in WHIMS ranged from 65-79 (mean age of 72), at least 15 years after menopause. In comparing the results of WHIMS to previous work examining the relationship between hormones and cognitive decline, it is evident that estrogen treatment is beneficial to cognition within a critical amount of time after estrogen depletion. Accordingly, the authors of WHIMS did not rule out the potential benefit of HRT in younger women. Together these studies suggest that the benefits of estrogen therapy may be dependent on the amount of time women remain in a hormone depleted state.

Beyond clinical studies, much of the evidence in favor of the beneficial effects of estrogen comes from animal studies. Rodents and non-human primates are useful models in the study of aging and estrogen. Non-human primates display a menstrual cycle, hormonal fluctuation, and menopausal state similar to that found in women (Gilardi et al., 1997; Goodman et al., 1977). Mice also exhibit similar hormonal modulation with irregular estrous cycles occurring during middle-age and subsequent drop off of estrogen levels. Rats exhibit an irregular estrous cycle at middle age; however, estrogen levels do not drop off immediately as seen in the human and mice.

As mentioned, non-human primates make a great model for estrogen research due to their similar menstrual cycle, hormonal fluctuation, and menopausal state found in women (Gilardi et al., 1997) (Goodman et al., 1977). Similar to clinical data, age-dependence for beneficial effects of estrogen has been observed in primates. Recently, it has been shown that aged female rhesus

monkeys display cognitive changes during fluctuation of the menstrual cycle (Lacreuse et al., 2001). Also, following natural or surgical menopause (Lacreuse et al., 2001; Roberts et al., 1997) aged female rhesus monkeys have displayed cognitive impairments. The results from estrogen therapy in monkeys indicate that estrogen can improve some aspects of cognition in aged monkeys. For example, estrogen improved spatial working memory in aged OVX rhesus monkeys that were without estrogen for at least 10 years (Lacreuse et al., 2002). Furthermore, cyclic estrogen treatment has also been shown to improve cognitive function in aged OVX rhesus monkeys (Rapp et al., 2003). Finally, Tinkler and Voytko (2005) compared results from young and middle-aged female rhesus monkeys and found effects of OVX and ERT on cognition that was dependent on age. These results indicate that estrogen has beneficial effects on cognition but these benefits may be decreasing with age or as the amount of time in an estrogen depleted state increases.

By far the bulk of research examining the role of estrogen in cognitive function comes from rodent studies. In a series of studies, Heikkinen and associates (2002, 2004) assessed the role of estropause in age-associated cognitive decline in female mice. In the first study, OVX animals at 3 months of age were deprived of estrogen for 4 months then placed on estrogen minipellets at 7 months of age, 40 days before behavior testing. In the second study, 5 month old mice were OVX and without estrogen for 19 months. These aged mice were placed on estrogen minipellets at 24 months of age, again 40 days prior to behavior testing. When the two groups (4 months vs 19 months of estrogen of deprivation) were compared the results showed that estrogen treatment was associated with improvement in cognitive performance in young mice compared to age-matched untreated mice. While aged mice did show estrogen effects in the same direction as their young counterparts, the results were not significant. In a similar study, rats OVX'd at

midlife were treated with either estrogen or vehicle 3 or 10 months post OVX (Gibbs, 2000). Those receiving estrogen 3 months after surgery preformed better on delayed spatial memory task (i.e. matching to position T-maze) compared to their vehicle treated counterparts. In contrast, when estrogen treatment was initiated 10 months after OVX, animals did not exhibit memory improvements. Gibbs (2000) suggested an important window between 3 and 10 months of estrogen withdrawal that is critical for the beneficial effects of estrogen. Taken together, the results from humans, primates, and rodents indicate that estrogen can have beneficial effects on cognition when treatment is administered soon after estrogen deprivation.

It has been shown that estrogen treatment administered with in a critical time period can delay cognitive impairments associated with aging. The work included in this dissertation set out to determine how the effect of estrogen changes with age. More specifically, how estrogen influences gene expression levels during aging and the role of estrogen receptors in gene expression response in the hippocampus.

Estrogen Receptors

Estrogen, a powerful pleiotropic steroid hormone, is produced by the aromatization of testosterone within the ovaries; however, aromatase activity is also present in the brain, including the hippocampus. Three types of estrogen are produced in cycling women; estradiol, estrone and estriol, with the primary estrogen being 17β-estradiol. Estrogen binds to nuclear receptors, the most prevalent being estrogen receptor alpha and estrogen receptor beta (ERα and ERβ, respectively). ERα and ERβ, share many structural and functional similarities but can also induce different responses.

Despite the fact that the two estrogen receptor subtypes have a similar structure, equivalent binding affinity for estrogen and share ~90% homolog, they seem to have unique roles. The N-

terminal domain of ERα and ERβ, which contains the AF-1 domain (ligand independent domain), shares only 20% amino acid homology and displays promoter and cell specific activity. The central domain of ERα and ERβ is highly conserved between the two receptors with 95% homology. The central domain contains the DNA binding domain, important for specific DNA binding and receptor dimerization. The ligand binding domain or E domain contains the AF-2 and shares 55% homology between ERα and ERβ . This domain has a similar 3D structure between the two receptors but contains different amino acids in the cavity of the domain resulting in a 20% smaller ligand-binding cavity in ERβ and may be important for receptor specificity. Studies have shown that ERβ has a weaker AF-1 domain and relies more on AF2 domain (Delaunay et al., 2000). These subtle differences in gene structures can lead to diverse actions on transcriptional regulation.

Results indicate that ERα and ERβ share a set of primary ERE but differ in their ability to associate with other EREs resulting in differences in transcriptional activation properties (Barkhem et al., 1998). Furthermore, the combination of receptor subtypes can have different or even display opposite effects on transcription related cellular activities (Paech et al., 1997). For example, ERα and ERβ show different effects on cyclin D promoter, with ERα inducing expression and ERβ repressing cyclin D expression (Liu et al., 2002). Patrone et al. (2000) provided evidence in neuroblastoma cells that estrogen activated specific signaling pathways dependent on the receptor subtype. The group showed that in SK-N-BE (neural cell derivatives), ERα activation increases length and neurite number, whereas ERβ activation only induces neurite elongation. These studies show that estrogen's vast impact may be due in part by specialization of its receptor subtypes. Through the use of chimeric receptors the authors further demonstrated that the presence of both transcription activation functions located in the NH2-

terminus and COOH-terminus are necessary for the differential biological activity. Their data highlight ERα activity in mature neural cells and displays the role of ERα in regulating neuronal morphology.

Although most of the focus of ER localization is in neurons, ER staining is found in non-neuronal cells. ERα staining has been found in select astrocytic processes, in close association with dendritic spines (Milner et al., 2005). Similarly, ERα labeling is shown in astrocytes in monolayer cultures (Garcia-Segura et al., 1999; Santagati et al., 1994) and gonadal steroids are shown to regulate glial morphology and the expression of glial fibrillary acid protein (Day et al., 1993). Furthermore, ERβ expression is found in glial cells, using double immunohistochemical localization of ERβ and the specific astroglial marker glial fibrillary acidic protein (GFAP), and ERβ-immunoreactive glial cells are found in all layers of CA1, CA2 and CA3 of male and female rats (Azcoitia et al., 1999; Bjornstrom and Sjoberg, 2005).

Furthermore, estrogen receptors have been associated with numerous subcellular structures. Chen et al., (2004a, 2004b) found that, not only are both ERα and ERβ present in mitochondria, but estrogen treatment significantly increased the level of mitochondria ERs in a time-and dose-dependent manner. Recently, aggregates of ERβ have been localized within mitochondria, endomembranes, and the plasma membrane (Milner et al., 2001). Work by Zhai et al., (2000), examined the effect of estrogen on the structure and function of mitochondria of wild type mice that were either sham or ovariectomized (OVX) and ERα knockout (KO) mice and found that the mitochondria of the OVX and ERαKO were abnormal in shape, with abnormal cristea and loss of matrix area, compared to the wild type counterparts. Together these studies display estrogen's potential fervent influence on cellular function.

Recent data from aortas indicate distinct pathways for ERα and ERβ signaling. ERα increases overall gene expression and ERβ decreases overall expression in aortic tissue (O'Lone et al., 2007). Similar results are seen in the regulation of apolipoprotein E, a protein believed to be involved in the accumulation of plaque formations in Alzheimer's disease. ERα increases levels of APOE mRNA and protein, whereas ERβ decreases the levels (Wang et al., 2006). Other studies have suggested opposition on ERα signaling by ERβ (Gonzalez et al., 2007). Gonzales et al., (2008) showed that in the presence of a specific ERα agonist the levels of the progesterone receptor were increased compared to estradiol alone. However, when an ERβ-selective agonist was used the levels of progesterone were attenuated. Together these results show that ER activation can lead to activation of distinct pathways and cross talk between the two, possible by inhibiting the activity of the other receptor.

As mentioned, estrogen receptors have been demonstrated at the plasma membrane (Kuroki et al., 2000; Milner et al., 2001; Ramirez et al., 1996). Toran-Allerand et al. (2002) has described a putative plasma membrane-associated ER-X, which is thought to be involved with estrogen-induced activation of the mitogen-activated protein kinase cascade. However, ER-X is believed to be expressed during development and after injury(Toran-Allerand et al., 2002). The role ER-X would have in the healthy adult brain is unknown. Interestingly, a G-protein coupled receptor, GPR30, is described to have estrogen specific binding(Funakoshi et al., 2006). Although controversial (Levin, 2009), GPR30 is believed to be a plasma membrane receptor that mediates some of the rapid effects of estrogen (GPR30 will be discussed further in a later section). Also, Lu et al. (2004) reported a change in compartmentalization of the ERβ that may characterize a regulator of intracellular signal transduction from membrane to cytosol in hippocampal neurons. Lastly, it is believed that non-nuclear and nuclear ERs may be the same

protein, but translocated to another cell compartment, and capable of producing nonclassical processes.

Estrogen Signaling Pathways

17β-Estradiol (EB) exerts many of these benefits through classical influences, as well as exhibiting rapid nonclassical effects. While both classical and nonclassical pathways have been found to be active within the hippocampus, the entire processes of both are not fully known(Bjornstrom and Sjoberg, 2005) .

Classical Processes

Estrogen can induce transcription through classic nuclear receptors. Estrogen binds to an ER and causes a conformational change in the receptor, promoting homodimerization or heterodimerization. Once dimerized, the classical mechanism involves the translocation ERs to the nucleus and binds to palindromic estrogen response elements (EREs). Binding to EREs results in the recruitment of specific coregulators that increase or decrease the transcription of genes. Each homodimer or heterodimer may have specific coregulators influencing the genes transcribed under each receptor. However, only a third of the genes in humans found to be regulated by EB contain ERE or ERE-like sequences (O'Lone et al., 2004). Moreover, the classical process of estrogen activation of transcription can take several hour (O'Lone et al., 2004) and estrogen has been found to induce rapid effect on signaling cascades within minutes of administration. Therefore, other nonclassical processes must also be involved in estrogen mediated gene transcription.

Nonclassical “Rapid” Processes

Within the last ten years researchers have discovered rapid estrogenic actions, not mediated through the classical ERE driven gene transcription. This dissertation refers to any estrogen signaling pathways not driving gene transcription through classical ER dimerization

and binding to ERE as "non-classical." These non-classical mechanisms can be characterized into three groups; ligand-independent, rapid non-genomic and ERE-Independent signaling (Bjornstrom and Sjoberg, 2005). Ligand-independent gene activation refers to the induction of second messenger pathways that lead to the activation of phosphate and kinase activity and go on to phosphorylate the estrogen receptor. Rapid-genomic activity includes membrane-associated receptors such as G-coupled protein receptors. ERE-independent signaling refers to direct protein-protein interactions between the ligand-bound ER and transcription factor complexes such as, c-Fos, SP-1 or NF-kB. Ligand-independent and rapid-genomic activity are characterized by the rapid activation of estrogenic responses (within minutes), ERE-independent activity can take several hours similar to classical processes. It is known that non-genomic activation of signaling is not mutually exclusive, cross-talk between genomic and nongenomic contribute to the diverse effects of estrogen (Foster, 2005). However, it is not known how the processes contribute to estrogenic properties and which effects are exclusive to each pathway and which are dependent on the contributions of each.

Ligand-independent: Estrogen most notably binds to the estrogen receptor, alters its configuration and influences transcriptional regulation by binding to ERE on promoter/enhancer regions of target genes; however, this process can also be initiated without ligand binding. Estrogen receptors can be phosphorylated by growth factors and signaling pathways to go on to bind to ERE on promoter/enhancer regions of target genes. Growth factors, such as epidermal growth factor (EGF) and its receptor, initiate signaling cascades leading to the phosphorylation of ERs and to subsequent binding to ERE (Leong et al., 2004). The signaling cascade ERK1/2 /MAPK has been found to activate ERα in a ligand-independent manner by phosphorylating serine 104 and 106. The biological relevance of ligand-independent activation of ER is still

under investigation. However, recent work has compared the temporal pattern of chromatin remodeling and mRNA transcription between cells treated with EB and EGF (Berno et al., 2008). Results show that EGF rapidly activates chromatin remodeling and mRNA transcription whereas EB has more of a cyclic effect. More work is needed to determine if ligand-dependent and ligand-independent mechanisms activate the same genes, influence the activation of each other or work independently.

Rapid non-genomic: Although still controversial, the existence of membrane-associated ERs have been isolated in several cell types (Kelly and Levin, 2001; Levin, 2002). Researchers have found that 5-10% of endogenous estrogen receptors are membrane associated (Pedram et al., 2006). This small proportion of estrogen receptors is believed to have a large impact on signaling cascades and estrogenic properties. Membrane-associated ERs initiate rapid effects by interacting with G-protein coupled receptors and Ca^{2+} signaling cascades, leading to (among others) increases in cAMP levels and ultimately altering the balance of phosphates and kinase activity (Sawai et al., 2002; Setalo et al., 2002; Shingo and Kito, 2002). Activation of the tyrosine kinase cascades and MAP kinase (MAPK) signaling by rapid calcium influx occurs within minutes of estrogen exposure (Kuroki et al., 2000; Singh et al., 1999). Once activated, MAPK phosphorylates CREB, an important component of hippocampal learning and memory and a key component of estrogen's neuroprotective signaling mechanisms (Murphy and Segal, 1997). Recently published work by Minano et al (2007) found the phosphorylation of CREB was dependent on SRC/RAS/ERK activity and went on to hypothesized that activation of the signaling cascade could have been initiated by Toran-Allerand's recently characterized putative membrane receptor, ER-X (Minano et al., 2008); however, involvement of ER-X needs further investigation due to findings that it is only activated during development or injury (Toran-

Allerand, 2004). The cellular responses to this activation include, adhesion, migration, survival, neurite maintenance and proliferation (Kelly and Levin, 2001; Minano et al., 2008).

Influence on CREB phosphorylation also occurs through the interaction and activation of metabotrophic glutamate receptors (mGluR) (Boulware et al., 2005). Through ERs at the plasma membrane, EB activates group I and group II mGluRs. Activation of group I receptors sets off a cascade of events including activation of PKC and MAPK, which phosporylate CREB. In contrast, activation of group II receptors decreases L-type calcium channel-dependent CREB phosphorylation by inhibiting adenylate cyclase and PKA. Interestingly, ERα has been found to solely interact with mGLuR1a receptors but both ERα and ERβ interact with mGlur2/3 receptors. Further studies are needed to better characterize ERs and other membrane receptors triggering these signal cascades. Recently, a novel G-protein-coupled receptor (GPR30) has been described as a membrane estrogen receptor. GPR30 has been localized to the plasma membrane and there is a report of the receptor being only localized to the endoplasmic reticulum (Raz et al., 2008). However, GPR30 was first isolated in MC7 breast cancer cells and high affinity binding by estrogen was confirmed in ER-negative breast SKBR3 cancer cells (Filardo et al., 2007). Even though SKBR3 cells show abundant expression of GPR30 they display low estrogen binding interaction and little cAMP response(Raz et al., 2008). It should be noted that ERα and ERβ might be able to tether to GPR30 through a currently unknown mechanism and mediate rapid estrogen effects. Given the interesting but limited knowledge of GPR30 more research is needed to determine its role in estrogen signaling.

ERE-independent signaling: ERE-independent signaling refers to ERs regulation of gene expression through direct protein-protein interactions with other transcription factors. Examples of these genotropic interactions include; c-Fos/c-Jun (AP-1), Sp1 and NF-kB (Jakacka et al.,

2002; Safe, 2001). The estrogen receptor binds to cofactors such as; SP1, AP-1, NF-kB and then to response element sites on DNA. Interestingly, genotropic effects can differ between ERα and ERβ resulting in differential regulation of genes. Estradiol induces transcription via AP-1 sites through ERα but inhibits transcription through ERβ. However, the anti-estrogenic compounds; raloxifene and tamoxifene induce transcription through ERβ and AP-1 interaction but minimal activation through ERα (Paech et al., 1997). ERs show high specificity or preferential binding to many of the transcription factors but further investigation is needed to determine which ER activates which transcription factors and which cofactors are involved.

Classical and Non-classical Crosstalk

Estrogen's classical and non-classical signaling events are not independent pathways. Much of estrogen's signaling results from the combination of classic and non-classic mechanisms. For example, brain derived neurotrophic factor (BDNF), a promoter of neuronal survival and regulator of synaptic plasticity, is a good example of estrogen's ability to influence gene transcription through the contribution of both mechanisms (Lu et al., 2003; Suzuki and Handa, 2004). BDNF is activated by EB through ERE elements, classic genomic events. BDNF is also activated via a cAMP response element in its promoter. CREB is phosphorylated by EB through cAMP/protien kinase A, MAPK, and CAMKII activity. Once phosphorylated CREB binds to CRE site and induced transcription of BDNF.

Dendritic spine formation is also sensitive to estrogen levels and responds to estrogen through numerous classical and non-classical mechanisms, including NMDA receptor activation. Recently, Morrissette et al. (2008), showed that the expression of NMDA receptor subunit N2B was dependent on genomic activation by ERα in the hippocampus (Morissette et al., 2008b). NMDA receptor function is also enhanced through tyrosine phosphorylation by c-src, which may

be activated by EB interaction with a membrane receptor (Murphy and Segal, 1996). Finally, estrogen has been shown to rapidly activate Akt, a pathway implicated in synatogenesis, spine number and morphology (Akama and McEwen, 2003). Phosphorylated Akt in the hippocmapus is increased during proestrus when compared to estrus, diestrus and ovariectomized animals (Znamensky et al., 2003). Akt may also be activated indirectly by estrogen through BDNFs activation of TrkB receptor. Estrogen's activation of the AKT pathway may be one-way estrogen is influencing new spine formation, transcription/translation of synaptic proteins, and cell survival (Du et al., 2004). Together these studies on signaling pathways show that classic and non-classic mechanism work together to produce estrogen's trophic neuroprotective effects.

The Hippocampus

For over 20 years research has focused on estrogen's affect on the hippocampus due to its well-established role in acquisition and consolidation of memory and due to the recent discovery of estrogen receptors within the hippocampus**.** The hippocampus has also been found to be morphologically, physiologically, and functionally responsive to estrogen treatment and estrogen removal (Foster, 2005). Therefore, the hippocampus is a prominent target to explorer estrogen's effect on cognition.

The rodent hippocampus is a curved structure located in the medial temporal lobe of the brain, within the limbic system. The hippocampus is generally broken down into a series of Cornu Ammonis (CA) areas: beginning with CA4, next CA3, followed by CA2 and finally CA1. The hippocampus formation also includes the dentate gyrus. These well-defined structures are easily distinguishable due to the pyramidal cells of the CA1 and CA3 and dentate gyrus granule cells. The major pathway of the hippocampus is the unidirectional perforant path, where axons from the entorhinal cortex (EC) enter the hippocampus via dentate gyrus and CA3 regions. There is also a projection from layer 3 of the EC to the CA1. Granule cells of the dentate gyrus send

their fibers to the CA3 and the axons of the pyramidal cells of CA3 send their axons to the CA1. Axons of the CA1 finish the loop by projecting to the subiculum and deep layers of the EC. The hippocampus also receives inputs from other brain areas including but not limited to; amygdala, thalamus, and areas of the lateral hypothalamus.

One of the early observations of hippocampal role in memory and consolidation came form the data of H.M. Patient HM suffered severe seizures and underwent surgery to remove the affected areas of his brain. The surgery left him with only 1/3 of his hippocampus and with extensive anterograde amnesia and partial retrograde amnesia. HM has an intact short-term memory but has a general inability to form long-term memories. Data obtained from HM over the years has helped pinpoint the role of the hippocampus in memory consolidation, declarative memory and regions of the hippocampus possibly involved in spatial memory (Gabrieli et al., 1988). Furthermore, studies using hippocampal ablation and lesion studies in rodents have ascertained the hippocampus's role in learning and memory. Morris et al., (1990) and initial studies using septo-hippocampal connection lesions found clear deficits in the ability to perform a spatial memory task (Olton, 1977).

Morris Water Maze

The gold standard for investigation of hippocampal function in rodents is the Morris water maze (MWM). The MWM was designed in 1984 by Richard G Morris to test spatial memory in rodents (Morris, 1984). The MWM uses a small pool in which a platform is hidden or unhidden. Animals are placed into the opaque colored warm pool and escape the pool by finding the platform using either the platform as a cue or finding the hidden platform using their spatial memory of the surrounding environment. In the cue discrimination task, the platform is visible with a flag attached to the top of the platform. The platform and release location is varied between the 4 quadrants (N, S, E,W). during testing. In our MWM protocol, we use the cue task

to test the animals' vision, ability to swim, and to learn the task. Five days after the cue discrimination task we perform the spatial discrimination task. During the spatial discrimination task the platform is hidden underneath the water. The platform is kept in the same quadrant (goal quadrant, can be either N, S, E, W) for the entire testing and the animal is placed in the water at any of the 4 quadrants. The animal must find the platform from spatial cue in the testing room (lamps, chairs, tables, posters). During spatial training (3 trails /4 blocks/day over three days) the amount of time and distance the animals takes to reach the platform is recorded and compared across groups. To test the animals' spatial memory, we use a probe test, where the platform is removed from the goal quadrant and the animal is released from the quadrant opposite the goal. For example if the goal quadrant is in quadrant "N" the animal would be released from quadrant "S" during the probe trial. During the probe test we record how much time the animal spend in the goal quadrant and how many time the animal crossed over the platform area. The probe trail is a direct measure of the animal's spatial memory.

The MWM is a hippocampal dependent spatial memory task and sensitive to memory deficits associated to hippocampal aging. Rapp et al (1987) found that aged hooded rats showed an acquisition deficit in locating a submerged platform. However, with subsequent training aged animals learned the task to the same extent as young and middle-aged (Rapp et al., 1987). Rapp and colleagues concluded that aged animals display deficit in the ability to utilize spatial information, suggesting an age-related impairment in hippocampal function.

Estrogen researchers have also utilized the MWM to test estrogen's effect on hippcampal-dependent spatial memory. Researchers have found that performance on the MWM fluctuates over the estrus cycle and the age-related impairments seen during the MWM is associated with the loss of circulating hormones during estropause (Frick, 2009; Healy et al., 1999). Therefore,

the MWM is a powerful tool to study estrogen's effect on hippocampal-dependent spatial memory during aging.

Estrogen Action in the Hippocampus

Both ERα and ERβ are present in the hippocampus but differ in their level of expression and distribution. Early ER localization studies found that the majority of nuclear ERα receptor expression was located in GABAergic interneurons throughout the hippocampus (Weiland et al., 1997). The evidence for ERβ in the hippocampus was hampered by the lack of a good antibody. However in 1999, Shughrue and associates showed the ERβ mRNA was translated into active protein in the brain (Shughrue et al., 1999). Shughrue and Merchenthaler (2000) also reported the existence of both ERα and ERβ mRNA in the neurons of pyramidal cells of CA1-CA3 in ovariectomized rat hippocampus; however, the maximum labeling was found localized to CA2-CA3 cells of the ventral hippocampus (Shughrue et al., 1999). Later, ERβ (protein and RNA) was found localized to only a few interneurons in the CA3 region (Shughrue and Merchenthaler, 2001). Mitra et al. (2003) went onto show that within the hippocampus there is an asymmetrical distribution of ERs, with more nuclear staining of ERβ in ventral hippocampus and more fiber staining in the fibers of the dorsal hippocampus and stronger ERα labeling in the ventral portion of the hippocampus than in the dorsal of ovariectomized mice. Recent reports indicate that ERα and ERβ are observed on dendritic spines, axon terminals and within axons of CA1 pyramidal cells (Adams et al., 2002; Milner et al., 2005; Milner et al., 2001; Romeo et al., 2005). Furthermore, ERβ displays extranuclear localization in the cell cytosol within the excitatory neurons of the CA3 region of the hippocampus; whereas, ERα is found primarily in the cell nuclei (Kalita et al., 2005). These regional differences in receptor density may contribute to the divergent signaling pathways and morphological responses.

A significant increase in the number of ERα immunoreactivity dendritic spines is observed in the hippocampus during proestrus females as compared to diestrus females and intact males (Romeo et al., 2005). Whereas, increased levels of ERβ mRNA and protein are found during estrous. These results are in parallel with earlier studies where the maximal level of nuclear ER expression is observed during diestrus, when estrogen levels are low to moderate (Weiland et al., 1997).

Even though remarkable progress has been made to determine localization of ERs, it is still unclear how ERs impact the function of the hippocampus. To determine some of the ways ERs impact the hippocampus, knockout (KO) animals have been employed to test behavioral and physiological changes due to the loss of the receptor. While ERαKO mice present severe impairment in reproductive behaviors, they also exhibit memory impairments on hippocampal dependent inhibitory avoidance tasks (Fugger et al., 2000). In contrast, ERβKO mice display only slight attenuation in reproductive behavior but pronounced morphological abnormalities in their brain (Krezel et al., 2001; Lubahn et al., 1993). Furthermore, ERβKO mice show deficits in hippocampus-mediated-fear-conditioning paradigm and attenuated hippocampal CA1 long-term potentiation (Day et al., 2005). It has also been reported that ERβKO display learning deficits on the MWM (Rissman et al., 2002) neuronal loss and shrinkage by 3 months of age, and cellular disorganization, astroglial proliferation, increased apoE and significant amyloid plaque deposition throughout the CNS by 12 months of age (Zhang et al., 2004). It is clear that ERα and ERβ can influence separate signaling pathways and have potentially different effects on behavior, however; it is not clear the role ERs have in cognitive impairments associated with the aging hippocampus.

Estrogen's Effect in the Aging Hippocampus

Many of the memory deficits found in natural aging affect hippocampus-dependent memory, through structural and functional changes within the hippocampus. Moreover, the hippocampus has been found to be particularly sensitive to aging and disease (Himeda et al., 2005; West et al., 2000). For example, a significant decrease in spine density in the CA1, decrease in spine length in the CA1 and dentate gyrus and subsequent deficits in hippocampus-dependent spatial memory tasks were found in aged mice (von Bohlen und Halbach et al., 2006). Aging has an impact on hippocampal plasticity due to the loss of synapses in dentate and CA1, decrease of NMDA-receptor mediated responses in dentate gyrus, and an alteration of CA2+ homeostasis in CA1 (Rosenzweig and Barnes, 2003). Moreover, synaptophysin has been found to decrease with age in the hippocampus and in patients with Alzheimer's disease (Sze et al., 1997). It is believed that the decrease in synaptophysin is correlated with impaired cognitive abilities (King and Arendash, 2002).

Several studies have demonstrated estrogen-reduced responsiveness in the hippocampus. For example, compared to young animals, aged rats show a smaller increase in axospinous density in the CA1 (Adams et al., 2001b) and estrogen fails to alter the distribution of ERα as seen in young animals (Adams et al., 2002). Estrogen treatment in aged animals does increase the levels of NMDA receptor 2B, a mediator of synaptic plasticity (Adams et al., 2004). Therefore, many of the mechanisms involved in EB effects on hippocampal function are attenuated in aging but other mechanisms or new mechanisms continue to compensate for the loss of neuroprotection. Furthermore, researchers are currently looking into a critical period hypothesis for estrogen treatment (Maki, 2006; Sherwin, 2007). Simply stated, estrogen treatment needs to be initiated within a critical time period (perimenopausal or immediately

following menopause) to be beneficial to cognitive function. The physiological, cellular and molecular changes occurring in the hippocampus and causing the truncated effects of EB treatment during this time are still under investigation but likely involve an age-related alteration of transcriptional regulation by EB.

The relative ratio of estrogen receptor is altered with age. Mehra et al., (2005) described a significant decrease in the number of ERα and ERβ positive neurons in both CA1 and CA3 along with a decrease in ERα and ERβ protein level in rats (the authors did not discuss or show results for DG). In the mouse, researchers found a decrease in ERβ mRNA and protein in the cortex while ERα levels remained constant during aging (Thakur and Sharma, 2007). A comparison of ER expression in the hippocampus of young and aged mice has not been done but similar results in the cortex have been reported in rats (Wilson et al., 2002). Moreover, it is believed that the loss of estrogen receptors is due to the loss of protein rather than loss of neurons. Rapp and Gallagher (1996) demonstrated that there is not a decrease in neuron numbers in the hippocampus of aged rats with spatial learning deficits. Therefore, the age related alteration of ERs in the hippocampus may leave it more susceptible to deterioration by certain excitotoxic and oxidative insults.

Together, the results suggest that EB can delay aging when initiated in middle-age. Therefore, the project presented in Chapter 2 was designed to test the hypothesis that EB will reverse age-related cognitive decline and gene expression in middle-age female mice. Furthermore, the critical period theory suggests that EB treatment initiated at middle-age will be beneficial to cognitive function but treatment initiated later in life may be detrimental to cognitive function. Chapter 3 was designed to test this theory by comparing hippocampal gene expression response to an acute EB treatment in young, middle-age, and aged female mice.

While the results of Chapter 2 and 3 found that EB treatment initiated in middle-aged maintained hippocampal response to EB, aged animals display a reduced response to EB treatment. In this regard, Chapter 4 was designed to test the theory that the loss of ERs is contributing to a reduced response to EB.

CHAPTER 2
ESTROGEN EFFECTS ON COGNITION AND HIPPOCAMPAL TRANSCRIPTION IN MIDDLE-AGED MICE

Introduction

The hypothesis that estrogen therapies slow cognitive decline in normal aging and Alzheimer's disease continues to draw debate. Research in humans (MacLennan et al., 2006; Sherwin, 2006), primates (Lacreuse et al., 2002; Rapp et al., 2003), and rodents (Foster et al., 2003; Frick et al., 2002; Gibbs, 2000; Markham et al., 2002; Markowska and Savonenko, 2002) indicate that estrogen replacement may prevent or delay memory impairments, if initiated during middle-age, while reduced benefits of estrogen replacement are observed with increasing age (Foster et al., 2003). This suggests that middle-age represents a time limited window for estrogen's protective influence.

Research indicates that estrogen can influence cell growth and synaptic connectivity of the hippocampus in a manner opposite that observed during aging (Adams and Morrison, 2003; Foster, 2005; Gibbs and Aggarwal, 1998; McEwen et al., 1997; Sandstrom and Williams, 2001). Interestingly, estrogen improves memory function examined several days after treatment, suggesting the involvement of long-term and possibly genomic mechanisms (Markowska and Savonenko, 2002; Rapp et al., 2003; Sandstrom and Williams, 2001). However, the examination of genomic regulation is complicated by the fact that estrogen can influence transcription through nuclear receptors (e.g. ERα and ERβ) and rapid signal transduction cascades which influence the activity of several transcription factors. Moreover, age dependent changes in the expression of estrogen receptors and signaling cascade activity further complicate the study of estrogen's genomic mechanisms (Foster, 2005). The overwhelming complexity of estrogen signaling emphasizes the limits of studies that focus on a single gene or a single age.

Gene array technology provides a powerful tool for examining multifaceted transcription processes through the ability to monitor parallel expression of thousands of genes. However, the power of this technique is limited by the increased chance for Type I error associated with multiple comparisons. The current study uses microarray technology to test the hypothesis that estrogen interacts with age to influence transcription in the hippocampus. We employed a system of filtering in order to limit Type I error and verified age and estrogen sensitivity for a subset of genes.

Material and Methods

Animals

Procedures involving animal subjects have been reviewed and approved by the Institutional Animal Care and Use Committee and were in accordance with guidelines established by the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. A total of sixty-seven female C57BL/6 mice (young: n = 28, 3-5 months; middle-aged: n = 39, 11-13 months) were obtained from National Institute of Aging. Animals were housed 3-5 per cage and maintained on 12:12 light: dark cycle. Following at least one week of habituation, mice were anesthetized (2 mg ketamine and 0.2 mg xylazine per 20 grams of body weight) and ovaries were removed through a small midline incision on the abdomen. All mice received ad lib access to food (Purina mouse chow, St Louis, MO) and water, until the surgery when they were placed on Casein based chow (Cincinnati Lab Supply, Cincinnati, OH), which is low in phytoestrogens found in soy based chow.

Hormone Administration

Following surgery, mice were separated into four groups: young receiving β-estradiol 3-benzoate (Sinagra et al.) (n = 10), young receiving oil (n = 18), middle-aged receiving EB (n = 18), middle-aged receiving oil (n = 21). EB (Sigma) was dissolved in light mineral oil (Fisher

Scientific, Pittsburgh, PA) to concentration of 0.5 mg/ml. Oil and EB (5µg) were injected subcutaneously at the nape of the neck in volumes of 0.05 ml. Injections were given on two continuous days of a five day cycle. The schedule for a series of eight cycles of injections and behavioral training is illustrated in Figure 2-1. Briefly, injections were initiated one week after surgeries and continued for a total of eight cycles during which animals were behaviorally tested on the water maze, starting 48 hr after completion of cycle five injections. A subset of animals employed for confirmation of cDNA microarray data received eight cycles of treatment in the absence of behavioral training. At 24 hr following the final injection of oil or EB, all animals were anesthetized with CO_2 and decapitated. The brain was quickly removed and placed in ice cold artificial cerebral spinal fluid. Both hippocampi were removed, frozen in liquid nitrogen, and stored at -80ºC. In some cases, uteri were also excised, excess tissue was removed and wet weight of uteri was immediately measured.

Water Maze

A circular black plastic pool (120 cm diameter) was filled with water (29 ±2°C, colored white with nontoxic white paint) to a level of 8 cm below the rim of the tank. The water maze task was located in a well-lit testing room. During testing on the cued discrimination task the pool was surrounded by a black curtain. The curtain was pulled back during spatial discrimination training in order to expose spatial cues in the room. A camera mounted above the center of the pool tracked the animal's movement.

Cue Discrimination Training

Behavioral training began when animals were approximately 5 months and 12 months of age and was preformed during the light phase of the cycle. The cued discrimination training began during week four of hormone replacement, 48 hr after the fifth set of injections. A white flag was attached to a circular escape platform (10 cm diameter). Initially, mice were given a

habituation swim in which they were placed in the pool and after 30 sec they were guided to the platform and permitted to climb up on the platform where they remained for 15 sec. Following habituation, mice received cued discrimination training consisting of four blocks with three trials per block. Each animal was released from one of four equally spaced starting locations (N, S, E, and W). The location of the escape platform and the release point were randomly assigned for each trial. The position of the escape platform and release point for each trial was kept consistent between animals. The mouse was allowed to swim until it located the escape platform. If the mouse failed to escape to the platform in 60 sec, it was then guided to the platform. After every trial, the animal remained on the platform for 15 sec. Between blocks, the animals were towel dried, placed back in the home cage, and warm air was blown over the cages. The inter-block interval was 15-20 min.

Spatial Discrimination Training

For spatial discrimination, the platform was localized just below the surface of the water and maintained in one quadrant (the goal quadrant) for the duration of testing. Training was conducted over three consecutive days and began in week five of the injection schedule, 48 hr after the sixth set of injections. Mice received four blocks of training per day, each block consisting of three trials. The release point for each trial was randomly assigned. The animal had 60 sec to escape during the trial. If the mouse did not escape within the allotted time they were guided to the platform and allowed to rest for 15 sec. A probe trial was performed during the penultimate trial for each day to determine the extent of learning. In addition, a probe trial to examine retention was conducted for the first trial on days two and three of spatial training. For probe trials, the platform was removed and the animal was released from the quadrant opposite the goal quadrant and allowed to swim freely for 60 sec. Following completion of each probe

trial, the platform was returned to the pool and the animal placed on the platform for 15 sec to rest.

Behavioral data for cue and spatial discrimination tasks were recorded and analyzed by Water Maze 4.31 (Columbus Instruments, Columbus, OH). Measures included latency and path length to escape from the pool and time spent near the pool wall (thigmotaxis) during each trial. For probe trials, the time spent searching the goal quadrant and number of goal platform location crossings was determined.

cDNA Microarray

Microarray analyses were performed on hippocampal tissues from each of the same behaviorally characterized animals (one chip per animal). Hippocampal RNA was isolated using Qiagen RNeasy Lipid Tissue Mini Kit (Qiagen, Germantown, MD) and RNA quality was analyzed on a subset of representative samples using Agilent Bioanalyzer (Santa Clara, CA). m17K mouse cDNA arrays were obtained from the JHU/NIA Microarray Facility (Bethesda, MD). Microarray procedure protocols used in the study are described in the National Institute on Aging Gene Expression and Genomic Unit website (http://www.daf.jhmi.edu/microarray/protocols.htm). Briefly, 5 μg total RNA from a single animal was reverse transcribed in the presence of ^{33}P-dCTP, labeled cDNA was purified using QIAquick Nucleotide Removal Kit (Qiagen), diluted in hybridization buffer and hybridized to the m17k array for 16–18 h at 55 °C with rotation. Hybridized arrays were washed with 2 X SSC and 0.1% SDS one to two times for 15 min each at 65ºC followed by one to two washes of 1 X SSC and 0.1% SDS at 65ºC for 15 min each. The arrays were exposed to phosphoimager screens for 48 hr and scanned in a Molecular Dynamics Storm Phosphor Imager (Molecular Dynamics, Sunnyvale, CA) at 50 μm resolution. Array-Pro Analyzer (Media Cybernetics, Silver Spring, MD) was used to extract the log of the net signal (raw data minus background) from the scanned

images. The data were exported into Microsoft Excel spreadsheets and converted to standard scores (z-score = (probe signal – mean signal for all probes across the array)/standard deviation of all probes across the array).

Oligonucleotide Array

Custom designed oligo GEArray® Microarrays were obtained form SuperArray Bioscience Corporation (Frederick, MD). The custom arrays were designed to contain two or three probes for each gene of interest. Custom array procedures were followed according to SuperArray protocol (User Manual part #1018A version 3.0, 2005). Briefly total of 3 μg of total RNA from a single animals was used to make cRNA. cRNA purification was performed using ArrayGrade cRNA Cleanup Kit (SuperArray). UV spectrophotometry was used to quantify and assess the quality of cRNA. A total of 2 μg of biotin-labeled cRNA was hybridized rotating in 0.75 ml of pre-warmed GEAhyb Hybridization Solution (SuperArray) over night at 60°C. After overnight hybridization, membranes were washed with 5 ml of 2X SSC and 1% SDS for 15 min at 60°C and with 0.1X SSC and 0.5% SDS for 15 min at 60°C and incubated with streptavidin-AP conjugate (SuperArray,) (1:8,000). Array images were developed using CDP-star chemiluminescent substrate and imaged using a CCD camera (Chemic Doc XRS, Bio-Rad Laboratories, Hercules, CA). Images were analyzed using Array-Pro and data were exported to Microsoft Excel spreadsheets. The background signal was subtracted from the probe signal to provide net expression. The net expression for each probe of a specific gene was averaged across probes for that gene and the averages were normalized to the averaged signal derived from an internal cyclophilin A standard on the same membrane. Thus, the expression was calculated using the following formula:

mRNA expression = [(average gene signal - background signal)/ (average cyclophilin A signal - background signal)].

RT-PCR

In some cases, RNA from animals employed in the oligonucleotide array study was used for RT-PCR. RNA was converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Briefly 3 μg of total RNA from a single animal was incubated with appropriate reagents at 25°C for 10 min and then heated to 37°C for 120 min using 7300 Fast Real Time PCR System (Applied Biosystems). For relative quantification of RNA, 2.5 μl of cDNA was added to 12.5 μl of TaqMan® Universal PCR Master Mix (2X), 1.25 μl of 20X Gene Expression Assay Mix, and 8.75 μl of nuclease-free water for a total volume of 25 μl. The TaqMan® probes used for RT-PCR were selected from the Applied Biosystems Library and included; Histone deacetylase 2 (Hdac2) (TCAGTTGCTGGGGCTGTGAAATTAA), assay identification number Mm00515108_m1), Longevity assurance homolog 2 (Lass2) (GCACCGGACGCCGAGATGCTCCAGA), assay identification number Mm00504086_m1), POU domain, class 3, transcription factor 1 (Pou3f1) (GCAGCGGTGCCTCCGGCGCGCAGTT), assay identification number Mm00843534_s1), and the probe for Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as an internal control (TGAACGGATTTGGCCGTAATTGGGCG), assay identification number Mm99999915_g1). Thermal cycle conditions were set at 2 min at 50°C, 10 min at 95°C and cycled between 15 sec at 95°C and 1 min at 60°C for 40 cycles. Relative quantification was determined with 7300 Fast Real-Time PCR System and SDS Software 1.3.1 analysis software (Applied Biosystems). Each sample was examined in triplicate and the relative quantities (Liu et al.) were normalized by the level of Gapdh. The three RQ values were averaged for each animal and the means for animals in the treated groups were normalized by the mean for the control samples (young oil treated) in order to derive the fold change.

Statistical Analysis

In general, repeated measures analyses of variance (ANOVAs) were used to establish main effects and interactions on behavioral measures. Follow-up ANOVAs either collapsing the data across days of training or examining behavior within each day were employed to localize specific differences.

For analysis of cDNA microarrays, the data underwent a two phase filtering process. The first phase was designed to exclude probe sets in which a substantial number of chips did not exhibit reliable detection/hybridization and remove probes with uncertain function. This was done by first calculating the mean and standard deviation of the blank probe sets for each microarray. A cut off was chosen as three standard deviations above the mean for the blanks. Scores below this value were considered as lacking specific hybridization. The probe was considered absent for a treatment group if the majority, greater than 60%, of the arrays in this group exhibited hybridization below this cut-off. Finally, the probe set was removed from further consideration if the probe set was judged absent for at least three of the four treatment groups. The remaining probe sets were further filtered to remove expressed sequence tags and probes for hypothetical proteins and pseudo genes that did not have an indication of biological or molecular function as expressed through gene ontology (GO). Finally, when a gene was represented by multiple probes, the probes for the gene were averaged for each animal and the averages were used as measures of expression.

To generate a list of likely age or estrogen sensitive genes, a second filter was employed using statistical sorting. The alpha level was set at 0.025 in accordance with previous studies (Blalock et al., 2003) and *t*-tests were used to detect differences associated with age or treatment. Once a subgroup of genes was identified as probable age sensitive, the stringency of the alpha value was reduced (e.g. $p < 0.01$) to examine the effects of the other variable (e.g. EB treatment)

for this subgroup of genes. False discovery rate (FDR) was calculated as the number of expected false positives/number of genes observed to change.

Results

Uterine Weight

Uterine weight was measured in a subset of animals (middle-aged EB treated (n = 14) 140 $\pm$ 6 mg; middle-aged oil treated (n = 14) 40 $\pm$ 6 mg; young EB treated (n = 5) 106 $\pm$ 7 mg; young oil treated (n = 13) 39 $\pm$ 4 mg). An ANOVA indicated a significant effect of age [$F(1,42) = 7.6$, $p < 0.01$] and treatment [$F(1,42) = 174.3$, $p < 0.0001$].

Cue Discrimination

Forty-seven mice received the treatment schedule outlined in Figure 1 (young EB = 10, young oil = 12, middle-aged EB = 11, middle-aged oil = 14) and were tested for cue and spatial discrimination. Figure 2-2 illustrates the performance on the cue task. Two-way repeated measures ANOVAs across the training blocks indicated a significant effect of training on escape latency [$F(3,129) = 32.6$, $p < 0.0001$] in the absence of age or treatment effects. A significant effect of training [$F(3,129) = 20.5$, $p < 0.0001$] on escape path length was observed in the absence of age or treatment effects. No age, treatment, or training effects were observed for swim speed (data not shown).

Spatial Discrimination

The behavioral measures indicated improved performance across days of training for all groups. In addition, age x treatment interactions were observed due to poorer performance by middle-aged oil treated mice. An ANOVA on escape latency (Figure 2-3A) indicated a significant effect of training [$F(2, 86) = 41.2$, $p < 0.0001$] (Figure 2-3A) and an interaction of day x age x treatment [$F(2, 86) = 4.5$, $p < 0.05$]. Examination within each day indicated a

tendency for an age x treatment interaction on Day 3 ($p = 0.06$), due to longer escape latencies for middle-aged oil treated mice.

Additional evidence for an age x treatment interaction was provided by analysis of escape path length (Figure 2-3B). An ANOVA revealed significant effects of training across days [$F(2, 86) = 35.0$, $p < 0.0001$] and a day x age x treatment interaction [$F(2, 86) = 6.7$, $p < 0.005$]. Examination within each day indicated an age x treatment interaction on Day 3 [$F(1, 43) = 5.0$, $p < 0.05$], again with the poorest poor performance observed in the middle-aged oil treated group. Finally examination of the percent of the escape latency time the animals spent along the wall (i.e. thigmotaxis) decreased over days of training $F(2, 86) = 27.0$, $p < 0.0001$] in the absence of age or treatment effects (Figure 2-3C).

Acquisition Probe Trials

Probe trial measures confirmed that middle-aged oil treated mice were impaired in acquiring the spatial discrimination. A repeated measures ANOVA for percent time in the goal quadrant for the three acquisition probe trials demonstrated a significant effect of training [$F(2,86) = 25.1$, $p < 0.0001$] and an interaction of day x age x treatment [$F(2,86) = 3.3$, $p < 0.05$]. ANOVAs within each day revealed an age x treatment interaction on Day 2 [$F(1,43) = 7.2$, $p < 0.01$] and ANOVAs within each age and treatment condition indicated an age difference for oil treated mice [$F(1,24) = 9.5$, $p < 0.01$] with poorer performance for middle-aged mice (Figure 2-4A).

A repeated measures ANOVA for platform crossings indicated a main effect of training [$F(2,86) = 22.6$, $p < 0.0001$] and age [$F(1,86) = 4.6$, $p < 0.05$] and a training x age x treatment interaction [$F(2,86) = 3.3$, $p < 0.05$]. ANOVAs within each day revealed an age x treatment interaction on Day 1 [$F(1,43) = 4.2$, $p < 0.05$] and Day 2 [$F(1,43) = 6.0$, $p < 0.05$] and ANOVAs within each age and treatment condition indicated an age difference for EB treated mice on Day

1 [$F(1,19) = 8.5$, $p < 0.01$] and confirmed poorer performance of middle-aged oil treated animals on Day 2 [$F(1,24) = 9.5$, $p < 0.005$] (Fig 4B).

Retention Probe Trials

An ANOVA examining the percent time in the goal quadrant during the retention probe trials delivered as the first trial on Days 2 and 3 indicated a significant effect of training [$F(1,43) = 5.3$, $p < 0.05$] and a significant age x treatment interaction [$F(1,43) = 5.1$, $p < 0.05$]. Analyses within each day revealed differences on Day 2 due to poorer performance by middle-aged oil treated mice such that treatment effects were observed for middle-aged animals [$F(1,23) = 5.1$, $p < 0.05$] and age difference were observed for oil treated mice [$F(1,24) = 8.9$, $p < 0.01$] (Figure 2-4C).

Examination of platform crossings during the retention probe trials confirmed age x treatment effects (Figure 2-4D). An ANOVA indicated a main effect of training [$F(1,42) = 6.7$, $p < 0.05$] and a tendency for an age x treatment interaction ($p = 0.07$). ANOVAs for each day localized an age x treatment interaction to the Day 2 probe trial [$F(1,42) = 8.8$, $p < 0.005$]. Analysis of treatment effects during the Day 2 retention probe trial indicated more crossings in middle-aged EB treated mice relative to aged matched oil treated mice [$F(1,22) = 4.7$, $p < 0.05$]. Examination of age differences indicated an age effect for oil treated mice [$F(1,24) = 9.9$, $p < 0.005$].

Microarray Results

Microarray analyses were performed on hippocampal RNA from each of the behaviorally characterized animals (one chip per animal). cDNA microarray data were not obtained for 10 animals due to poor hybridization or poor quality of RNA. The remaining 37 microarrays (young EB = 10, young oil = 8, middle-aged EB = 9, middle-aged oil = 10) were submitted to the first phase filtering process to eliminate probes with low hybridization and uncertain biological

function. This initial filtering process resulted in 4217 probes that were eligible for the second phase of filtering to identify likely genes that are sensitive to age and treatment effects. Our previous work and that of others suggests that a substantial number of genes are altered between young and middle-aged animals. In order to examine the relative influence of age and treatment, *t*-tests with alpha set at 0.025 were used to estimate the number of genes influenced by age regardless of treatment. An age difference was observed for 567 probes. In contrast, with alpha set at 0.025, only 187 probes exhibited a treatment effect regardless of age. An examination of treatment effects within each age group suggested that treatment effects were more common in middle-aged animals. Only 58 probes exhibited differences between oil and EB treatment in young animals, while 244 probes were influenced by EB treatment in middle-aged animals.

Our interest is for genes which exhibit EB effects in a manner opposite that observed during aging. If EB has effects opposite that of age, it is likely that the probes of interest are missed by collapsing across treatments or ages. Accordingly, in order to identify candidate aging genes for further analysis, we used statistical sorting according to expression differences between middle-aged oil treated and young oil treated mice, before examining EB effects only in middle-aged mice. Using *t*-tests with alpha set at 0.025, the number of probes expected to reach significance by chance alone is 105. A comparison indicated that 570 probes (FDR = 0.18) were differentially expressed in oil treated middle-aged mice relative to oil treated young mice. These 570 probes were classified as potential age-related genes, and this set of genes was used to test for EB effects. Because we were only interested in effects opposite that of aging, we employed one tailed *t*-tests (alpha set at 0.025) with the direction specified as opposite that observed for aging and compared middle-aged oil treated and middle-aged EB treated mice. For the 570 potential age-related genes, 132 probes (FDR = 0.1) were observed to exhibit transcription

changes in a manner opposite that observed for aging. Gene expression profiles of the 132 age-EB sensitive genes were examined using the Unigene EST Profile Viewer from the National Center for Biotechnology Information and a cut off for expression for the gene of interest was set at $\geq 10^{-4}$ out of every one million transcripts normally found in neural tissue, brain or dorsal root ganglion. This procedure resulted in 119 age-EB sensitive genes that are moderately to highly expressed in the brain (Table 2-1&2-2). Using gene ontology, Swiss-Prot protein knowledgebase, and literature searches, the age-EB sensitive genes were characterized according to likely biological processes. In most cases, genes could be categorized as involved in more than one biological function; however, the majority could be classified as involved in transcription, apoptosis/cell health, receptor/cell signaling pathways, cell growth/structural organization, cholesterol/lipid metabolism, and protein metabolism.

In order to provide some validation of the findings, custom oligonucleotide arrays containing probes for 10 representative age-EB sensitive genes and a cyclophillin A (Ppia) control were constructed. The genes were selected in order to have a balance of those that exhibited an increase (5 genes) and decrease (5 genes) with age. In addition, genes were selected to represent the various biological processes including transcription (Ldb2, Hdac2, Pou3f1), apoptosis/cell health (Foxo3a), receptor/cell signaling (Kctd3, Cbln1), cell growth/structural organization (Ppfia1), cholesterol/lipid metabolism (Lass2), and protein metabolism (Fbxw8, Ube2r2). RNA was obtained from an independent set of young oil (n = 6), middle-aged oil (n = 7), and middle-aged EB treated (n = 7) mice which received the same ovariectomy-injection schedule (see Fig 1) in the absence of behavioral training and cRNA was hybridized to the oligonucleotide arrays.

For two probes (Ppfia1, Ube2r2) hybridization of at least 8 of the 20 chips exhibited an absence of signal and data were considered unreliable for parametric statistical analysis. The signal for the remaining genes was normalized by the level of Ppia on the array. These normalized scores were then divided by the mean for young animals in order to determine directional changes and fold changes. Predicted direction of change due to age or EB treatment was based on results of the cDNA arrays. For the remaining 8 genes, the predicted direction of change associated with age, increasing or decreasing in middle-aged oil treated mice relative to young oil treated mice, was confirmed for all genes except Pou3f1, in which the mean response for middle-aged oil treated mice was elevated relative to young oil treated mice (Figure 2-5). Furthermore, the predicted direction of change for EB treatment in middle-aged EB treated mice relative to middle-aged oil treated animals was observed in 8 of the 8 genes. Thus, for the set of eight genes, the relative direction of change due to age and EB treatment could be predicted correctly ~94 % of the time.

Genes were separated according to the expected directional influence of age and EB and repeated measures ANOVAs were employed to examine group effects. A repeated measures ANOVA across the 5 genes expected to increase with age and decrease with EB treatment (Ldb2, Foxo3a, Hdac2, Lass2, Kctd3) indicated a difference across groups [$F(2,68) = 4.10$, $p < 0.05$]. Subsequent ANOVAs comparing young oil treated relative to middle-aged oil treated indicated a significant [$F(1,44) = 6.91$, $p < 0.05$] increase in expression with age. Although, the mean for each gene was reduced in the middle-aged EB treated group relative to middle-aged oil treated animals, an ANOVA examining EB treatment effects in middle-aged animals indicated no difference. For three genes (Fbxw8, Pou3f1, Cbln1) expected to decrease with age and increase with EB treatment, a repeated measures ANOVA indicated a difference across the genes

[$F(2,34) = 5.24$, $p < 0.05$] and subsequent ANOVAs indicated a treatment effect in aged animals due to an increased expression associated with EB treatment [$F(1,24) = 10.67$, $p < 0.01$].

An examination of the oligonucleotide arrays revealed several genes that were expected to exhibit at least a 2 fold increase in middle-aged oil or EB treated animals compared to young oil treated mice (e.g. Hdac2, Lass2). Thus, RNA isolated for the oligonucleotide array study was also employed for RT-PCR studies (3-4 animals per group) to further validate the gene profiling results (Table 2-3). The results confirmed that histone deacetylase 2 and LAG1 longevity assurance homolog 2 increased by at least 2 fold in middle-aged oil animals. Furthermore, RT-PCR of octamer-binding transcription factor 6 (Pou3f1) confirmed an increased expression in middle-aged animals treated with EB relative to young oil treated mice.

Discussion

While middle-aged oil treated mice exhibited learning over the course of training, age and treatment interactions were observed for spatial discrimination escape latency and path length, as well as probe trial measures due in part to poorer performance by middle-aged oil treated mice. In contrast, the performance of middle-aged EB treated animals was similar to young mice and treatment effects were not observed for younger animals. The poor performance of middle-aged oil treated mice was not due to sensory-motor deficits since there was no difference in latency or swim speed on the cue discrimination task. Furthermore, no age or treatment effects were observed for thigmotaxis, measured as the percent time swimming along the pool wall, suggesting that the disparity in performance was not due to differences in anxiety.

The results are consistent with previous studies in humans (Foster, 2006) and rodents (Verbitsky et al., 2004; Ziegler and Gallagher, 2005), which indicate mild cognitive impairments emerge in mid-life and cognitive weakening continues with advancing age. Further, age-related impairments may be enhanced by hormone deprivation, and estrogen treatment in humans

(MacLennan et al., 2006; Sherwin, 2005) and animals (Daniel et al., 2006; Foster et al., 2003; Frick et al., 2002; Markham et al., 2002; Markowska and Savonenko, 2002) may delay the decline of certain cognitive processes. Interestingly, beneficial effects of estrogen treatment may not be evident if the treatment is initiated after long-term hormone deprivation or the behavioral demands of the task do not reveal an age difference (Daniel et al., 2006; Markowska and Savonenko, 2002; Ziegler and Gallagher, 2005). The interaction of aging and hormonal status on cognition suggests that middle-age may provide an important window for examining hormonal influences on markers of brain aging.

Gene Profiles Associated with Aging and Estradiol Treatment

For cDNA arrays examined across treatment groups, 567 probes exhibited age differences; confirming that a considerable number of genes alter their expression during middle-age. In contrast, only 187 genes exhibited altered expression in response to EB treatment, independent of age, suggesting that a relatively low number of hippocampal genes are sensitive to our EB treatment. However, the effects of age are relatively chronic, and the number of EB responsive genes, and magnitude of EB effects, is likely to be a function of the time between treatment and sample collection. Furthermore, when EB effects were examined within each age group, the number of probes increased four fold between middle-aged animals relative to young animals (244 versus 58 probes) indicating that older animals are more sensitive to hormonal status.

Treatment Effects on Transcription in the Aging Hippocampus

While the set of EB responsive genes in aged animals is likely to be important for determining treatment effects on hippocampal function, the current study focused on a subset that were age and EB responsive. Age-EB responsive genes were distinct from those normally seen with learning, which often involve synapse specific molecules (Cavallaro et al., 2002; D'Agata and Cavallaro, 2003; Irwin, 2001; Leil et al., 2003; Luo et al., 2001). The difference

might be due to the fact that hippocampi were harvested several days after training when training effects may have dissipated. Thus, the set of age-EB genes may contribute to overall hippocampal function rather than being induced by behavioral training. Estrogen has enduring effects on synaptic plasticity, memory, the growth, and vitality of neurons, processes which depend on transcriptional regulation. Microarray results indicate hormonal status of middle-aged animals regulates transcriptional mechanisms; including an EB associated reduction in transcription repressors (Hdac2, Zik1, Sap18) and an increase in products which enable transcription/translation (Gtf3c2, Bop1, Sfrs7, Tcf12) including Mms19l, a possible estrogen receptor coactivator of transcription (Wu et al., 2001). The findings suggest EB influenced the production of components of the transcription apparatus.

Estrogen modulates structural plasticity of dendritic spines (Gould et al., 1990; Li et al., 2004), which maybe age specific (Miranda et al., 1999). EB treatment was associated with genes for biosynthesis (Gmppa, Axot, Rpl23), protein folding (Tcp1, Cct6a, Erp29), and vesicle/protein transport (Copg, Vps28, Vps33a, Rab11fip2, Kifc2). An increase was observed for genes associated with growth (Nrp1, Emp1, Sema3e) (Pozas et al., 2001; Wulf and Suter, 1999), structural organization (Bsg, Lrtm1, Mical2, Flnb) (Fan et al., 1998; Lauren et al., 2003; Naruhashi et al., 1997; Sheen et al., 2002; Terman et al., 2002; Zhang et al., 1998), regulation of neural connections and glutamate receptor trafficking (Pcdhgc3, Lmtk2, Ppfia1) (Dunah et al., 2005; Kawa et al., 2004; Ko et al., 2003; Serra-Pages et al., 1998; Wu and Maniatis, 1999). Finally, neurogenesis is altered by aging and estrogen (Galea et al., 2006; Kempermann et al., 2004; Saravia et al., 2007) and we observed a shift in expression of genes supporting neurogenesis, dendritic formation, and synaptogenesis (Pou3f1, Vldlr, Adam19, Tcf12, Emp1) (Frantz et al., 1994; Kurisaki et al., 2002; Niu et al., 2004; Sinagra et al., 2005; Uittenbogaard

and Chiaramello, 2002; Wulf and Suter, 1999). Together, these results suggest estrogen's actions on hippocampal function in middle-age include trophic influences.

Brain aging involves enhanced inflammation and stress responses (Prolla, 2002; Verbitsky et al., 2004) and EB treatment is neuroprotective (Singh et al., 2006). Middle-aged mice exhibited reduced expression of genes for preventing oxidative (Akr1c13) and ischemic damage (Egln2), as well as genes preventing damage from inflammation (C1qbp). EB increased transcription of these protective proteins, and reversed an elevation in the expression of genes associated with stress (Foxo3a, Rps9, Myd116) and inflammation (Socs7). EB also increased the expression of mRNA coding for enzymes involved in DNA repair (Mms19l, Giyd2, Polb), ubiquitin ligase activity, and protein degradation (Derl3, Fbxw8, Psmd3, Psmb5, Ube2r2). This effect is consistent with studies indicating that improved performance in aged animals is associated with transcriptional up-regulation of genes associated with the proteasome (Blalock et al., 2003; Burger et al., 2007). Furthermore, an increased expression of Fbxw8 has been associated with memory consolidation in young animals (Cavallaro et al., 2001). Thus, an EB dependent increase in neuroprotective genes may contribute to maintenance of hippocampal function with age.

Some aging-EB responsive genes have been linked to premature aging (Tbl2, Il13ra1), transcriptional changes in the hippocampus of senescence-accelerated mice (Dusp12) (Cheng et al., 2007; Kyng et al., 2003; Meng et al., 1998), and aging in different organisms (Lass2) (Obeid and Hannun, 2003), or organs (Peli1) (Chelvarajan et al., 2006; Cheng et al., 2004). Some genes may be associated with biological markers of aging including altered lipid/cholesterol metabolism (Abca2, Vldlr, Acsl5, Srebf1, Apoa1) (Foster, 2006), lipid composition (Ptdss2) (Giusto et al., 2002), mitochondrial function (Atp5g1, Aco2) (Poon et al., 2006), and structural

changes (Ctsb) (Bednarski et al., 1997). Indeed, specific genes may be markers for an aging hippocampus. Similar changes were found in CA1 of aged rats, including decreased Ilkap (i.e. protein phosphatase 2C), Polb, Bsg, and Cct6a and an increase in Nrp1(Blalock et al., 2003). Importantly, this transcriptional profile was reversed by EB treatment in middle-aged females.

Many of the aging-EB responsive genes can be classified under one of the following cell signaling processes: cytokine signal transduction (Socs7, Il13ra1, Peli1, Irak4), G-protein coupled receptor signaling (Adcyap1r1, Gpr125, Cbln1, Tmem11, Trh), and phosphatase/kinase activity (Ilkap, Lmtk2, Adck1, Hs1bp3, Dusp12, Irak4, Nagk, Pace4, Ppfia1, Ppp1r2). Age-related changes in these signaling cascades may contribute to altered synaptic plasticity and memory impairments (Foster, 1999; Lynch, 1998). Thus, estrogen influences on these signaling pathways may preserve physiological processes involved in memory (Foster, 2005).

EB treatment of ovariectomized middle-age mice reversed transcriptional markers of brain aging, which have been previously described. It may be important that altered transcription emerges by middle-age, prior to cognitive decline, possibly in response to oxidative stress, or altered neural activity (Blalock et al., 2003; Lu et al., 2004). Unchecked, these processes may initiate cascades for altered cell signaling and transcriptional regulation, resulting in a decrease in neuronal growth and enhanced inflammation. Thus, middle age may be a critical period for treatments that can delay aging processes that begin in middle-age and cumulate over time to weaken cognition. In this regard, it may be important the EB treatment of middle-aged animals counteracts changes in transcription for proteins associated with cell signaling cascades, and reverses a decline in markers for neuroprotection, biosynthesis, and neurite growth.

Table 2-1 Microarray results for genes increasing with age and decreasing with EB treatment

Gene Name	Gene Symbol	Mean Z-Score MA-EB	Mean Z-Score MA-Oil	Mean Z-Score Y-EB	Mean Z-Score Y-Oil	Age Effect	P value	EB Effect	P value
General transcription factor IIH, polypeptide 3	Gtf2h3	-0.49	0.15	-0.46	-0.81	Up	0.00001	Down	0.02
ATP-binding cassette, sub-family A, member 2	Abca2	-0.73	-0.09	-0.88	-0.92	Up	0.0001	Down	0.0025
Methylcrotonoyl-Coenzyme A carboxylase 2	Mccc2	-0.54	-0.01	-0.6	-0.8	Up	0.0001	Down	0.01
Zinc finger protein interacting with K protein 1	Zik1	-0.39	0.4	-0.39	-0.57	Up	0.0002	Down	0.0025
Transducin (beta)-like 2	Tbl2	0.39	0.7	-0.08	-0.03	Up	0.0002	Down	0.01
Translocase of inner mitochondrial membrane 50 homolog	Timm50	-0.96	0.07	-0.25	-1.12	Up	0.0002	Down	0.0025
Sin3-associated polypeptide 18	Sap18	-0.66	0.01	-0.76	-0.84	Up	0.0002	Down	0.01
Histone deacetylase 2	Hdac2	-0.56	-0.02	-0.61	-0.66	Up	0.0004	Down	0.001
Cleavage stimulation factor, 3' pre-RNA, subunit 1	Cstf1	0.52	0.73	0.04	0.09	Up	0.0005	Down	0.02
Protein tyrosine phosphatase, receptor type, f polypeptide, interacting protein, alpha 1	Ppfia1	-0.53	-0.03	-0.72	-0.76	Up	0.0008	Down	0.02
Calcitonin gene-related peptide-receptor component protein	Crcp	-0.36	0.4	-0.29	-0.4	Up	0.0009	Down	0.01
Potassium channel tetramerisation domain containing 3	Kctd3	-0.03	0.35	-0.21	-0.16	Up	0.0009	Down	0.02
N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1	Ndst1	-0.93	0.09	-0.72	-0.77	Up	0.0009	Down	0.001
Adenylate cyclase activating polypeptide 1 receptor 1	Adcyap1r1	-0.66	-0.13	-0.56	-0.64	Up	0.001	Down	0.015
Myeloid differentiation primary response gene 116	Myd116	-0.9	-0.2	-0.84	-0.99	Up	0.001	Down	0.015
Forkhead box O3a	Foxo3a	-0.87	-0.31	-0.72	-0.9	Up	0.002	Down	0.02
LIM domain binding 2	Ldb2	-0.51	0.31	-0.45	-0.41	Up	0.002	Down	0.01
Fatso	Fto	-0.47	-0.09	-0.61	-0.29	Up	0.003	Down	0.0035

Table 2-1 Continued

Gene Name	Gene Symbol	Mean Z-Score MA-EB	Mean Z-Score MA-Oil	Mean Z-Score Y-EB	Mean Z-Score Y-Oil	Age Effect	P value	EB Effect	P value
Ribosomal protein S9	Rps9	-0.03	0.38	0.02	-0.11	Up	0.006	Down	0.02
G protein-coupled receptor 125	Gpr125	1.18	1.52	1.06	1.2	Up	0.01	Down	0.0035
TBC1 domain family, member 1	Tbc1d15	-0.11	0.29	-0.29	-0.06	Up	0.01	Down	0.005
RCC1 and BTB domain-containing protein 2	Rcbtb2	0.34	0.6	0.2	0.25	Up	0.01	Down	0.0025
Longevity assurance homolog 2	Lass2	-0.29	0.09	-0.09	-0.38	Up	0.02	Down	0.015
Suppressor of cytokine signaling 7	Socs7	0.83	1.17	0.73	0.66	Up	0.02	Down	0.02
Protocadherin gamma subfamily C, 3	Pcdhgc3	0.71	0.96	0.63	0.64	Up	0.02	Down	0.0025
Phosphatidylserine synthase 2	Ptdss2	2.08	2.67	2.08	2.19	Up	0.02	Down	0.00004
Ubiquitin specific peptidase 53	Usp53	-0.22	0.16	-0.28	-0.2	Up	0.02	Down	0.02
General transcription factor IIIC, polypeptide 4,	Gtf3c4	-0.29	0.27	-0.35	-0.21	Up	0.02	Down	0.02
Purkinje cell protein 4-like 1	Pcp4l1	-0.07	0.26	-0.13	-0.09	Up	0.02	Down	0.02
FGF receptor activating protein 1	Frag1	-0.27	-0.04	-0.26	-0.41	Up	0.02	Down	0.005
Zinc finger protein 426	Zfp426	0.02	0.31	-0.2	-0.26	Up	0.02	Down	0.005
Ankyrin repeat domain 25	Ankrd25	-0.33	0.2	-0.1	-0.33	Up	0.02	Down	0.015
Protein phosphatase 1, regulatory subunit 2	Ppp1r2	-1.07	-0.39	-0.77	-0.97	Up	0.02	Down	0.01

Table 2-2 Microarray results for genes decreasing with age and increasing with EB treatment

Gene Name	Gene Symbol	Mean Z-Score MA-EB	Mean Z-Score MA-Oil	Mean Z-Score Y-EB	Mean Z-Score Y-Oil	Age Effect	P value	EB Effect	P value
Interleukin-1 receptor-associated kinase 4	Irak4	1.36	0.51	1.35	1.22	Down	0.00008	Up	0.00035
EGL nine homolog 2	Egln2	1.5	0.78	1.63	1.49	Down	0.0002	Up	0.01
Natural killer tumor recognition sequence	Nktr	0.45	-0.08	0.11	0.43	Down	0.0002	Up	0.0004
Ubiquitin-conjugating enzyme E2R 2	Ube2r2	1.03	-0.17	0.76	1.16	Down	0.0002	Up	0.0015
Aldo-keto reductase family 1, member C13	Akr1c13	1.51	1.08	1.83	1.75	Down	0.0002	Up	0.02
Apolipoprotein A-I	Apoa1	1.66	1.03	1.76	1.78	Down	0.0003	Up	0.015
Cerebellin 1 precursor protein	Cbln1	1.46	0.74	1.56	1.47	Down	0.0008	Up	0.01
Histocompatibility 2, K region	H2-K1	2.25	1.88	2.32	2.3	Down	0.001	Up	0.02
Proteasome subunit, beta type 5	Psmb5	1.54	0.94	1.36	1.51	Down	0.001	Up	0.015
CNDP dipeptidase 2	Cndp2	1.86	1.31	2.09	1.9	Down	0.001	Up	0.02
Block of proliferation 1	Bop1	1.19	0.61	1.29	1.15	Down	0.001	Up	0.02
POU domain, class 3, transcription factor 1	Pou3f1	0.99	0.41	1.12	1.16	Down	0.001	Up	0.015
Glycerol-3-phosphate dehydrogenase 1-like	Gpd1l	0.65	0.16	0.75	0.76	Down	0.001	Up	0.01
RAN GTPase activating protein 1	Rangap1	1.52	0.58	1.59	1.37	Down	0.002	Up	0.002
Integrin-linked kinase-associated serine/threonine phosphatase 2C	Ilkap	1.98	1.35	2.06	2.14	Down	0.002	Up	0.025
Interleukin 13 receptor, alpha 1	Il13ra1	1.07	0.48	0.98	1.02	Down	0.002	Up	0.01
Thyrotropin releasing hormone	Trh	1.77	1.09	1.96	1.88	Down	0.002	Up	0.015
Nudix type motif 14	Nudt14	2.18	1.31	1.86	1.98	Down	0.002	Up	0.003
T-complex protein 1	Tcp1	1.42	0.71	1.58	1.47	Down	0.002	Up	0.01

Table 2-2 Continued

Gene Name	Gene Symbol	Mean Z-Score MA-EB	Mean Z-Score MA-Oil	Mean Z-Score Y-EB	Mean Z-Score Y-Oil	Age Effect	P value	EB Effect	P value
Polymerase beta	Polb	1.14	0.43	1.18	1.14	Down	0.003	Up	0.015
Leucine-rich repeats and transmembrane domains 1	Lrtm1	1.37	0.69	1.55	1.3	Down	0.003	Up	0.015
Semaphorin 3E	Sema3e	1.18	0.74	1.45	1.41	Down	0.003	Up	0.025
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c, isoform 1	Atp5g1	0.97	0.24	0.75	0.91	Down	0.003	Up	0.0003
Aconitase 2, mitochondrial	Aco2	1.22	-0.03	1.07	1.03	Down	0.003	Up	0.0035
Surfeit gene 5	Surf5	0.25	-0.18	0.33	0.3	Down	0.004	Up	0.01
Basal cell adhesion molecule	Bcam	1.64	0.65	1.31	1.57	Down	0.004	Up	0.01
Dual specificity phosphatase 12	Dusp12	1.6	0.88	1.67	1.62	Down	0.004	Up	0.02
Microtubule associated monoxygenase, calponin and LIM domain containing 2	Mical2	1.45	0.83	1.6	1.51	Down	0.004	Up	0.02
Ribosomal protein L23	Rpl23	1.01	0.79	1.08	1.03	Down	0.004	Up	0.005
Proprotein convertase subtilisin/kexin type 6	Pace4	0.87	0.36	0.78	0.84	Down	0.004	Up	0.015
Lupus brain antigen 1	Lba1	1.45	0.73	1.48	1.26	Down	0.004	Up	0.0035
Vacuolar protein sorting 28	Vps28	0.91	0.37	0.88	0.89	Down	0.004	Up	0.02
RAB11 family interacting protein 2	Rab11fip2	1.65	0.99	1.38	1.58	Down	0.004	Up	0.015
Neuropilin	Nrp1	0.27	-0.02	0.33	0.46	Down	0.005	Up	0.015
Ubiquitously transcribed tetratricopeptide repeat gene, X chromosome	Utx	1.33	0.96	1.48	1.46	Down	0.005	Up	0.025
Solute carrier family 21, member 2	Slco2a1	0.68	-0.34	0.65	0.72	Down	0.005	Up	0.02
F-box and WD-40 domain protein 8	Fbxw8	0.9	0.26	0.95	0.92	Down	0.006	Up	0.015

Table 2-2 Continued

Gene Name	Gene Symbol	Mean Z-Score MA-EB	Mean Z-Score MA-Oil	Mean Z-Score Y-EB	Mean Z-Score Y-Oil	Age Effect	P value	EB Effect	P value
Chaperonin subunit 6a; T-complex protein 1, zeta subunit	Cct6a	1.46	0.61	1.51	1.24	Down	0.007	Up	0.005
Histidine triad nucleotide binding protein	Hint1	1.31	0.73	1.34	1.2	Down	0.008	Up	0.015
Splicing factor, arginine/serine-rich 7	Sfrs7	0.66	0.22	0.58	0.7	Down	0.008	Up	0.02
MMS19-like	Mms19l	0.49	-0.07	0.35	0.4	Down	0.008	Up	0.00045
Transcription factor 12	Tcf12	0.66	0.22	0.58	0.7	Down	0.008	Up	0.015
AarF domain containing kinase 1	Adck1	-0.07	-0.53	-0.03	0.01	Down	0.01	Up	0.01
Pellino 1	Peli1	0.29	-0.17	0.29	0.32	Down	0.01	Up	0.01
GIY-YIG domain containing 2	Giyd2	1.08	0.25	0.87	0.75	Down	0.01	Up	0.0025
General transcription factor IIIC, polypeptide 2, beta	Gtf3c2	1.04	0.19	0.88	0.97	Down	0.01	Up	0.025
Filamin, beta	Flnb	1.1	0.47	1.13	1.05	Down	0.01	Up	0.01
Solute carrier family 35, member B4	Slc35b4	1.28	1.01	1.28	1.35	Down	0.01	Up	0.02
Endoplasmic reticulum protein 29	Erp29	0.91	0.43	0.86	0.82	Down	0.01	Up	0.025
Cathepsin B	Ctsb	0.88	0.47	0.88	0.75	Down	0.01	Up	0.01
ERBB receptor feedback inhibitor 1	Errfi1	1.44	0.83	1.43	1.33	Down	0.01	Up	0.01
Homeobox containing 1	Hmbox1	1.02	-0.64	0.86	0.9	Down	0.01	Up	0.01
Teashirt zinc finger family member 2	Tshz2	0.74	-0.92	-0.32	-0.08	Down	0.01	Up	0.00005
Nucleoporin 205	Nup205	1.67	1.15	1.21	1.61	Down	0.01	Up	0.01
Coatomer protein complex, subunit gamma 1	Copg	0.83	0.37	0.9	0.83	Down	0.01	Up	0.025

Table 2-2 Continued

Gene Name	Gene Symbol	Mean Z-Score MA-EB	Mean Z-Score MA-Oil	Mean Z-Score Y-EB	Mean Z-Score Y-Oil	Age Effect	P value	EB Effect	P value
Transmembrane protein 11	Tmem11	1.2	0.81	1.2	1.22	Down	0.02	Up	0.015
Limb region 1	Lmbr1	1.24	0.6	0.89	1.02	Down	0.02	Up	0.01
Very low density lipoprotein receptor	Vldlr	1.06	0.23	0.9	0.67	Down	0.02	Up	0.0035
Mannoside acetylglucosaminyltransferase 1	Mgat1	1.53	0.25	1.51	1.2	Down	0.02	Up	0.005
Epithelial membrane protein 1	Emp1	0.87	0.09	0.77	0.65	Down	0.02	Up	0.0045
Transmembrane protein 34	Tmem34	-0.22	-0.75	-0.39	-0.17	Down	0.02	Up	0.02
A disintegrin and metalloproteinase domain 19	Adam19	0.74	0.08	0.39	0.53	Down	0.02	Up	0.0025
N-acetylglucosamine kinase	Nagk	1.88	1.25	1.49	1.78	Down	0.02	Up	0.025
Complement component 1, q subcomponent binding protein	C1qbp	0.81	0.16	0.64	0.61	Down	0.02	Up	0.01
Proteasome 26S subunit, non-ATPase, 3	Psmd3	0.67	0.13	0.63	0.61	Down	0.02	Up	0.01
Axotrophin	Axot	0.99	0.11	1.07	0.96	Down	0.02	Up	0.02
Tubulin tyrosine ligase-like family, member 4	Ttll4	1.08	0.02	1.01	0.8	Down	0.02	Up	0.01
Der1-like domain family, member 3	Derl3	1.84	1.2	1.64	1.73	Down	0.02	Up	0.025
LPS-responsive beige-like anchor	Lrba	0.63	0.15	0.52	0.51	Down	0.02	Up	0.01
PHD finger protein 23	Phf23	-0.04	-0.62	-0.19	-0.06	Down	0.02	Up	0.005
Suppression of tumorigenicity 7-like	St7l	1.74	1.2	1.68	1.6	Down	0.02	Up	0.005
Vacuolar protein sorting 33A	Vps33a	0.97	-0.16	1.07	0.82	Down	0.02	Up	0.02

Table 2-2 Continued

Gene Name	Gene Symbol	Mean Z-Score MA-EB	Mean Z-Score MA-Oil	Mean Z-Score Y-EB	Mean Z-Score Y-Oil	Age Effect	P value	EB Effect	P value
Trophoblast glycoprotein	Tpbg	0.98	0.32	0.86	0.88	Down	0.006	Up	0.02
GDP-mannose pyrophosphorylase A	Gmppa	2.02	1.22	1.91	1.95	Down	0.01	Up	0.01
Kinesin family member C2	Kifc2	1.74	0.99	1.67	1.51	Down	0.01	Up	0.01
ZW10 interactor	Zwint	1.13	0.93	1.34	1.37	Down	0.002	Up	0.025

Table 2-3 Oligonucleotide array and RT-PCR fold changes

Gene Symbol	Array Fold change Y vs MA-Oil	RT-PCR Fold Change Y vs MA-Oil	Array Fold change Y vs MA-EB	RT-PCR Fold Change Y vs MA-EB
Hdac2	3.62 ± 0.77	2.57 ± 1.00	2.77 ± 0.66	1.83 ± 0.30
Lass2	3.58 ± 0.38	3.83 ± 0.97	2.78 ± 1.08	3.42 ± 0.92
Pou3f1	1.30 ± 0.13	1.28 ± 0.66	2.84 ± 0.29	2.25 ± 0.44

(± SEM) for genes increased during aging or increased by estrogen treatment.

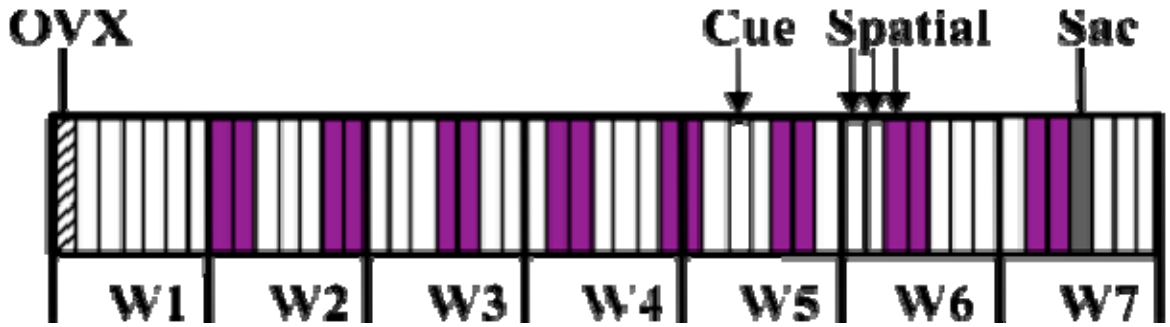

Figure 2-1. Schedule of injections and behavioral testing. The bars represent days starting with the day of ovariectomy (OVX, striped bar). Injections (light grey bars) of EB (5μ/0.05ml) or vehicle began one week after ovariectomy and took place on two consecutive days of a five day cycle. Animals received eight sets of injections over the course of the study. Arrows indicate behavioral training days. Cue discrimination training began in week 5 (W5), 48hr after the fifth set of injections. Spatial discrimination training began in week 6 (W6), 48 hr after the sixth set of injections and continued for three consecutive days. The seventh set of injections was initiated after completion of the third day of spatial training. Animals were sacrificed (Sac, dark grey bar) in week 7 (W7), 24hr after the eighth set of injections.

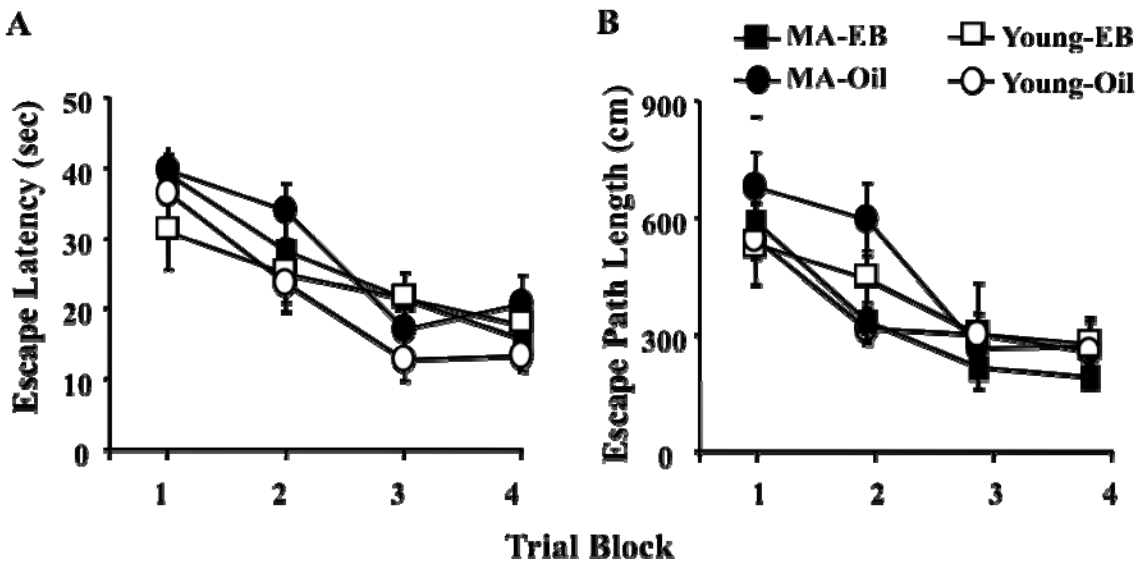

Figure 2-2. Cue Discrimination Training. Mean escape latency (A) and escape path length (B) for middle-aged (MA, filled symbols) and young (open symbols) mice receiving either EB (squares) or oil (circles) across the four trial blocks (1-4) of cue discrimination training.

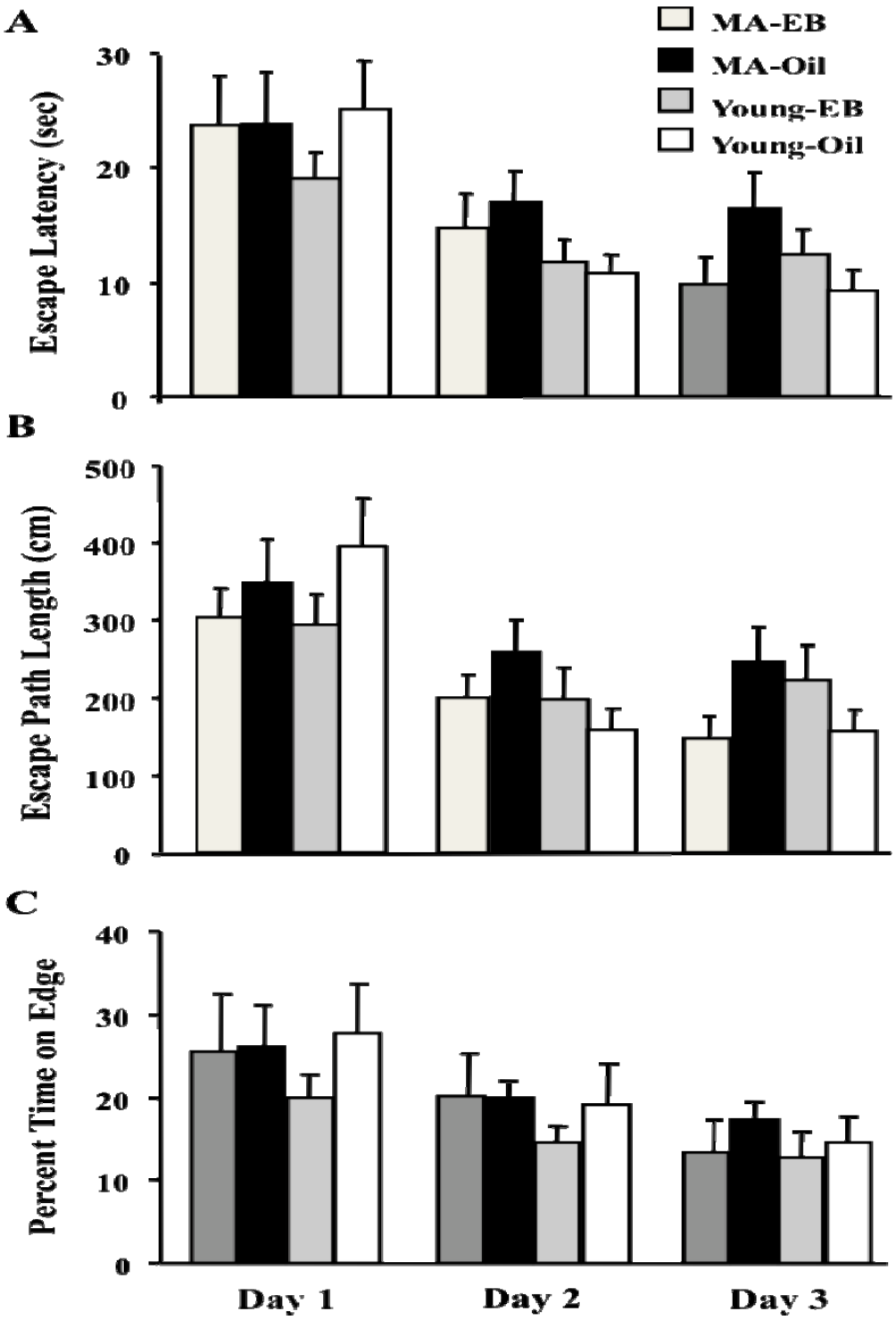

Figure 2-3. Spatial Discrimination Training. Mean escape latency A) escape path length B) and percent time on the edge of the pool (C) across days of spatial discrimination training for middle-aged EB (MA-EB, dark grey bars), middle-aged oil (MA-Oil, black bars), young EB (Young-EB light grey bars), and young oil (Young-Oil open bars).

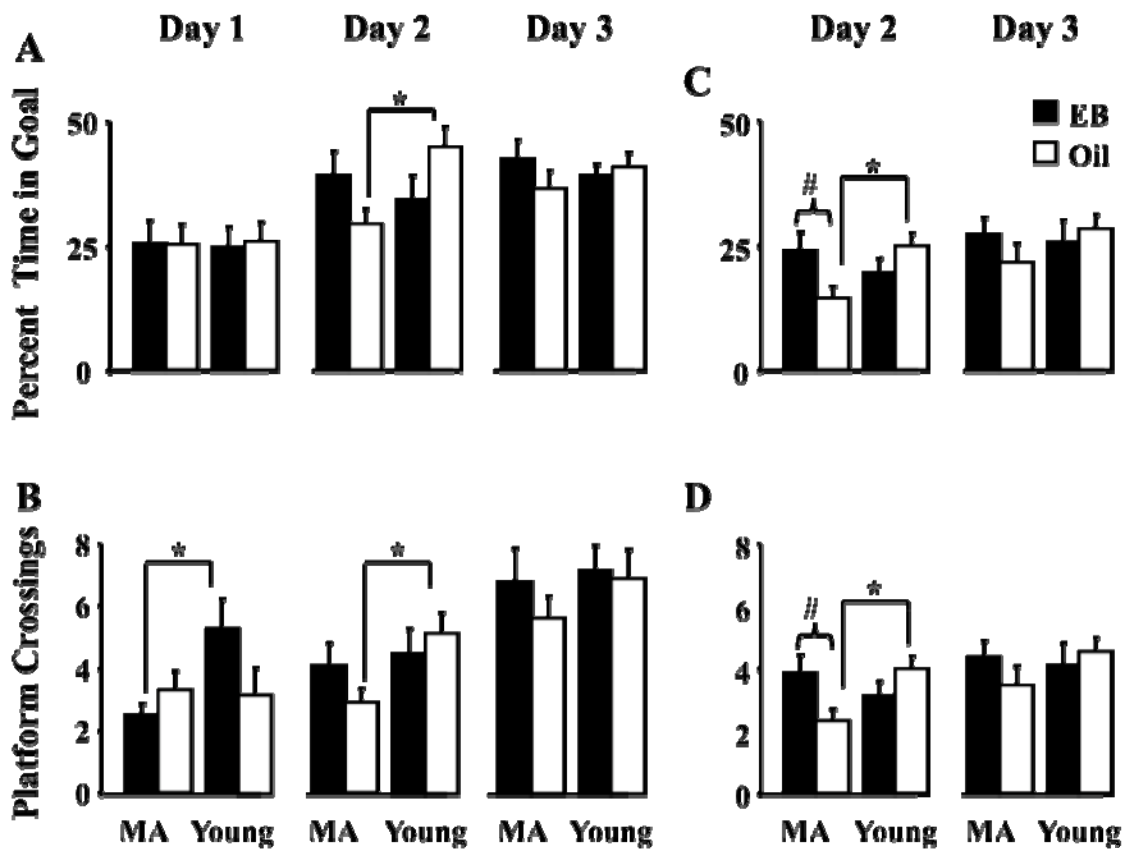

Figure 2-4. Probe trials delivered during the last block of training each day (Acquisition) and on the first trial of Day 2 and 3 (Retention) were used to measure the percent time searching the goal quadrant during Acquisition (A) and Retention (C) trials and number of platform crossings during Acquisition (B) and Retention (D) trials. The bars represent the means + SEM for middle-aged (MA) and young (Young) mice treated with oil (open bars) or EB (filled bars). In general middle-aged oil treated mice exhibit reduced time in the goal quadrant and reduced platform crossings on testing Days 2 and 3 and significant differences are noted for Day 2. Pound signs indicate a difference due to treatment within the same age group($p < 0.05$) and asterisks indicate a difference across age within the same treatment group ($p<0.05$).

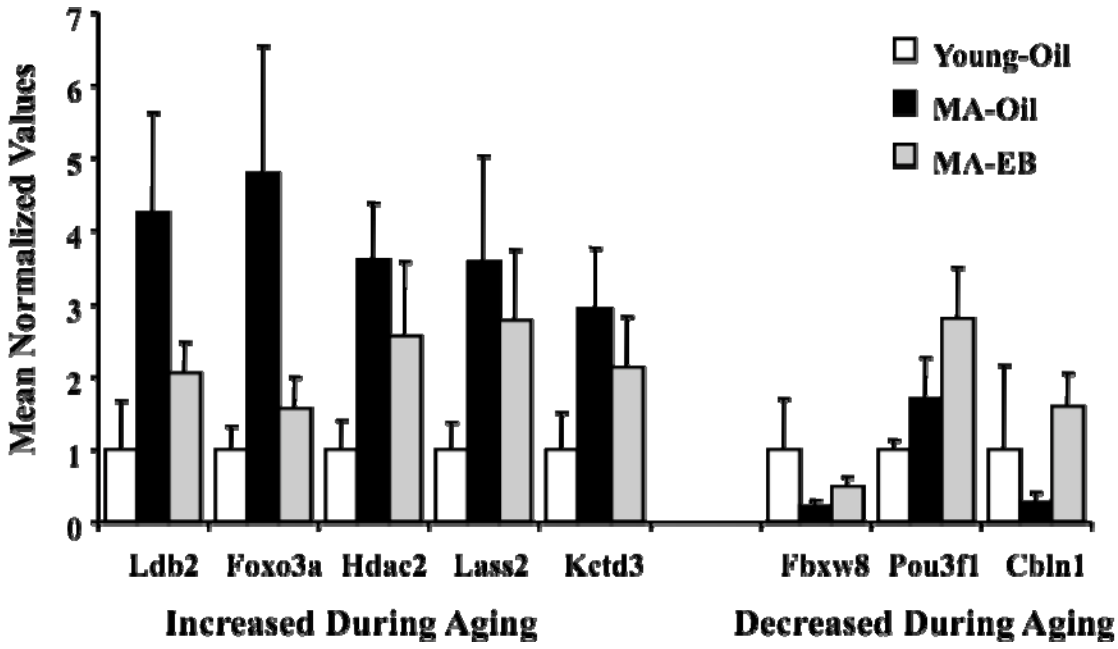

Figure 2-5. Confirmation of age and EB treatment effects using oligonucleotide array. Expression for a subset of eight genes was normalized by the means for young mice

CHAPTER 3
AGING ALTERS THE EXPRESSION OF GENES FOR NEUROPROTECTION AND SYNAPTIC FUNCTION FOLLOWING ACUTE ESTRADIOL TREATMENT

Introduction

In humans, age-related impairments in hippocampal-dependent memory begin in middle-age and cognitive weakening continues with advancing age (Foster, 2006; Small et al., 1999). Estrogen treatment in women (Sherwin, 2006), nonhuman primates (Lacreuse et al., 2002; Rapp et al., 2003), and rodents(Aenlle et al., 2009; Blalock et al., 2003; Markham et al., 2002) has been shown to protect against cognitive decline. However, it is becoming apparent that estradiol treatment initiated late in life is less effective (Adams et al., 2001a; Daniel et al., 2006; Foster et al., 2003; Sherwin and Henry, 2008).

The mechanism for differential estradiol effects across the lifespan is unclear. In younger animals, estradiol has numerous effects on the hippocampus that could provide a mechanism for improved cognition. For example, estradiol can rapidly activate signaling pathways for neuroprotection (Guerra et al., 2004; Jover-Mengual et al., 2007; Kuroki et al., 2001; Sarkar et al., 2008; Wu et al., 2005) and synaptogenesis (Akama and McEwen, 2003; Mukai et al., 2007) and estradiol effects on neuroprotection and synaptogenesis may be impaired in aged animals (Adams et al., 2001a; Brinton, 2008; Miranda et al., 1999; Yildirim et al., 2008) suggesting a possible breakdown in estrogen signaling.

To determine whether the age differences in synaptogenesis and neuroprotection result from a weakening of the signaling pathways, we investigated differences in gene expression following an acute estradiol treatment. *In vitro* (Carroll et al., 2006) (Schnoes et al., 2008) and *in vivo* studies (Fertuck et al., 2003; Naciff et al., 2007; Pechenino and Frick, 2009) have provided evidence for distinct temporal patterns of estrogen-mediated gene expression. In general, genes related to the regulation of transcription are altered within the first 2 hrs of treatment. Protein

changes associated with this early transcription contribute to the amplification in the number of altered genes occurring between 4-12 hr. Furthermore, this second wave of altered genes expression, between 4-12 hr, includes genes related to the functional effects of estrogen treatment for specific cell systems. Therefore, 17β-estradiol was injected in ovariectomized mice and estradiol-responsive genes were identified by transcript profiling at 6 and 12 hr after treatment. Pathway analysis of estradiol-responsive genes identified age-related differences in functional pathways related to oxidative phosphorylation, synaptic plasticity, and estrogen responsive signaling cascades.

Materials and Methods

Subjects

Procedures involving animal subjects have been reviewed and approved by the Institutional Animal Care and Use Committee at the University of Florida and were in accordance with guidelines established by the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. Initially 85 female C57/BL6 mice were obtained from National Institute of Aging for gene array analysis, with one gene chip per animal. However, quality controls for gene arrays indicate that 5 chips were outliers and the data for these animals was removed from further analysis. Therefore, a total of 80 female mice (young: n = 26, 4 months; middle-aged: n = 26, 12 months; aged: n = 28, 18 months) were employed in this study. Animals were housed 3-5 per cage and maintained on 12:12 light:dark cycle (lights on at 6 am). Following one-week habituation, mice were anesthetized (2 mg ketamine and 0.2 mg xylazine per 20 gm of body weight) and ovaries were removed through a small midline incision on the abdomen. All mice received ad lib access to food (Purina mouse chow, St Louis, MO) and water, until the surgery when they were placed on Casein based chow (Cincinnati Lab Supply, Cincinnati, OH), which is low in phytoestrogens found in soy based chow.

Hormone Administration

Briefly, a single injection of 17β-estradiol (Sigma Chemical Co, St Louis, MO) or mineral oil was initiated 10 days after ovariectomy (OVX) at 10 pm or 4 am. To control for time of day effects, all animal were sacrificed between 10 - 11 am, ~4 hr after lights on and either 6 hrs (injection at 4 am) or 12 hrs (injection at 10 pm) following the injection of estradiol or oil. Estradiol was dissolved in light mineral oil (Fisher Scientific, Pittsburgh, PA) to concentration of 0.1 mg/ml. Oil or estradiol (5μg) in oil was injected subcutaneously at the nape of the neck in volumes of 0.05 ml. The groups included: young receiving oil and sacrificed 6 hr (n = 5) and 12 hr (n = 3) later; young receiving estradiol and sacrificed 6 hr (n = 10) and 12 hr (n = 8) later; middle-aged receiving oil and sacrificed 6 hr (n = 5) and 12 hr (n = 6) later; middle-aged receiving estradiol and sacrificed 6 hr (n = 9) and 12 hr (n = 6) later; aged receiving oil and sacrificed 6 hr (n = 5) and 12 hr (n = 7) later; aged receiving estradiol and sacrificed 6 hr (n = 9) and 12 hr (n = 7) later. To determine effectiveness of estradiol treatment, uteri were excised at the time of sacrifice and weighed immediately. An analysis of variance (ANOVA) was used to compare main effects on uterine weight.

At the time of sacrifice, each animal was anesthetized with CO_2 and decapitated. The brain was quickly removed and placed in ice-cold artificial cerebral spinal fluid. Both hippocampus were removed, frozen in liquid nitrogen, and stored at -80ºC. RNA was isolated from each sample using Qiagen RNeasy Lipid Tissue Mini Kit (Qiagen, Germantown, MD). RNA concentration was determined using spectrophometer and a subset of samples was examined using Agilent 2100 Bioanalyzer (Santa Clara, CA). Microarray analysis was performed for individual animals (one chip per animal).

Microarray Hybridization and Signal Detection

An amount of 5µg of total RNA was synthesized to cRNA using Affymetrix amplification kit following the manufacture's protocol. Hybridization of cRNA was carried out by the Interdisciplinary Center for Biotechnology Research Microarray Core, University of Florida. Hybridization of Affymetrix Mouse 430 2.0 Arrays occurred for 17 hours at 60°C in accordance with manufacture's instructions and arrays were scanned using an Affymetrix Microarray scanner. Images were analyzed using Affymetrix Gene Chip Operating System software (GCOS version 1.1) and scaled to 500. Hybridization signal intensities between GeneChips were normalized using dChip's (Li and Wong, 2001) model-based expression index with the PM-only model. The model was used to set thresholds for identify outlier probe sets. Arrays with a large number of outlier probe sets (> 5% of total) were removed from further analysis. Data was then transferred into Microsoft excel for further analysis. Probe sets were annotated using Affymetrix® NetAffx™ (12/2007).

RT-PCR

Real time PCR (RT-PCR) was performed to verify microarray results. RNA from each group was treated with Turbo DNAfree (Ambion, Austin TX) to remove any remaining genomic DNA. RNA was then converted to cDNA using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Primers and probes for WDFY1, GABRA2, NNT, PPARGC1A, AFT4, ENTPD4 and GAPDH were purchased from Applied Biosystems. Briefly, 3µg of total RNA from a single animal was incubated with appropriate reagents at 25°C for 10 min and then heated to 37°C for 120 min using 7300 Fast Real-Time PCR System (Applied Biosystems). For relative quantification of RNA, 100ng in 2.5µl of cDNA was added to 12.5µl of Taqman® Universal PCR Master Mix (2X), 1.25µl of 20X Gene Expression Assay Mix, a probe specific

primer mixture (Table 3-1), and 8.75µl of nuclease-free water for a total volume of 25µl. Thermal cycler conditions were set at 2 min at 50°C , 10 min at 95°C and cycles 15s at 95°C and 1 min at 60°C for 40 cycles. The point at which the fluorescence crosses the threshold (Ct) was determined using 7300 Real-Time PCR System and SDS Software 1.3.1 analysis software (Applied Biosystems). Each sample was in triplicate and normalized to corresponding GAPDH values (ΔCt_{sample}) and then compared to normalized young oil ($\Delta Ct_{reference}$). The mean normalized values were compared using ΔΔCt method as described by Applied Biosystems to derive fold change (Aenlle et al., 2009), where $\Delta\Delta Ct=(\Delta Ct_{sample}) -(\Delta Ct_{reference})$.

Statistical Analysis

Probe set filtering and initial statistical analysis was performed according to our previously published work (Aenlle et al., 2009; Blalock et al., 2003). Briefly, the number of present calls for each probe was determined across all chips and the probed was removed if fewer than 80% of the chips exhibited a present call for the probe. For all studies, differential expression was determined using two-tailed *t*-tests with the alpha level set at 0.025 in accordance with our previous studies (Aenlle et al., 2009; Blalock et al., 2003). The probes sets that exhibited an increase or decrease in expression following treatment were submitted to Ingenuity Pathway Analysis's (IPA; Ingenuity Systems). With alpha set at $p<0.025$ we were able to obtain >800 molecules for generating networks, in accordance with IPA best practices for pathway analysis. The IPA program uses a right-tailed Fisher's Exact Test to compute the likelihood that the relationship between the list of submitted genes and a set of genes representing a given pathway is due to chance. A similar procedure was employed for determining overrepresentation of genes related to synaptic structure using the Expression Analysis Systematic Explorer (EASE) through the NIH DAVID Bioinformatics Resources (Hosack et al., 2003).

Results

For each age group, all animals treated with oil (young = 8, MA = 11, aged = 12) were used as controls to determine effects of treatment for age matched animals sacrificed 6 hr (young = 10, MA = 9, aged = 9) or 12 hr (young = 8, MA = 6, aged = 7) after a single estradiol injection. To determine the effectiveness of the estradiol treatment uterine weight was compared across groups (young oil 20 $\pm$ 5 mg; young estradiol 6 hr 54 $\pm$ 3 mg; young estradiol 12 hr 50 $\pm$ 3 mg; middle-aged oil 30 $\pm$ 6 mg; middle-aged estradiol 6 hr 52 $\pm$ 3 mg; middle-aged estradiol 12 hr 43 $\pm$ 10 mg; aged oil 34 $\pm$ 4 mg; aged estradiol 6 hr 56 $\pm$ 3 mg; aged estradiol 12 hr 56 $\pm$ 4 mg). An ANOVA indicated an overall treatment effect ($p < 0.0001$) in the absence of an age differences and post hoc FLSD tests indicated a significant increase in uterine weight at 6 hr ($p < 0.0001$) and 12 hr ($p < 0.0001$) following treatment relative to oil treated controls.

Age Differences in Estradiol-Responsive Genes for Synaptogenesis, and Neuroprotection 6hr After Treatment

Figure 3-1 illustrates the number of estradiol-responsive probes for the 6 and 12 hr time points. At the 6 hr time point the MA mice exhibited the greatest shift in gene expression with approximately twice as many probes exhibiting altered expression relative to young animals and approximately a ten fold increase in the number of altered probes relative to aged mice. Age-related differences in the pattern of estradiol-responsive gene expression were also apparent. For probes that were observed to change expression at 6 hrs, young and MA mice exhibited increased expression for ~60% of the probes, while the majority (64%) of estradiol-responsive probes were decreased in aged animals. A few probes were altered in the same direction across the different age groups; however, in some cases estradiol effects were in the opposite direction (Figure 3-1B).

To examine markers of synaptic components, estradiol-responsive genes were grouped according to age and whether the genes increased or decreased expression. The gene groups were submitted to DAVID Bioinformatics Resources to determine overrepresentation of genes related to the gene ontology classification for synapse cellular components (GO: 0045202). The results indicate that estradiol treatment was associated with increased expression of synaptic genes only for young (17 genes, $p < 0.0005$) and MA animals (26 genes, $p < 0.00005$) at the 6 hr time point. Four of the genes (ENAH, GRIA4, PJA2, GRIP1, GRIA1) were increased in both age groups (Table 3-2). A significant clustering was not observed for synaptic component genes that decreased expression (young: 4 genes; MA: 6 genes). Furthermore, aged animals did not exhibit altered expression, increasing or decreasing, for genes related to synaptic components.

Estrogen responsive genes were submitted to IPA to determine whether expression changes were associated with gene-enrichment for signaling pathways. Table 3-3 shows the pathways that exhibited significant ($p < 0.01$) overrepresentation. For genes that increased expression at the 6 hr time point, only young animals exhibited overrepresentation in specific signaling pathways including PPAR/RAR signaling, which has been linked to neuroprotection (Martin et al., 2006; Rosa et al., 2008; Sanguino et al., 2006; Santos et al., 2005). Interestingly, while significant gene enrichment was not observed for the PPAR/RAR pathway in MA mice, three of the six genes that increased in MA mice were common for the young group (CLOCK, GNAQ, NCOR1). For genes that decreased expression at the 6 hr time point, young and MA animals exhibited clustering of genes for oxidative phosphorylation and mitochondrial dysfunction (Table 4), with two genes NDUFV1 and NDUFV2 decreased in both age groups.

Four genes that increased (WDFY1, GABRA2, NNT, PPARGC1) and two that decreased (ATF4, ENTPD4) in young mice treated with estradiol were selected for validation of microarray

results using RT-PCR. These genes were selected because they exhibited altered expression in the same direction at 6 hr and 12 hr after treatment at $p < 0.025$, except for PPARGC1 which was increased at 12 hr for $p < 0.05$. RNA isolated from oil treated mice ($n = 3$) was used as the control to calculate the fold change for mice ($n = 3$) treated 6 hr earlier with estradiol. Similarly, the fold change was calculated for values on the microarray for young oil and estradiol (6 hr) treated mice. Figure 3-2 illustrates that the direction and extent of altered transcription was similar for the microarray and RT-PCR.

Age Differences in Estradiol-Responsive Genes 12hr After Treatment

The number of probes influenced by treatment decreased from the 6 to 12 hr time points for young and MA mice. In contrast, aged animals exhibited approximately a five fold increase in the number of estradiol-responsive probes at 12 hr relative to the 6 hr time point (Fig 3-1A). Most of the probes (67%) for aged animals exhibited decreased expression at 12 hr. When estradiol-responsive genes were compared across age groups, common probes were usually altered in the opposite direction in aged animals compared to the other two groups (Fig 1C) and include a number of genes involved in the regulation of transcription (Table 3-7).

To examine overrepresentation in functional categories, the list of significantly altered genes was submitted to DAVID Bioinformatics Resources for examination of synaptic components. For all age groups, overrepresentation of synaptic component genes was not observed. Data were then submitted to IPA for determination of over representation in functional pathways ($p < 0.01$). No significant clustering was observed for genes that exhibited decreased expression, regardless of age group (Table 3-5). In the case of increased expression, only aged animals exhibited gene enrichment which was largely focused on signaling pathways that are rapidly influenced by estrogen including a-adrenergic signaling (Aydin et al., 2008; Bowman et al., 2002; Favit et al., 1991; Heikkinen et al., 2002), Ca^{2+} signaling (Brewer et al., 2006; Foster,

2005; Zhao and Brinton, 2007), synaptic plasticity (Cordoba Montoya and Carrer, 1997; Smith and McMahon, 2005; Warren et al., 1995), and IGF-1 signaling (Azcoitia et al., 1999; Donahue et al., 2006; Perez-Martin et al., 2003). Several of the genes interact with multiple signaling pathways (Table 3-6). Finally, the PPAR pathway was increased at 6 hr in young an 12 hr in aged mice; however, only one gene, CHUK, was common for young 6 hr and aged 12 hr groups.

The increase in the number of altered genes at 12 hr for aged animals suggests that gene changes observed in younger mice may have been delayed in older animals. To examine this possibility we employed the gene expression data for 6 hr in young and MA mice and compared it to gene expression in aged mice at 6 and 12 hr to determine the number of genes that changed in the same direction. As illustrated in Figure 3-1B for age mice, the number of genes that changes in the same direction at 6 hr was 13 compared to young and 18 compared to MA. When we used the gene expression from age mice at 12 hr, we expected to observe an increase of ~4 fold, since the number of genes for the aged group increase from 198 to 940. However, relative to the young 6 hr group we saw a small increase from 13 to 19 and relative to MA animals the number of genes decreased from 18 to 4.

Discussion

Age Differences in Estradiol-Responsive Gene Signatures 6hr After Treatment

The current study examined altered gene expression in the hippocampus following estradiol treatment over the course of aging. The results reveal that aged animals were less responsive to estradiol treatment examined 6 hr after an acute treatment. In young and MA animals, estradiol treatment reduced expression for genes involved in oxidative phosphorylation and mitochondrial dysfunction. The decreased expression may represent feedback regulation due estradiol effects on oxidative phosphorylation. Altered oxidative phosphorylation is a major outcome of estradiol treatment in young animals and recent work indicates that in the brain,

estradiol can enhance mitochondrial efficiency and decrease oxidative stress (Irwin et al., 2008; Massart et al., 2002; Nilsen et al., 2007; Stirone et al., 2005; Zheng and Ramirez, 1999). It is unclear whether estradiol influences oxidative phosphorylation to the same extent in aged animals. This point is important since previous research indicates that regulation of mitochondrial function and oxidative phosphorylation may constitute a corner stone for estrogen's neuroprotective effects (Simpkins and Dykens, 2008). The results of the current study suggest that the acute effects of estradiol on oxidative phosphorylation may be less pronounced with advanced age.

Age differences in genes that increased expression were also apparent. Estradiol treatment is associated with an increase in dendritic spines in the hippocampus of young adult rats (Gould et al., 1990; Woolley et al., 1990; Woolley and McEwen, 1992; Woolley and McEwen, 1993; Woolley et al., 1996), and this process is impaired in older rats (Adams et al., 2001a; Miranda et al., 1999; Yildirim et al., 2008). We observed that young and MA, but not aged mice, exhibited an increase in expression of genes related to the synapse 6 hr after acute estradiol treatment. Young mice exhibited an increase in genes related to PPAR signaling. Although, MA did not exhibit a significant number of genes in this pathway, for the 6 genes that increased, 3 were common to young and MA animals. Aged animals exhibited increased expression of genes related to the PPAR pathway at 12 hr post treatment suggesting that the interaction of estrogen and PPAR signaling is maintained in advanced age. The ability of estrogen to increase gene expression of this pathway may be important for hippocampal aging since PPAR signaling has been implicated in the progression of Alzheimer's disease (Dupuy et al., 2001) and neuroprotection from inflammation (Kapadia et al., 2008; Vegeto et al., 2008).

Furthermore, age and sex specific changes have been noted for hippocampal PPAR signaling (Sanguino et al., 2006), suggesting that older females may be at greater risk.

Age Differences in Estradiol-Responsive Gene Signatures 12hr After Treatment

In contrast to young and MA mice, which exhibited a decline in the number of altered gene between 6 and 12 hr, the number of genes with altered expression increased during this time in aged animals. For aged mice at the 12 hr time point after an acute injection, gene expression increased in signaling pathways that are rapidly influenced by estradiol including; Ca^{2+} signaling (Brewer et al., 2006; Foster, 2005; Zhao and Brinton, 2007), cAMP signaling (Gu and Moss, 1996), IGF-1 signaling (Azcoitia et al., 1999), and synaptic plasticity (Foy et al., 2008a; Sharrow et al., 2002). The rapid activation of these pathways by estrogen is due to membrane interactions and not the result of classic transcriptional regulation. However, there is some indication for a reciprocal interaction between rapid membrane effects of estrogen on Ca^{2+}, G-protein coupled receptor, and trophic factor signaling and estrogenic modulation of genes in these pathways (Foster, 2005).

Mechanisms for Age-Related Differences in Estradiol-Responsive Gene Signatures

In the case of increased expression of genes for rapid signaling cascades, it may be important that these same signaling cascades decline during aging (Foster, 2005). Thus, aged cells may be differentially sensitive to estradiol influences due to age-related changes in baseline transcriptional activity and the activation of rapid signaling cascades by estradiol. Estradiol effects on Ca^{2+} signaling provides a prime example. Estradiol rapidly influences Ca^{2+} signaling (Sarkar et al., 2008; Wu et al., 2005). In turn, Ca^{2+} signaling can regulate gene expression through "non-classical" transcriptional regulation, independent of estrogen nuclear receptor mechanisms (Bading et al., 1993; Foster, 2005). Aged hippocampal neurons exhibit altered Ca^{2+} homeostasis and estrogen has effects on Ca^{2+}-dependent processes, which are opposite that

observed during aging (Foster, 2007). For example, the Ca^{2+}-dependent afterhyperpolarization is increased with age and estradiol reduces the afterhyperpolarization (Kumar and Foster, 2002). Furthermore, estradiol pretreatment may have a greater effect on Ca^{2+} regulation in aged cells (Brewer et al., 2006; Brewer et al., 2009). Together the results indicate that age differences in gene expression for rapid signaling pathways may relate to disparity in basal pathway activity and estrogen mediated activation of rapid signaling cascades.

In addition, to age-related changes in rapid signaling cascades, it is likely that changes in estrogen receptors contribute to differences in gene expression. In brain regions, like the hippocampus, that express both estrogen receptor alpha (ERα) and beta (ERβ), the magnitude and direction of gene regulation will depend on the relative expression of each receptor and the interaction of receptors (Gonzalez et al., 2007; Gottfried-Blackmore et al., 2007). While it is unclear how estrogen receptor expression changes in the hippocampus of mice, aging female mice exhibit a decrease in the transcription and expression of ERβ in the cortex (Thakur and Sharma, 2007). In contrast, an age-related shift in the hippocampal expression of ERα splice variants may reduce the sensitivity to estrogen treatment in women (Ishunina and Swaab, 2007). In the hippocampus of rats, expression of both ERα and ERβ declines during aging (Mehra et al., 2005) and the loss of ERα is associated with the decreased responsiveness of hippocampal synapses to estradiol (Adams et al., 2002). Indeed, previous work indicates an important role for ERα in the estrogen-mediated increase in synaptic markers (Jelks et al., 2007; Morissette et al., 2008b; Mukai et al., 2006). However, several of these studies report a similar, though usually blunted effect of ERβ activation (Jelks et al., 2007; Morissette et al., 2008b; Patrone et al., 2000), suggesting that ERβ may be less active but have similar effects on transcription (Lindberg et al., 2003).

An age-related change in ERα and ERβ or a decline in rapid signaling pathways could have reduced or delayed estradiol induced signaling and gene regulation. In the current study there appears to be a delay in the expression of PPAR genes. However, only one gene was common for young 6 hr and aged 12 hr groups. Furthermore, IPA analysis indicated that many more pathways were differentially influenced across young 6 hr and aged 12 hr groups. Similarly, synaptic component genes did not increase in aged animals for either time point. Finally, examination of all genes indicated little correspondence in the gene changes between young 6 hr and aged 12 hr groups. Together, the results indicate that delayed activation is not responsible for most of the age differences in altered gene expression between 6 and 12 hr.

It is possible that gene changes observed in aged animals could act as a priming response for successive estradiol induced changes beyond the 12 hr time point. For example, estradiol application to hippocampal slices rapidly increases ERK/MAPK activation and NMDA receptor function (Bi et al., 2003) and the magnitude of LTP (Foy et al., 2008b) in young, but not aged animals. In contrast, *in vivo* priming with estradiol 48 hr prior to sacrifice can enhance LTP in slices from young and aged animals (Smith and McMahon, 2005; Yun et al., 2007). Previous works suggests that both estrogen receptors and rapid signaling cascades are involved in the estradiol-mediated spine growth and synaptogenesis (Akama and McEwen, 2003; Lee et al., 2004; Mukai et al., 2007; Murphy and Segal, 1996; Murphy and Segal, 1997; Yildirim et al., 2008; Znamensky et al., 2003). Thus, while young mice exhibit a rapid increase in the expression of the synaptic marker synaptophysin, an increase in synaptophysin can also be observed in aged mice following treatment with estradiol over several days (Frick et al., 2002; Spencer et al., 2008a). Similarly, behavioral studies suggest that a single estradiol injection delivered after training can improve memory in young and MA animals, but not aged animals (Frick, 2009),

consistent with the idea that aged animals are less responsive to a single injection. Estradiol treatment for several days prior to training reliably improves memory in middle-aged animals (Foster, 2005; Frick, 2009); however, the effects in aged animals can vary across species. Treatment prior to training improves memory in mice (Frick et al., 2002; Heikkinen et al., 2004; Vaucher et al., 2002), and is less effective in aged rats (Foster et al., 2003; Savonenko and Markowska, 2003; Talboom et al., 2008). Similar differences in responsiveness are noted for estradiol effects on synaptic markers, which can be increased in aged mice (Frick et al., 2002; Spencer et al., 2008a), but not in aged rats (Adams et al., 2001a; Miranda et al., 1999; Yildirim et al., 2008). The difference in rats and mice may be due to differences in the expression of estrogen receptors during aging. Regardless, the results indicate that aged animals are less responsive to a single injection of estradiol, however; depending on the species, estradiol priming may rescue estrogen responsiveness. It would be enlightening to determine whether an increase in the expression of estrogen receptors or an enhancement of rapid signaling would ameliorate age-related differences in gene changes, synaptic plasticity and memory following estradiol treatment.

Table 3-1 Context Sequence of Genes for RT-PCR Analysis

Gene Symbol	Assay ID	Context Sequence
WDFY1	Mm00840455_m1	GGGGTGTGATGGAATTTCACGTTT
GABRA2	Mm01211683_m1	CGGGAAGAGTGTAGTCAATGACAAG
NNT	Mm01298455_m1	GCCAACATCTCTGGTTATAAGGCTG
PPARGC1A	Mm00447183_m1	CGCAACATGCTCAAGCCAAACCAAC
ATF4	Mm00515324_m1	GCCATGGCGCTCTTCACGAAATCCA
ENTPD4	Mm00491888_m1	TTCCTGCCCTTGAGAGACATCCGGC
GAPDH	Mm99999915_g1	GAACGGATTTGGCCGTATTGGGCGC

Table 3-2 Synaptic Component Genes Increased at 6 hr in Young and MA Mice

Affymetrix	Symbol	Description	p-value	Fold
Young				
1458298_at	CADPS	Ca^{2+} dependent activator protein for secretion	1.58E-02	1.20
1443876_at	CAMK2A	Calcium/calmodulin-dependent protein kinase II alpha	1.00E-02	1.19
1423286_at	CBLN1	Cerebellin 1 precursor protein	1.33E-02	1.53
1433607_at	CBLN4	Cerebellin 4 precursor protein	9.86E-03	1.38
1433451_at	CDK5R1	Cyclin-dependent kinase 5, regulatory subunit (p35) 1	1.49E-03	1.26
1422887_a_at	CTBP2	c-terminal binding protein 2	9.81E-03	1.20
1442223_at	ENAH	Enabled homolog (drosophila)	9.46E-03	1.20
1455444_at	GABRA2	Gamma-aminobutyric acid receptor, subunit alpha 2	1.06E-06	2.75
1434098_at	GLRA2	Glycine receptor, alpha 2 subunit	1.29E-02	1.34
1458285_at	GRIA1	Glutamate receptor, ionotropic, ampa1 (alpha 1)	5.61E-03	1.34
1440891_at	GRIA4	Glutamate receptor, ionotropic, ampa4 (alpha 4)	1.55E-02	1.48
1436575_at	GRIN3A	Glutamate receptor ionotropic, nmda3a	1.35E-02	1.20
1435951_at	GRIP1	Glutamate receptor interacting protein 1	4.60E-03	1.20
1437363_at	HOMER1	Homer homolog 1 (drosophila)	1.94E-02	1.19
1417376_a_at	IGSF4A	Immunoglobulin superfamily, member 4a	1.19E-02	1.22
1450435_at	L1CAM	L1 cell adhesion molecule	1.18E-02	1.15
1452328_s_at	PJA2	Praja 2, ring-h2 motif containing	1.78E-02	1.41
Middle-age				
1439220_at	ANK3	Ankyrin 3, epithelial	1.93E-02	2.01
1445798_at	DLGH1	Discs, large homolog 1 (drosophila)	1.95E-02	1.74
1446585_at	DLGH2	Discs, large homolog 2 (drosophila)	1.32E-02	1.37
1429768_at	DTNA	Dystrobrevin alpha	5.52E-03	1.27
1445329_at	DTNB	Dystrobrevin, beta	5.79E-03	1.66
1446426_at	ENAH	Enabled homolog (drosophila)	4.17E-03	1.85
1454022_at	EPHB2	Eph receptor b2	9.43E-03	1.58
1458285_at	GRIA1	Glutamate receptor, ionotropic, ampa1 (alpha 1)	1.36E-02	1.37
1453098_at	GRIA2	Glutamate receptor, ionotropic, ampa2 (alpha 2)	1.27E-02	1.75

Table 3-2 Continued

Affymetrix	Symbol	Description	p-value	Fold
1443285_at	GRIA4	Glutamate receptor, ionotropic,	4.53E-03	2.08
1440602_at	GRIK2	Glutamate receptor, ionotropic, kainate 2 (beta 2)	6.52E-03	2.57
1421350_a_at	GRIP1	Glutamate receptor interacting protein 1	1.69E-02	1.57
1458861_at	GRM7	Glutamate receptor, metabotropic 7	2.12E-02	1.42
1440637_at	ITSN1	Intersectin 1 (sh3 domain protein 1a)	1.58E-02	1.75
1424848_at	KCNMA1	Potassium large conductance calcium-activated channel	9.27E-03	2.99
1440807_at	MAGI2	Membrane associated guanylate kinase	1.97E-03	2.90
1420171_s_at	MYH9	Myosin, heavy polypeptide 9, non-muscle	1.25E-02	1.34
1422520_at	NEF3	Neurofilament 3, medium	2.28E-02	1.13
1447216_at	NRXN1	Neurexin I	1.37E-02	2.10
1457212_at	NRXN3	Neurexin III	9.69E-04	2.11
1444126_at	PJA2	Praja 2, ring-h2 motif containing	1.52E-04	2.07
1442620_at	PSD3	Pleckstrin and sec7 domain containing 3	3.20E-04	2.12
1438282_at	SYT1	Synaptotagmin I	2.99E-03	2.02
1429729_at	SYT11	Synaptotagmin 11	1.22E-02	1.88
1459009_at	UTRN	Utrophin	2.27E-02	1.44

The Affymetrix probe identifier, gene symbol, gene description, *t*-test p-value and fold change are provided for genes of synaptic components that increase 6 hr following treatment in young and MA mice.

Table 3-3 Estradiol-Responsive Pathways 6 hr Post Treatment

Increasing	p-value	Genes	Decreasing	p-value	Genes
Young					
PPAR/RARa activation	5.E-03	15	Oxidative phosphorylation	5.E-05	17
Glutamate receptor signaling	5.E-03	8	Mytochondrial dysfunction	1.E-03	14
Circadian rhythm signaling	1.E-02	4			
Middle-age					
None			Oxidative phosphorylation	1.E-05	24
			Mytochondrial dysfunction	5.E-03	18
			Protein ubiquination pathway	1.E-02	25
Aged					
None			None		

Pathways with overrepresentation of genes that were observed to increase or decrease expression 6 hr following treatment. The p-value is calculated from a right-tailed Fisher's Exact Test. The number of altered genes is also provided.

Table 3-4 Oxidative Phosphorylation and Mitochondrial Dysfunction Genes Altered at 6 hr in Young and Middle-Aged Mice

Affymetrix	Symbol	Description	p-value	FC
Young				
1417607_at	COX6A2	cytochrome c oxidase subunit VIa polypeptide 2	4.53E-03	-1.37
1424364_a_at	UCRC	ubiquinol-cytochrome c reductase complex (7.2 kD)	1.86E-02	-1.31
1416057_at	NDUFB11	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 11, 17.3kDa	1.98E-02	-1.28
1417286_at	NDUFA5	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5, 13kDa	9.06E-03	-1.27
1454716_x_at	COX5B	cytochrome c oxidase subunit Vb	2.13E-02	-1.25
1437680_x_at	GLRX2	glutaredoxin 2	7.65E-03	-1.22
1428360_x_at	NDUFA7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex	1.42E-02	-1.16

Table 3-4 Continued

Affymetrix	Symbol	Description	p-value	FC
1423676_at	ATP5H (includes	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit	2.48E-02	-1.17
1416495_s_at	NDUFS5	NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15kDa (NADH-coenzyme Q reductase)	2.01E-03	-1.14
1428322_a_at	NDUFB10 (includes EG:4716)	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa	3.85E-03	-1.14
1455283_x_at	NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase)	1.12E-02	-1.14
1415980_at	ATP5G2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C2 (subunit 9)	1.45E-02	-1.13
1428075_at	NDUFB4	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 4, 15kDa	3.76E-03	-1.13
1426689_s_at	SDHA	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	1.11E-02	-1.11
1428179_at	NDUFV2	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa	9.55E-03	-1.10
1415966_a_at	NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	1.02E-02	-1.07
1449622_s_at	ATP6AP1	ATPase, H+ transporting, lysosomal accessory protein 1	1.66E-02	-1.07
Middle-age				
1429329_at	COX10	COX10 homolog, cytochrome c oxidase assembly protein, heme A: farnesyltransferase (yeast)	7.36E-04	-1.38
1419544_at	ATP6V1C1	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit c1	1.98E-02	-1.32
1426742_at	ATP5F1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit B1	9.93E-03	-1.30
1415967_at	NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	3.37E-03	-1.30
1428782_a_at	UQCRC1	ubiquinol-cytochrome c reductase core protein I	3.35E-03	-1.29
1417799_at	ATP6V1G2	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G2	1.13E-02	-1.25

Table 3-4. Continued

Affymetrix	Symbol	Description	p-value	FC
1417799_at	ATP6V1G2	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G2	1.13E-02	-1.25
1455640_a_at	TXN2	thioredoxin 2	7.96E-03	-1.29
1423711_at	NDUFAF1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 1	1.42E-03	-1.27
1423737_at	NDUFS3	NADH dehydrogenase (	5.67E-04	-1.24
1424488_a_at	PPA2	pyrophosphatase (inorganic) 2	4.49E-04	-1.23
1448331_at	NDUFB7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa	8.45E-03	-1.22
1450968_at	UQCRFS1	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	1.08E-02	-1.22
1437013_x_at	ATP6V0B	ATPase, H+ transporting, lysosomal 21kDa, V0 subunit b	4.57E-03	-1.21
1432264_x_at	COX7A2L	cytochrome c oxidase subunit VIIa polypeptide 2 like	1.44E-02	-1.21
1448153_at	COX5A	cytochrome c oxidase subunit Va	2.60E-05	-1.20
1448292_at	UQCR	ubiquinol-cytochrome c reductase 6.4kDa subunit	2.33E-02	-1.20
1448286_at	HSD17B10	hydroxysteroid (17-beta) dehydrogenase 10	4.13E-03	-1.19
1448589_at	NDUFB5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5, 16kDa	1.29E-03	-1.18
1451096_at	NDUFS2	NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa (NADH-coenzyme Q reductase)	4.95E-03	-1.18
1428631_a_at		ubiquinol-cytochrome c reductasecore protein II	2.34E-02	-1.17
1428179_at	NDUFV2	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa	1.60E-03	-1.16
1416663_at	NDUFA9 (includes EG:4704)	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9, 39kDa	2.01E-03	-1.16
1416952_at	ATP6V1D	ATPase, H+ transporting, lysosomal 34kDa, V1 subunit D	1.41E-02	-1.16
1448203_at	ATP5L	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit G	7.34E-03	-1.15
1415671_at	ATP6V0D1	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d1	1.19E-03	-1.13
1428075_at	NDUFB4	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 4, 15kDa	1.56E-02	-1.12

The Affymetrix probe identifier, gene symbol, gene description, *t*-test p-value and fold change (FC) are provided for genes of in the oxidative phosphorylation and mitochondrial dysfunction pathways that were decrease 6 hr following treatment in young and MA mice.

Table 3-5 Estradiol-Responsive Pathways 12 hr Post Treatment

Increasing	p-value	Genes	Decreasing	p-value	Genes
Young					
None			None		
MA					
None			None		
Aged					
□-Adrenergic signaling	5.E-05	13	None		
Calcium signaling	1.E-04	16			
Long-term depression signaling	5.E-04	13			
Long-term potentiation signaling	1.E-03	12			
G-protein coupled receptor signaling	5.E-03	13			
cAMP-mediated signaling	1.E-02	11			
IGF-1 signaling	1.E-02	10			
PPAR signaling	1.E-02	7			
Neuregulin signaling	1.E-02	8			

Pathways with overrepresentation of genes with altered expression 12 hr following treatment. The p-value is calculated from a right-tailed Fisher's Exact Test. The number of altered genes is also provided.

Table 3-6 Signaling Genes Altered at 12 hr in Aged Mice

					Signaling Pathways								
AffyID	Symbol	Description	p-value	Fold	□-Adrenergic	Calcium	LTD	LTP	G-Protein	cAMP	IGF	PPAR	Neuregulin
1426585_s_at	MAPK1	mitogen-activated protein kinase 1	1.47 E-02	1.14	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**
1416351_at	MAP2K1	mitogen-activated protein kinase kinase 1	1.08 E-02	1.11	**x**		**x**	**x**	**x**	**x**	**x**	**x**	**x**
1453419_at	MRAS	muscle RAS oncogene homolog	1.27 E-02	1.25	**x**		**x**	**x**	**x**		**x**	**x**	**x**
1452032_at	PRKAR1A	protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)	4.08 E-05	1.12	**x**	**x**		**x**	**x**	**x**	**x**		
1440132_s_at	PRKAR1B	protein kinase, cAMP-dependent, regulatory, type I, beta	5.23 E-03	1.14	**x**	**x**		**x**	**x**	**x**	**x**		
1460419_a_at	PRKCB	protein kinase C, beta	2.03 E-02	1.16	**x**		**x**	**x**	**x**				**x**
1418754_at	ADCY8	adenylate cyclase 8 (brain)	1.26 E-02	1.24	**x**		**x**	**x**	**x**	**x**			
1426582_at	ATF2	activating transcription factor 2	2.39 E-02	1.28		**x**		**x**	**x**	**x**			
1433592_at	CALM1	calmodulin 1 (phosphorylase kinase, delta)	3.37 E-03	1.18	**x**	**x**		**x**		**x**			
1434440_at	GNAI1	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	2.30 E-02	1.12	**x**		**x**		**x**	**x**			

Table 3-6 Continued

AffyID	Symbol	Description	p-value	Fold	□-Adrenergic	Calcium	LTD	LTP	G-Protein	cAMP	IGF	PPAR	Neuregulin
1422103_a_at	STAT5B	signal transducer and activator of transcription 5B	2.07 E-02	1.16								**x**	**x**
1450186_s	GNAS	GNAS complex	1.9	1.14	**x**		**x**		**x**	**x**			
1417091_at	CHUK	conserved helix-loop-helix ubiquitous kinase	1.42 E-03	1.26					**x**			**x**	
1421622_a_at	RAPGEF4	Rap guanine nucleotide exchange factor (GEF) 4	2.09 E-02	1.49					**x**	**x**			
1416286_at	RGS4	regulator of G-protein signaling 4	5.89 E-04	1.23					**x**	**x**			
1450202_at	GRIN1	glutamate receptor, ionotropic, N-methyl D-aspartate 1	4.12 E-03	1.35		**x**		**x**					
1452533_at	RYR3	ryanodine receptor 3	1.02 E-03	1.36		**x**	**x**						
1440962_at	SLC8A3	solute carrier family 8 (sodium/calcium exchanger), member 3	3.51 E-03	1.26	**x**	**x**							
1450655_at	PTEN	phosphatase and tensin homolog	2.15 E-02	1.28									**x**
1419073_at	TMEFF2	transmembrane protein with EGF-like and two follistatin-like domains 2	1.08 E-02	1.16									**x**

Table 3-6 Continued

AffyID	Symbol	Description	p-value	Fold	□-Adrenergic	Calcium	LTD	LTP	G-Protein	cAMP	IGF	PPAR	Neuregulin
1422313_at	IGFBP5	insulin-like growth factor binding protein 5	1.65 E-03	1.57							**x**		
1418099_at	TNFRSF1B	tumor necrosis factor receptor	5.29 E-03	1.26								**x**	
1417933_at	IGFBP6	insulin-like growth factor binding protein 6	8.90 E-03	1.35							**x**		
1450431_at	NEDD4	neural precursor cell expressed, developmentally down-regulated 4	1.33 E-02	1.10							**x**		
1452046_at	PPP1CC	protein phosphatase 1, catalytic subunit, gamma isoform	1.95 E-02	1.15				**x**					
1420534_at	GUCY1A3	guanylate cyclase 1, soluble, alpha 3	8.46 E-03	1.56			**x**						
1420871_at	GUCY1B3	guanylate cyclase 1, soluble, beta 3	3.69 E-03	1.43			**x**						
1453260_at	PPP2R2A	protein phosphatase 2 (formerly 2A), regulatory subunit B, alpha isoform	2.23 E-02	1.23			**x**						
1452788_at	PPP2R5E	protein phosphatase 2, regulatory subunit B', epsilon isoform	1.44 E-02	1.87			**x**						
1417943_at	GNG4	guanine nucleotide binding protein (G protein), gamma 4	4.75 E-04	1.30	**x**								

Table 3-6. Continued

AffyID	Symbol	Description	p-value	Fold	□-Adrenergic	Calcium	LTD	LTP	G-Protein	cAMP	IGF	PPAR	Neuregulin
1424852_at	MEF2C	myocyte enhancer factor 2C	6.59 E-03	1.27		**x**							
1452363_a_at	ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	1.76 E-03	1.15		**x**							
1450243_a_at	RCAN2	regulator of calcineurin 2	1.57 E-02	1.50		**x**							
1423721_at	TPM1	tropomyosin 1 (alpha)	1.41 E-02	1.13		**x**							

The Affymetrix probe identifier, gene symbol, gene description, *t*-test p-value and fold increase are provided for genes increased 12 hr following treatment in signaling pathways in aged mice. An x indicates that the gene is a member of the pathway.

Table 3-7 Genes from aged mice which exhibited expression opposite young or MA mice at 12 hr

			p-value			Fold Change		
Affy ID	Gene	Description	Y	MA	A	Y	MA	A
1452369_at	MAGI1	MEMBRANE ASSOCIATED GUANYLATE KINASE, WW AND PDZ DOMAIN CONTAINING 1	6.3E-04	4.4E-01	3.8E-03	-1.29	-1.10	1.51
1434008_at	SCN4B	SODIUM CHANNEL, TYPE IV, BETA	2.2E-02	3.9E-01	3.9E-03	-1.26	-1.16	1.47
1419008_at	NPY5R	NEUROPEPTIDE Y RECEPTOR Y5	9.2E-01	2.1E-02	5.3E-05	1.01	-1.52	1.45
1426495_at	2410042D21RIK	RIKEN CDNA 2410042D21 GENE	5.2E-01	1.2E-02	1.9E-03	1.08	-1.51	1.41
1448795_a_at	TBRG4	TRANSFORMING GROWTH FACTOR BETA REGULATED GENE 4	6.8E-01	1.6E-02	5.3E-03	-1.05	-1.34	1.33
1427329_a_at	IGH-6	IMMUNOGLOBULIN HEAVY CHAIN 6 (HEAVY CHAIN OF IGM)	5.0E-02	2.3E-02	1.3E-02	-1.41	-1.27	1.32
1455277_at	HHIP	HEDGEHOG-INTERACTING PROTEIN	8.4E-05	7.1E-01	1.1E-02	-1.45	1.06	1.29
1426582_at	ATF2	ACTIVATING TRANSCRIPTION FACTOR 2	1.4E-01	2.1E-02	2.4E-02	1.27	-1.45	1.28
1429249_at	4833424O15RIK	RIKEN CDNA 4833424O15 GENE	7.1E-01	1.9E-02	5.9E-03	1.04	-1.58	1.27
1456904_at	EST		8.0E-01	1.0E-02	2.2E-02	1.02	-1.18	1.26
1426806_at	OBFC2A	OLIGONUCLEOTIDE BINDING FOLD CONTAINING 2A	2.6E-01	1.9E-02	1.9E-02	1.15	-1.51	1.25
1428429_at	RGMB	RGM DOMAIN FAMILY, MEMBER B	2.9E-01	2.2E-02	1.3E-02	1.13	-1.43	1.25
1435165_at	CNTN2	CONTACTIN 2	2.1E-02	4.4E-01	1.1E-02	-1.23	-1.09	1.25
1416286_at	RGS4	REGULATOR OF G-PROTEIN SIGNALING 4	4.9E-01	2.3E-02	5.9E-04	1.05	-1.13	1.23

Table 3-7 Continued

			p-value			Fold Change		
Affy ID	Gene	Description	Y	MA	A	Y	MA	A
1433719_at	SLC9A9	SOLUTE CARRIER FAMILY 9 (SODIUM/HYDROGEN EXCHANGER), ISOFORM 9	2.3E-02	4.4E-01	3.5E-03	-1.18	-1.07	1.21
1455734_at	CRBN	CEREBLON	2.1E-01	2.4E-02	2.3E-03	1.09	-1.15	1.20
1436056_at	KIF13B	KINESIN FAMILY MEMBER 13B	7.6E-03	6.6E-01	2.4E-02	-1.23	1.04	1.20
1419184_a_at	FHL2	FOUR AND A HALF LIM DOMAINS 2	4.7E-03	5.6E-01	1.7E-02	-1.17	-1.03	1.20
1456967_at	TRIM66	KIAA0298 HYPOTHETICAL PROTEIN (HUMAN)	1.2E-02	7.4E-01	1.8E-02	-1.24	1.04	1.19
1448752_at	CAR2	CARBONIC ANHYDRASE 2	7.9E-01	2.4E-02	9.7E-03	1.02	-1.25	1.16
1449164_at	CD68	CD68 ANTIGEN	9.2E-01	1.3E-02	7.7E-03	-1.00	-1.33	1.15
1428903_at	3110037I16RIK	RIKEN CDNA 3110037I16 GENE	9.8E-03	9.8E-01	1.2E-02	-1.13	-1.00	1.14
1429227_x_at	NAP1L1	NUCLEOSOME ASSEMBLY PROTEIN-1	2.1E-01	1.5E-02	2.4E-03	1.09	-1.25	1.13
1424801_at	ENAH	ENABLED HOMOLOG (DROSOPHILA)	3.8E-01	1.9E-02	2.5E-02	1.05	-1.20	1.12
1416458_at	ARF2	ADP-RIBOSYLATION FACTOR 2	9.6E-01	1.4E-02	2.0E-02	-1.00	-1.26	1.12
1434440_at	GNAI1	GUANINE NUCLEOTIDE BINDING PROTEIN, ALPHA INHIBITING 1	6.2E-01	1.4E-02	2.3E-02	1.05	-1.10	1.12
1424594_at	LGALS7	LECTIN, GALACTOSE BINDING, SOLUBLE 7	1.0E-02	3.1E-01	6.3E-03	-1.12	-1.05	1.12
1455403_at	MANEA	MANNOSIDASE, ENDO-ALPHA	1.8E-02	9.3E-01	2.3E-02	-1.12	1.01	1.11

Table 3-7 Continued

			p-value			Fold Change		
Affy ID	Gene	Description	Y	MA	A	Y	MA	A
1448963_at	NFYC	NUCLEAR TRANSCRIPTION FACTOR-Y GAMMA	1.8E-02	4.5E-01	1.9E-03	-1.10	-1.05	1.11
1434612_s_at	SBNO1	SNO, STRAWBERRY NOTCH HOMOLOG 1 (DROSOPHILA)	8.1E-01	2.2E-02	2.4E-02	1.01	-1.17	1.11
1455011_at	STARD4	RIKEN CDNA 4632419C16 GENE	9.2E-03	4.4E-03	2.1E-02	-1.23	-1.17	1.10
1417364_at	EEF1G	EUKARYOTIC TRANSLATION ELONGATION FACTOR 1 GAMMA	8.8E-01	2.4E-02	1.2E-02	-1.01	-1.13	1.09
1452159_at	2310001A20RIK	RIKEN CDNA 2310001A20 GENE	2.1E-04	2.4E-01	2.2E-02	1.17	-1.10	-1.10
1417252_at	NT5C	5',3'-NUCLEOTIDASE, CYTOSOLIC	1.4E-03	1.2E-01	2.9E-03	1.32	-1.17	-1.12
1429048_at	BLOC1S2	BIOGENESIS OF LYSOSOME-RELATED ORGANELLES COMPLEX-1, SUBUNIT 2	2.1E-02	2.4E-02	1.5E-02	1.24	-1.28	-1.14
1434521_at	RFXDC2	REGULATORY FACTOR X DOMAIN CONTAINING 2 HOMOLOG (HUMAN)	7.8E-01	9.3E-03	7.9E-03	-1.01	1.20	-1.15
1459874_s_at	MTMR4	MYOTUBULARIN RELATED PROTEIN 4	4.9E-03	1.9E-01	1.3E-02	1.22	1.14	-1.16
1434745_at	CCND2	CYCLIN D2	1.1E-03	2.3E-01	1.5E-02	1.23	-1.09	-1.16

Table 3-7. Continued

			p-value			Fold Change		
Affy ID	Gene	Description	Y	MA	A	Y	MA	A
1426858_at	INHBB	INHIBIN BETA-B	1.5E-02	5.6E-01	2.2E-02	1.20	-1.05	-1.17
1456748_a_at	NIPSNAP1	4-NITROPHENYLPHOSPHATASE DOMAIN AND NON-NEURONAL SNAP25-LIKE PROTEIN H...	1.3E-02	6.0E-01	2.2E-02	1.21	1.04	-1.17
1455940_x_at	WDR6	WD REPEAT DOMAIN 6	2.4E-02	3.6E-01	2.0E-02	1.16	1.14	-1.20
1447320_x_at	RPO1-3	RNA POLYMERASE 1-3	7.7E-03	1.4E-01	1.3E-02	1.29	-1.20	-1.20
1436443_a_at	KDELC1	KDEL (LYS-ASP-GLU-LEU) CONTAINING 1	7.4E-03	9.5E-01	1.6E-02	1.31	-1.01	-1.21
1448694_at	JUN	JUN ONCOGENE	3.4E-01	1.4E-02	2.8E-03	-1.05	1.13	-1.22
1436114_at	Rnf165	Ring finger protein 165	2.3E-02	2.4E-01	1.3E-03	1.19	1.11	-1.25
1455039_a_at	SIN3B	TRANSCRIPTIONAL REGULATOR, SIN3B (YEAST)	1.8E-02	6.7E-01	4.5E-03	1.23	1.04	-1.30
1441727_s_at	ZFP467	HYPOTHETICAL PROTEIN, MNCB-3350	1.6E-02	3.1E-01	5.6E-03	1.34	1.10	-1.33
1456573_x_at	NNT	NICOTINAMIDE NUCLEOTIDE TRANSHYDROGENASE	2.0E-03	5.8E-01	1.7E-02	1.77	1.09	-1.33
1434210_s_at	LRIG1	LEUCINE-RICH REPEATS AND IMMUNOGLOBULIN-LIKE DOMAINS 1	2.1E-03	9.9E-01	2.1E-02	1.42	-1.00	-1.34

Table 3-7. Continued

			p-value			Fold Change		
Affy ID	Gene	Description	Y	MA	A	Y	MA	A
1446464_at	PSME4	PROTEASOME (PROSOME, MACROPAIN) ACTIVATOR SUBUNIT 4	9.3E-01	5.4E-03	1.5E-02	-1.02	2.11	-1.58
1438157_s_at	NFKBIA	NUCLEAR FACTOR OF KAPPA LIGHT CHAIN GENE ENHANCER IN B-CELLS INHIBITOR	1.4E-02	3.0E-01	2.4E-03	1.29	1.14	-1.35
1439422_a_at	C1QDC2	C1Q DOMAIN CONTAINING 2	2.4E-02	4.1E-01	3.2E-03	1.25	-1.06	-1.37
1429372_at	SOX11	SRY-BOX CONTAINING GENE 11	3.5E-03	6.7E-01	1.3E-02	1.51	1.06	-1.39
1454869_at	WDR40B	WD REPEAT DOMAIN 40B	8.2E-01	2.1E-02	1.3E-03	-1.07	1.59	-1.74

Genes from aged mice which exhibited expression opposite young or MA mice at 12 hr. The Affymetrix probe identifier, gene symbol, gene description, *t*-test p-value and fold change are provided for genes in young (Y), MA, and aged (A) mice 12 hr following treatment. The p-values are all < 0.025 for aged mice and for either young or MA mice

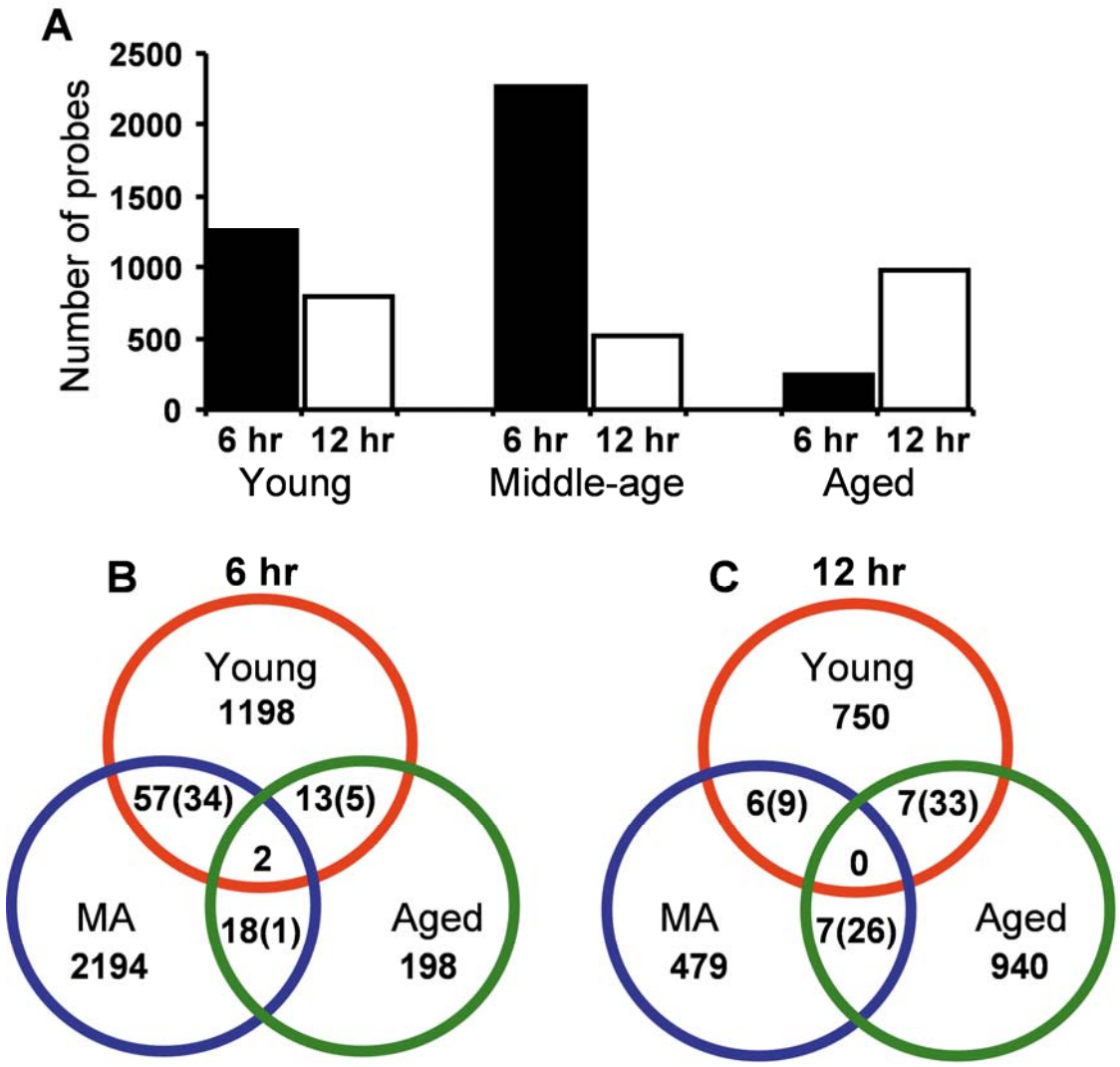

Figure 3-1. Estradiol-responsive gene expression is altered over the course of aging. A) Illustration of the number of probes that were increased or decreased by estradiol treatment in young (Young), middle-aged (MA) and aged (Aged) animals at 6 hr (filled bars) or 12 hr (open bars) after a single estradiol injection. MA animals exhibited over two times the number of altered probes at 6 hr relative to the other two age groups. The number of altered probes decreased at 12 hr relative to 6 hr for young and MA animals. In contrast, aged animals exhibited approximately a five fold increase in the number of estradiol-responsive probes during this time period. B&C) Venn diagrams of the number of differentially expressed probes in response to estradiol treatment at B) 6 hr and C) 12 hr. The numbers in parentheses represent probes that changes in opposite directions.

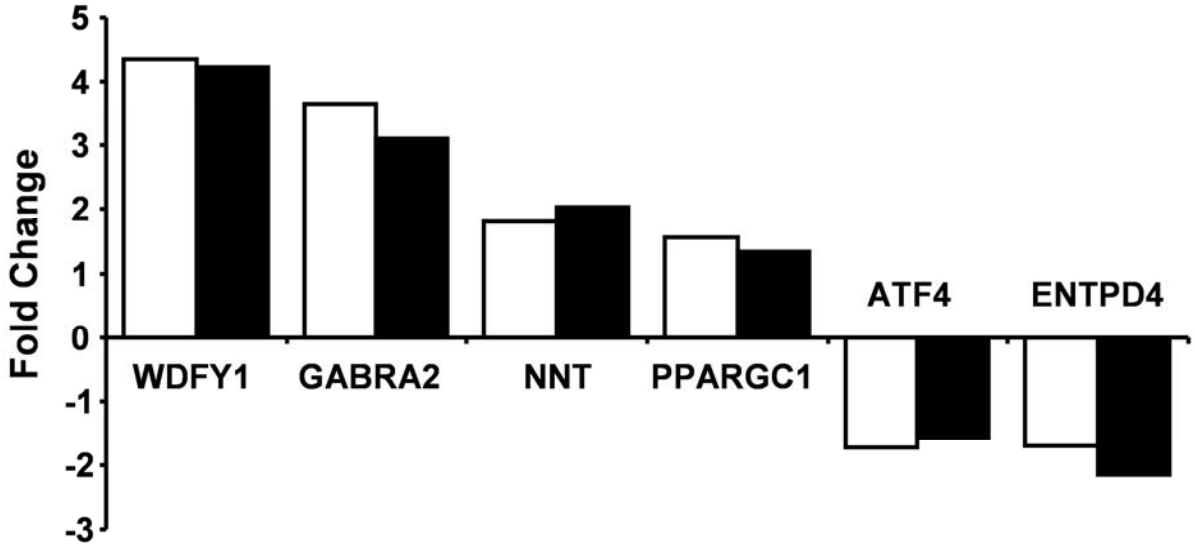

Figure 3-2. Validation of estradiol treatment effects in young animals at 6 hr for six genes using RT-PCR. The bars represent the mean fold change in gene expression for young mice 6 hr following estradiol treatment compared to the mean of age-matched oil treated animals using RT-PCR (open bars) and for microarray measures (filled bars)..

CHAPTER 4
ANALYSIS OF HIPPOCAMPAL GENE EXPRESSION IN ESTROGEN RECEPTOR ALPHA AND ESTROGEN RECEPTOR BETA KNOCKOUT MICE AFTER ACUTE ESTRADIOL TREATMENT

Introduction

Steroid hormones and their receptors play important roles in development, sexual, non-sexual behaviors and neuronal function. In particular, estradiol (the most potent form of estrogen) and its two main receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), have powerful neurotrophic and protective effects within the central nervous system (CNS). Since the discovery of ERs in the hippocampus researchers have examined the role estrogen on hippocampal function (Foster, 2005; McEwen and Alves, 1999; Spencer et al., 2008b). Estrogen has powerful effects on hippocampal dendritic spine density by increasing the density of spines across the estrus cycle and after EB treatment in ovariectomized rats(Gould et al., 1990; Woolley et al., 1990; Woolley and McEwen, 1992). Moreover, research suggests that estrogen receptors influence neuronal physiology and hippocampal dependent memory (Woolley, 2007). However, it is not clear how estrogen receptors are involved in regulating hippocampal function. Thus, a better understanding of estrogen receptors and the mechanisms supporting its influence on hippocampal function must be established.

Despite the fact that ERα and ERβ have a similar structure, binding affinity for estrogen and share ~90% homolog, their behavior can be quite different (Nilsson et al., 2001). The N-terminal domain of ERα and ERβ, which contains the AF-1 domain (ligand independent domain), shares only 20% amino acid homology and displays promoter and cell specific activity. The central domain of ERα and ERβ is highly conserved between the two receptors with 95% homology and contains the DNA binding domain, important for specific DNA binding and receptor dimerization (Enmark et al., 1997). The ligand binding domain or E domain contains the

AF-2 domain and shares 55% homology between ERα and ERβ. This domain has a similar 3D structure between the two receptors but contains different amino acids in the cavity of the domain resulting in a 20% smaller ligand-binding cavity in ERβ. This may be an important region of receptor specificity. Studies have shown that ERβ has a weaker AF-1 domain and relies more on AF-2 domain (Delaunay et al., 2000). These subtle differences in gene structures can lead to diverse actions on transcriptional regulation.

The current study was designed to test the effect of the loss of ERβ and ERα on hippocampal gene expression with and without EB treatment using ERα knockout (KO) and ERβ knockout (KO) female mice. Using microarray analysis we found distinct differences in the hippocampal response to loss of functional ERα and ERβ and response to EB treatment. However, estrogen's ability to promote neuroprotection is maintained after the loss of ERs with and without EB treatment. Our results highlight the adaptable relationship between the two receptors and suggest that even as the ratio of ERs are altered, young mice are still able to maintain transcriptional response to an acute EB treatment.

Materials and Methods

Subject

ERα-/- (ERαKO)(Lubahn et al., 1993) and ERβ-/- (ERβKO)(Krege et al., 1998) were created from heterozygous mouse colonies. Mice were screened using PCR amplification as previously described (Krege et al., 1998; Lubahn et al., 1993). Wildtype (Adams et al.) liter mates were obtained from the same ERαKO and ERβKO colonies and combined into one WT group. All procedures involving animal subjects have been reviewed and approved by the Institutional Animal Care and Use Committee at the University of Florida and were in accordance with guidelines established by the U.S. Public Health Service Policy on Humane

Care and Use of Laboratory Animals. A total of 35 female mice (WT: n=14, 4months; ERαKO n=10, 4 months; ERβKO n=11, 4 months) were employed in this study. Animals were housed 3-5 per cage and maintained on 12:12 light: dark cycle (lights on at 6 am). After 3 months of age, mice were anesthetized (2 mg ketamine and 0.2 mg xylazine per 20 grams of body weight) and ovaries were removed through a small midline incision on the abdomen. All mice received ad lib access to food (Purina mouse chow, St Louis, MO) and water, until the surgery when they were placed on Casein based chow (Cincinnati Lab Supply, Cincinnati, OH), which is low in phytoestrogens found in soy based chow.

Hormone Administration

Briefly, ten days after ovariectomy (OVX) animals received either a single injection of estradiol or oil and were sacrificed 6 hrs after injection. Estradiol (17 β-estradiol benzoate, EB) (Sigma Chemical Co, St Louis MO) was dissolved in light mineral oil (Fisher Scientific, Pittsburgh, PA) to a concentration of 0.1 mg/ml. Oil and EB (5μg) was injected subcutaneously at the nape of the neck in a final volume of 0.05ml. Animals were separated into groups including WT receiving EB (n=7), WT receiving Oil (n=7), ERαKO receiving EB (n=5), ERαKO receiving Oil (n=5), ERβKO receiving EB (n=6) and ERβKO receiving Oil (n=5).

At the time of sacrifice, animals were anesthetized with CO2 and decapitated. The brain was quickly removed and placed in ice-cold artificial cerebral spinal fluid. Both hippocampi were removed, frozen in liquid nitrogen, and stored in -80°C until processing. RNA was isolated from each sample using Qiagen RNeasy Lipid Tissue Mini Kit (Qiagen, Germantown, MD). RNA concentration was determined using spectrophometer and a subset of samples was examined using Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA).

Microarray Hybridization and Signal Detection

One mouse per array was used for all microarray procedures. An amount of 5μg of total RNA was synthesized to cRNA using Affymetrix amplification kit following the manufacture's protocol. Hybridization of cRNA was carried out by the Interdisciplinary Center for Biotechnology Research Microarray Core, University of Florida. Hybridization of Affymetrix Mouse 430 2.0 Arrays (one chip per animal) occurred for 17 hours at 60°C in accordance with manufacture's instructions and arrays were scanned using an Affymetrix Microarray scanner. Images were analyzed using Affymetrix Gene Chip Operating System software (GCOS version 1.1) and hybridization signal intensity levels between GeneChips were normalized using dChip (Li & Hung Wong, 2001). Data was then transferred into Microsoft excel for further analysis.

Data Filtering and Statistical Analysis

Probe sets were annotated using Affymetrix® NetAffx™ (12/2007). The detection of signal (presence/absence) was determined by MAS5.0 (Affymetrix). The number of present calls for each probe set was determined across all arrays by setting criteria that more than 80% of the chips had to exhibit a present call for that probe. The remaining probe sets were submitted to Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA) to determine the number of genes available for generating networks. This filtering procedure resulted in a total data set of 9602 genes. These 9602 genes comprised the total data set used for all statistical comparisons. Two-tailed *t*-tests were used to determine differentially expressed probe sets and alpha level cut-off was set at 0.025 according to previously published work (Aenlle et al., 2007; Blalock et al., 2003). For each set of comparisons, the false discovery rate (FDR) was calculated as the expected number of false positives from multiple testing/total observations. Probe sets with a FDR > 0.5 were considered as unacceptable for pathway investigation and no further

analysis was performed (Ewens and Grant, 2005). Acceptable data sets were submitted to IPA for pathway mapping of differentially expressed transcripts. IPA uses a right-tailed Fisher's Exact Test to compute the likelihood that the relationship between the list of submitted genes and a set of genes representing a given pathway is due to chance. In accordance with best practices, we attempted to limit the IPA analysis to ~800 molecules or less for generating networks. A similar procedure was employed for determining overrepresentation of genes related to synaptic structure using the Expression Analysis Systematic Explorer (EASE) through the NIH DAVID Bioinformatics Resources (Hosack et al., 2003).

RT-PCR

RNA from each group was treated with Turbo DNAfree (Ambion, Austin TX) to remove any remaining genomic DNA. Total RNA (3µg) was then converted to cDNA using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). For relative quantification of RNA, 100ng in 2.5µl of cDNA was added to 12.5µl of Taqman® Universal PCR Master Mix (2X), 1.25µl of 20X Gene Expression Assay Mix (primer sequences can be found in Table 4), and 8.75µl of nuclease-free water for a total volume of 25µl. Thermal cycler conditions were set at 2 min at 50°C, 10 min at 95°C and cycles 15s at 95°C and 1 min at 60°C for 40 cycles. The point at which the fluorescence crosses the threshold (Ct) was determined using 7300 Real-Time PCR System and SDS Software 1.3.1 analysis software (Applied Biosystems). Each sample was in triplicate and normalized to corresponding GAPDH values (ΔCt_{sample}) and then compared to normalized young oil ($\Delta Ct_{reference}$). The mean normalized values were compared using $\Delta\Delta Ct$ method as described by Applied Biosystems to derive fold change (Aenlle et al., 2009), where $\Delta\Delta Ct=(\Delta Ct_{sample}) -(\Delta Ct_{reference})$.

Results

Alteration of Basal Gene Expression Levels in ERKO Mice

To determine differences in basal gene expression we compared estrogen receptor knockout (ERKO) Oil treated animals to WT Oil treated controls. Comparisons between WT Oil treated and ERαKO Oil treated mice indicated 181 genes with altered expression (Figure 4-1). The FDR was > 0.5; therefore, no further analysis was conducted on these genes. In contrast to ERαKO mice, comparisons of WT Oil treated and ERβKO Oil treated animals revealed 674 differentially expressed genes (FDR = 0.35). The majority of the genes (543, 80%) increased expression in ERβKO Oil treated animals (Figure 4-1). Moreover, 353 genes exhibited an increase greater than 1.5 fold (Figure 4-2). For the 131 genes that decreased expression only four genes displayed a greater than 1.5 fold decrease in expression.

The genes from ERβKO Oil treated animals that displayed a significant change in expression from baseline were submitted to IPA to determine whether expression changes were restricted to specific signaling pathways. For the 543 genes that were increased in ERβKO Oil treated animals, IPA indicated overrepresentation in several pathways (Table 4-1). The top five pathways included IGF-1 signaling ($p<0.001$), PPAR signaling (0.005), RAR activation ($p<0.005$), Clarthrin-mediated endocytosis ($p<0.007$), and Wnt/β-catenin signaling ($p<0.007$). For the 131 genes that decreased expression in ERβKO Oil treated mice, two pathways exhibited gene clustering including, SAP/JNK signaling ($p<0.03$), and Calcium signaling ($p<0.04$).

Previous research indicates a role for estrogen in regulating and the expression of synaptic proteins and synapse number. Therefore, we submitted the 674 genes that were differentially expressed in ERβKO Oil treated mice to the Expression Analysis Systematic Explorer (EASE) through the NIH DAVID Bioinformatics Resources (Huang, 2009; Dennis

2003) to examine overrepresentation of genes related to the synapse (GO: 0045202). The analysis indicate significant ($p<0.004$) clustering of genes for synaptic components, with 19 genes increasing in expression and 3 genes decreasing in expression relative to WT Oil treated mice (Table 4-2).

Alteration of Gene Expression After Acute EB Treatment

To examine differential gene expression following EB treatment we determined the number of altered genes between Oil and EB treated within each genotype (Figure 4-3). A total of 937 (FDR=0.25) genes were differentially expressed between WT EB and WT Oil treated mice. Within the 937 genes, 436 (46%) genes increased in expression and 501 (54%) genes decreased in expression. Further, 65 genes had a fold change of greater than 1.5, with 41 increasing and 24 decreasing. Compared to WT animals, ERKO mice exhibited reduced responsiveness to acute EB treatment (Figure 4-3). Two-tailed t-tests of ERαKO EB treated relative to ERαKO Oil treated mice and ERβKO EB treated relative to ERβKO Oil treated mice revealed 171 and 121 genes, respectively, were altered after EB treatment. In both cases the FDR was >0.5.

The apparent lack of EB effects in ERKO animals suggests that both receptors are required for EB influences on gene expression. Alternatively, the lack of responsiveness may be due to the altered basal gene expression in ERKO mice, attributable to the release of inhibition and thus enhanced activity of the remaining receptor (Pettersson et al., 2000; Williams et al., 2008). Interestingly, of the 937 genes differentially expressed in WT Oil vs WT EB treated mice, 191 were also differentially expressed in ERβKO Oil treated mice compared to WT Oil (Figure 4-4). Most of the genes were differentially express in the same direction (136 increasing, 49 decreasing) (Table 4-3,Table 4-4), suggesting ceiling/floor effects of EB induced gene

expression due to uninhibited ERα activity. If the shift in basal gene expression is due to enhanced activity of the remaining receptor, then EB treatment may eliminate gene expression differences. Indeed, a comparison between WT EB and ERαKO EB treated mice reveal only 87 differentially expressed genes. Similarly, 196 genes were differentially expressed between ERβKO EB and WT EB treated mice (Figure 4-5). Finally, analysis of gene expression differences between ERαKO EB and ERβKO EB treated mice found 100 differentially expressed genes. Therefore, at least for the 6 hr time point examined, ERKO mice and WT mice display similar gene expression following a single EB treatment.

The idea that the removal of one receptor will enhance activity of the remaining receptors was further tested by comparing the gene expression levels between WT Oil treated and ERKO EB treated mice. For this analysis genes were selected that exhibited a significant difference ($p < 0.025$) between WT Oil and ERKO EB treated mice. Furthermore, we set a criterion that expression change had to be monotonic with respect to the presumed increased receptor activation, such that for genes that increased expression following EB treatment, the level of expression had to follow the pattern of WT Oil treated< ERKO Oil treated< ERKO EB treated. A similar criterion was set for genes that decreased in expression following EB treatment (ERKO EB treated< ERKO Oil treated< WT Oil treated). Comparison of WT Oil treated versus ERαKO EB treated animals revealed 1182 (FDR=0.20) genes out of the total data set were altered between WT Oil and ERαKO EB treated animals and exhibited a directional change across the three groups (Figure 4-6). The number of differentially expressed genes was 6.5 fold greater than the number of genes differentially expressed for WT Oil versus ERαKO Oil treated animals. Similar gene expression changes were seen in ERβKO EB treated mice with a total of 1164 (FDR=0.20) genes out of the total data set altered between WT Oil and ERβKO EB treated mice

(Figure 4-6), which was a 1.7 fold increase in the number of genes altered relative to comparisons between WT Oil and ERβKO Oil treated animals.

A total of 276 genes were differentially expressed in all three genotypes treated with EB compared to WT Oil. Figure 4-7 displays the average percent change in gene expression in WT, ERαKO and ERβKO relative to WT Oil and indicates that the maximum response to EB treatment across genes is similar across genotypes. Furthermore, for those genes that increased with EB treatment, the baseline expression (i.e. in Oil treated animals) was increased in ERβKO mice.

Estrogen Receptor Alpha Gene Expression Levels

Interestingly, a significant increase ($p<0.0001$) in ERα expression was found in ERαKO EB and Oil treated compared to WT and ERβKO in both EB and Oil treated animals (Figure 4-8). There was no significant difference in ERα gene expression levels between ERαKO EB and Oil treated animals or between WT (EB or Oil treated) to ERβKO (EB or Oil treated). At least, a 3-fold increase in expression was found in ERαKO compared to WT and ERβKO animals regardless of treatment. No significant differences between groups were found in ERβ gene expression levels (data not shown).

RT-PCR

Validations of microarray results were preformed on 7 genes. Of the 7 primers used to confirm microarray results; 2 genes (Camk2a, Atp2b1) were significantly altered by the loss of the ERβ (WT Oil versus ERβKO Oil), 4 genes (Sqk1, Fut, March7, Eif2s1) were influenced by EB treatment in WT animals and ESR1 which was found to be increased in ERαKO animals. All 7 primers showed at least a 1.25 fold increase in expression, confirming microarray results (Figure 4-8 and 4-9).

Discussion

Our analyses reveal distinct roles of ERα and ERβ on hippocampal gene expression with and without EB treatment. The absence of functional ERβ appears to have a greater impact on basal hippocampal gene expression. Relative to WT Oil treated mice, ERαKO mice treated with Oil exhibited few gene changes. In contrast, ERβKO mice treated with Oil exhibited a relatively large number of altered genes, with 80% of the genes increasing expression. Moreover, the magnitude of expression was conspicuous such that 52% of the differentially expressed genes exhibited increased expression greater than 1.5 fold.

The shift in basal hippocampal gene expression in ERβKO animals is likely due to the loss of ERβ inhibition on transcription normally induced by ERα activity. In a number of systems, ERβ acts as a dominate inhibitory regulator by forming heterodimer complexes to limit ERα binding to estrogen response elements (Gonzalez et al., 2007; Hall and McDonnell, 1999; Pettersson et al., 2000; Strom et al., 2004; Williams et al., 2008; Zhao et al., 2007). Gene profiling studies in bone (Lindberg et al., 2003) and aortic tissue (O'Lone et al., 2007) of ERαKO and ERβKO mice demonstrate increased transcriptional activity associated with ERα activation, which is enhanced in ERβKO mice. It is important to note that these studies observed differences in gene expression after EB treatment. In contrast, we observed significant alteration of basal gene expression in ERβKO relative to WT mice. The difference in basal expression found in ovariectomized animals is likely due to endogenous hippocampal estrogen driving ERα mediated changes in gene expression. Even after the removal of gonadal hormones, the level of locally synthesized estrogen in the hippocampal is ~1 nM, a level which will preferentially activate ERα (Barkhem et al., 1998; Pettersson et al., 2000). Thus, in the absence of exogenous hormone,

hippocampal ERβ appears to act mainly as an inhibitory regulator of gene expression driven by ERα.

Estrogen is neuroprotective against ischemia and neurodegenerative diseases, although the exact mechanisms are unclear. The results of the current study indicate that in the absence of a functional ERβ, ERα increases the expression of genes involved in several neuroprotection pathways including; IGF-1 signaling, PPAR signaling, RAR activation, and Wnt/β-catenin signaling. The link between ERα and IGF-I signaling has been well established using both *in vitro* and *in vivo* methods (Azcoitia et al., 1999; Cardona-Gomez et al., 2000; Garcia-Segura et al., 2007; Garcia-Segura et al., 2006; Mendez et al., 2005). Recent work suggests estrogen enhances neuroprotection by interacting with the Wnt/β-catenin pathway (Zhang et al., 2008) and increasing the expression of genes for components of PPAR signaling (Aenlle and Foster, 2009). The enhancement of neuroprotective pathways by unopposed ERα activity may explain why ERβKO animals displayed less infarct volume after middle cerebral artery occlusion compared to ERαKOs (Dubal et al., 2006; Dubal et al., 2001) and the differential effects of ERα and ERβ agonisits in protecting hippocampal neurons (Dai et al., 2007). Together the results indicate that ERα activity, unopposed by ERβ, acts on gene expression to augment several neuroprotective pathways,

In addition, the results of the current study indicate that ERα plays a prominent role in regulating synapses. Estrogen induces the growth of hippocampal dendritic spines (Gould et al., 1990; Woolley et al., 1990; Woolley and McEwen, 1992) and synaptogenesis (Akama and McEwen, 2003; Choi et al., 2003; Jelks et al., 2007; Kretz et al., 2004; Rune and Frotscher, 2005). We observed an increased in the basal expression of synaptic component genes in ERβKO Oil treated mice compared to WT Oil treated mice. The results are consistent with

several previous studies indicating that local estrogen synthesis can drive the maintenance of synapses (Kretz et al., 2004), and ERα activity mediates an increase in synaptic markers (Jelks et al., 2007; Morissette et al., 2008a; Murakami et al., 2006; Patrone et al., 2000; Romeo et al., 2005). However, it should be noted that several of these studies report a similar, though usually blunted effect of ERβ activation (Jelks et al., 2007; Liu et al., 2008; Morissette et al., 2008a; Patrone et al., 2000), suggesting that ERβ may be less active but have similar effects (Lindberg et al., 2003).

Acute Estrogen Treatment Differentially Alters Hippocampal Gene Expression in WT, ERαKO and ERβKO Mice

Our results reveal that EB treatment of ERαKO and ERβKO mice enhanced differences in gene expression relative to WT Oil treated mice indicating that both receptors are responsive to EB. Furthermore, a similar pattern of gene expression was observed in all three genotypes, WT, ERβKO, and ERαKO, treated with EB indicating that EB having similar effects on gene expression regardless of receptor type. It possible that, rather than the classical ER genomic signaling, the similarity in gene expression observed 6 hr after EB treatment was mediated through activation of ERs on the membrane which then activate signaling cascades which are common to multiple membrane receptors (Foster, 2005). Nevertheless, the fact that EB treatment was required to observed changes in ERαKO mice, which were evident under basal conditions in ERβKO mice, indicates that membrane ERα may be predominately activated under conditions of low estrogen or genomic ERα induced transcription has feed-forward effects to promote elements involved in rapid estrogen-mediated processes (Aenlle and Foster, 2009)

Together our results indicate that when circulating estrogen levels are relatively low, ERα is the principal receptor regulating gene expression. In several systems that express both receptors, ERβ acts as an inhibitory regulator of gene expression driven by ERα possibly by

forming heterodimer complexes to limit ERα binding to estrogen response elements (Hall and McDonnell, 1999; Strom et al., 2004; Zhao et al., 2007) (Pettersson et al., 2000; Williams et al., 2008). This is consistent with the notion that higher levels of estrogen are required to affect behavior when ERβ is present (Rissman, 2008).

Our results indicate that ERβ activation can have effects similar to that observed for ERα; however, in ovariectomized mice, locally synthesized estrogen may not reach a level needed to activate ERβ; therefore, activation of ERβ may be observed following estrogen treatment. Accordingly, the effect of EB treatment on hippocampal functions, including memory, is likely dependent on the level of ongoing ER activity. Previous research indicates that, in the absence of estrogen treatment, ovariectomized ERβKO mice exhibit normal learning and memory (Fugger et al., 2000; Liu et al., 2008; Rissman et al., 2002), possibly due to unopposed ERα activity acting to maintain or increase genes for synaptic growth and remodeling. We observed that, due to enhanced baseline expression, subsequent EB treatment had minimal effect on gene expression in ERβKO mice. As such we might expect that estrogen treatment of ERβKO mice would have minimal influence on memory (Liu et al., 2008) or could possibly impair learning if over activation of ERα resulted in a feed back reduction in ERα expression (Rissman et al., 2002) or an uncoupling of receptor-transcription activity (Foster, 2005). In contrast to ERβKO mice, ovariectomized ERαKO mice exhibit impaired learning and memory relative to WT controls, possibly due to the inability of locally synthesized estrogen to activate ERβ. This impairment can be rescued by viral mediated delivery of ERα (Foster et al., 2008) or estrogen treatment (Fugger et al., 2000), which would be expected to drive ERβ to activate pathways normally induced by ERα. The idea that ERα activity maintain hippocampal function and that estrogen treatment is required when ERα activity is disrupted may extend to women in which

ERα polymorphisms are associated with memory deficits that emerge postmenopausally (Corbo et al., 2006; Ji et al., 2000; Olsen et al., 2006; Yaffe et al., 2009).

For animals that express both receptors in the hippocampus, the effect of EB treatment is likely to reflect the relative expression and function of each receptor. Whereby, ERs can act antagonistically or synergistically to promote or inhibit the expression of genes. Furthermore, these effects are tissue and state dependent, as the ratio of receptors differ according to brain region and adjust to changes in the environment such as after injury. Work by Wise and colleagues have found that ERβKO mice display less total infarct after middle-cerebral artery occlusion (MCAO) and WT mice show an initial increase in ERα immediately following injury (Dubal et al., 2006; Dubal et al., 2001). Suggesting an upregulation of ERα or unopposed ERα promotes neuroprotective pathways.

Conclusion

Finally, our results have important implications on the effects of estrogen to treat age-related cognitive impairments. The effect of estrogen decreases with age with a possible critical period where treatment should be initiated during middle-age (Craig and Murphy, 2008; Maki, 2006; Sherwin and Henry, 2008). This critical time period coincides with estropause in mice and menopause in humans, a time of fluctuating hormones before cessation of the menstrual cycle. Similar to humans, rodents show a decrease in circulating hormones, decrease in responsiveness to EB and an age-related alteration in estrogen receptors (Adams and Morrison, 2003; Espeland et al., 2004; Foster, 2005; Shumaker et al., 2004). A better understanding of the behavior of estrogen receptors and how a change in the ratio of estrogen receptors affects hippocampal function, may provide better treatment options during this critical period.

Table 4-1 The top pathways affected in ERβKO Oil treated mice

Increasing IGF-1 Signaling		Affymetrix ID	p-value	FC
CSNK2A1	casein kinase 2, alpha 1 polypeptide	1419038_at	0.009	5.56
CSNK2A2	casein kinase 2, alpha prime polypeptide	1453099_at	0.022	2.16
CTGF	connective tissue growth factor	1416953_at	0.013	1.51
IGF1R	insulin-like growth factor 1 receptor	1428967_at	0.012	1.56
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	1434000_at	0.013	1.08
NOV	nephroblastoma overexpressed gene	1426852_x_at	0.014	1.19
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	1425514_at	0.003	1.69
RASA1	RAS p21 protein activator (GTPase activating protein) 1	1426478_at	0.017	1.43
RPS6KB1	ribosomal protein S6 kinase, 70kDa, polypeptide 1	1460705_at	0.020	1.42
YWHAQ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide	1460621_x_at	0.023	1.07
PPAR Signaling		**Affymetrix ID**	**p-value**	**FC**
CREBBP	CREB binding protein	1435224_at	0.014	1.22
HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 1	1437497_a_at	0.022	1.37
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	1434000_at	0.013	1.08
MAP3K7	mitogen-activated protein kinase kinase kinase 7	1426627_at	0.007	1.244
MED1	mediator complex subunit 1	1421907_at	0.004	3.64
NCOR1	nuclear receptor co-repressor 1	1423201_at	0.014	2.78
NRIP1	nuclear receptor interacting protein 1	1418469_at	0.006	2.11
PDGFD	platelet derived growth factor D	1426319_at	0.012	1.30
PPARGC1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	1437751_at	0.010	1.35
RAR Activation		**Affymetrix ID**	**p-value**	**FC**
CREBBP	CREB binding protein	1435224_at	0.014	1.22
CSNK2A1	casein kinase 2, alpha 1 polypeptide	1419038_at	0.009	5.56
CSNK2A2	casein kinase 2, alpha prime polypeptide	1453099_at	0.022	2.16
DUSP1	dual specificity phosphatase 1	1448830_at	0.022	1.54
MED1	mediator complex subunit 1	1421907_a	0.004	3.63

		t		
NCOR1	nuclear receptor co-repressor 1	1423201_at	0.014	2.78
NRIP1	nuclear receptor interacting protein 1	1418469_at	0.006	2.11
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	1425514_at	0.003	1.69
PNRC1	proline-rich nuclear receptor coactivator 1	1433668_at	0.005	1.26
PPARGC1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	1437751_at	0.010	1.352
PTEN	phosphatase and tensin homolog	1422553_at	0.003	3.84
SMAD1	SMAD family member 1	1416081_at	0.009	1.58
SMAD4	SMAD family member 4	1422485_at	0.015	1.42

Table 4-1. Continued

Clathrin-mediated Endocytosis		Affymetrix ID	p-value	FC
AP2B1	adaptor-related protein complex 2, beta 1 subunit	1427077_a_at	0.005	1.52
CSNK2A1	casein kinase 2, alpha 1 polypeptide	1419038_a_at	0.009	5.56
CSNK2A2	casein kinase 2, alpha prime polypeptide	1453099_at	0.022	2.16
DNM3	dynamin 3	1446265_at	0.012	2.1
FGF12	fibroblast growth factor 12	1451693_a_at	0.004	1.27
FGF14	fibroblast growth factor 14	1435747_at	0.006	1.23
FGF9	fibroblast growth factor 9 (glia-activating factor)	1438718_at	0.017	1.27
HIP1	huntingtin interacting protein 1	1424755_at	0.005	1.24
HSPA8	heat shock 70kDa protein 8	1420622_a_at	0.004	1.12
PDGFD	platelet derived growth factor D	1426319_at	0.012	1.30
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	1425514_at	0.003	1.69
WASL	Wiskott-Aldrich syndrome-like	1426777_a_at	0.007	2.79

Wnt/β-catenin Signaling		Affymetrix ID	p-value	FC
CDH2	cadherin 2, type 1, N-cadherin	1418815_at	0.010	1.36
CREBBP	CREB binding protein	1435224_at	0.014	1.22
CSNK1A1	casein kinase 1, alpha 1	1424827_a_at	0.008	1.20
CSNK1E	casein kinase 1, epsilon	1417176_at	0.012	2.09
CSNK2A1	casein kinase 2, alpha 1 polypeptide	1419038_a_at	0.009	5.56
CSNK2A2	casein kinase 2, alpha prime polypeptide	1453099_a	0.022	2.16

		t		
GSK3B	glycogen synthase kinase 3 beta	1437001_at	0.009	6.41
MAP3K7	mitogen-activated protein kinase kinase kinase 7	1426627_at	0.007	1.24
NLK	nemo-like kinase	1419112_at	0.004	1.97
PPM1L	protein phosphatase 1 (formerly 2C)-like	1438012_at	0.007	2.24
PPP2R5C	protein phosphatase 2, regulatory subunit B', gamma isoform	1434206_s_at	0.008	1.44
PPP2R5E	protein phosphatase 2, regulatory subunit B', epsilon isoform	1428463_a_at	0.006	1.47
SOX11	SRY (sex determining region Y)-box 11	1436790_a_at	0.012	1.14

Decreasing

SAPK/JNK Signaling		Affymetrix ID	p-value	FC
FADD	Fas (TNFRSF6)-associated via death domain	1416888_at	0.002	-1.40
GNG11	guanine nucleotide binding protein (G protein), gamma 11	1448942_at	0.006	-1.15
RIPK1	receptor (TNFRSF)-interacting serine-threonine kinase 1	1449485_at	0.008	-1.14

Table 4-1 Continued

Calcium Signaling		Affymetrix ID	p-value	FC
ATP2B3	ATPase, Ca++ transporting, plasma membrane 3	1442645_at	0.009	-1.2
CHRNA5	cholinergic receptor, nicotinic, alpha 5	1442035_at	0.016	-1.18
HTR3A	5-hydroxytryptamine (serotonin) receptor 3A	1418268_at	0.022	-1.12
RYR1	ryanodine receptor 1	1427306_at	0.008	-1.19

The pathways with overrepresentation of genes that were observed to increase or decrease expression in ERβKO Oil treated mice. The p-valuse is calculated from a right-talied Fosher's Exact T

Table 4-2 The 22 genes involved in synapse in ERβKO Oil treated mice

Symbol	Description	Affymetrix	p-value	Fold Change
CADM1	cell adhesion molecule 1	1417378_at	0.014	1.16
CAMK2A	calcium/calmodulin-dependent protein kinase II alpha	1442707_at	0.018	2.66

CDH2	cadherin 2, type 1, N-cadherin (neuronal)	1418815_at	0.010	1.36
CHRNA5	cholinergic receptor, nicotinic, alpha 5	1442035_at	0.016	-1.18
DLG2	discs, large homolog 2 (Drosophila)	1437927_at	0.011	1.35
DOK7	docking protein 7	1434812_s_at	0.008	-1.13
DTNA	dystrobrevin, alpha	1425292_at	0.001	1.29
GABRA4	gamma-aminobutyric acid (GABA) A receptor, alpha 4	1429330_at	0.005	2.04
GPHN	gephyrin	1426462_at	0.015	1.18
GRIA1	glutamate receptor, ionotropic, AMPA 1	1448972_at	0.011	2.20
GRM3	glutamate receptor, metabotropic 3	1430136_at	0.002	1.52
HTR3A	5-hydroxytryptamine (serotonin) receptor 3A	1418268_at	0.022	-1.12
ITSN1	intersectin 1 (SH3 domain protein)	1425899_a_at	0.009	1.17
KCNMA1	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	1424848_at	0.012	4.12
MYH9	myosin, heavy chain 9, non-muscle	1417472_at	0.008	1.25
NLGN1	neuroligin 1	1437160_at	0.022	1.31
NRXN1	neurexin 1	1428240_at	0.021	2.06
PCLO	piccolo (presynaptic cytomatrix protein)	1452423_at	0.015	1.46
RIMS1	regulating synaptic membrane exocytosis 1	1438305_at	0.020	1.46
RPS6KB1	ribosomal protein S6 kinase, 70kDa, polypeptide 1	1460705_at	0.020	1.42
SNAP29	synaptosomal-associated protein, 29kDa	1423356_at	0.008	1.21

The Affymetrix probe identifier, gene symbol, gene description, *t*-test p-value and fold change are provided for genes of synaptic components that are increasing and decreasing in ERβKO Oil treated mice

Table 4-3 Genes involved in RAR Activation and PPAR that are significantly altered in WT EB treated and ERβKO Oil treated mice

	WT Oil vs. WT EB		WT Oil vs. ERβKO Oil	
RAR Activation		p-value		p-value
	CREBBP	0.009	CREBBP	0.014
	PIK3R1	0.020	PIK3R1	0.003
	SNW1	0.023	MED1	0.004
	SMAD4	0.012	SMAD4	0.015
	NCOR1	0.021	NCOR1	0.014
	PRKCA	0.003	SMAD1	0.009
	PPARGC1A	0.025	PPARGC1A	0.010
	CSNK2A2	0.020	CSNK2A2	0.022
	PNRC1	0.020	PNRC1	0.005

	JUN	0.004	CSNK2A1	0.009
	NCOA1	0.005	DUSP1	0.022
	NRIP1	0.017	NRIP1	0.006
	PRKACB	0.021	PTEN	0.003
PPAR Signaling		p-value		p-value
	CREBBP	0.009	CREBBP	0.014
	JUN	0.004	MED1	0.004
	NCOR1	0.021	NCOR1	0.014
	MAPK1	0.024	HSP90AA1	0.022
	NCOA1	0.005	PDGFD	0.012
	PDGFB	0.021	KRAS	0.013
	PPARGC1A	0.025	PPARGC1A	0.010
	NRIP1	0.017	NRIP1	0.006
	MAP3K7	0.020	MAP3K7	0.007

Genes involved in RAR Activation and PPAR that are significantly altered in WT EB treated and ERβKO Oil treated mice

Table 4-4 The 191 genes significant in WT EB treated and ERβKO Oil treated mice

		WT Oil vs. WT EB		WT Oil vs. ERβKO Oil	
Symbol	Description	p-value	FC	p-value	FC
ADSL	adenylosuccinate lyase	0.005	-1.14	0.014	-1.15
ALKBH3	alkB, alkylation repair homolog 3 (E. coli)	0.022	-1.13	0.007	-1.18
APRT	adenine phosphoribosyltransferase	0.024	-1.10	0.006	-1.14
ARHGAP5	Rho GTPase activating protein 5	0.021	-1.15	0.020	1.67
ASCC1	activating signal cointegrator 1 complex subunit 1	0.014	-1.19	0.013	-1.18
ATXN10	ataxin 10	0.018	-1.09	0.006	-1.108
BLVRB	biliverdin reductase B (flavin reductase (NADPH))	0.019	-1.13	0.019	-1.144
C19ORF62	chromosome 19 open reading frame 62	0.004	-1.14	0.022	-1.11

Table 4-4 Continued

		WT Oil vs. WT EB		WT Oil vs. ERβKO Oil	
Symbol	Description	p-value	FC	p-value	FC
C1QTNF2	C1q and tumor necrosis factor related protein 2	0.004	-1.32	0.008	-1.34
C1ORF35	chromosome 1 open reading frame 35	0.023	-1.13	0.020	-1.18
C6ORF134	chromosome 6 open reading frame 134	0.006	-1.34	0.020	-1.20
C9ORF64	chromosome 9 open reading frame 64	0.004	-1.12	0.023	-1.12
CBX5	chromobox homolog 5 (HP1 alpha homolog, Drosophila)	0.002	-1.12	0.017	1.48
CCBL1	cysteine conjugate-beta lyase, cytoplasmic	0.008	-1.26	0.021	-1.26
CCDC134	coiled-coil domain containing 134	0.021	-1.07	0.013	-1.10
CCNT2	cyclin T2	0.004	-1.55	0.004	-1.51
CDON	Cdon homolog (mouse)	0.001	-1.19	0.006	-1.20
CYB561D2	cytochrome b-561 domain containing 2	0.018	-1.13	0.022	-1.14
DHX35	DEAH (Asp-Glu-Ala-His) box polypeptide 35	0.006	-1.18	0.015	-1.19
EGFL7	EGF-like-domain, multiple 7	0.005	-1.23	0.022	-1.22

FADD	Fas (TNFRSF6)-associated via death domain	0.003	-1.30	0.002	-1.40
FAM113A	family with sequence similarity 113, member A	0.007	-1.12	0.024	-1.13
GJB2	gap junction protein, beta 2, 26kDa	0.003	-1.56	0.009	-1.44
GMPPA	GDP-mannose pyrophosphorylase A	0.017	-1.13	0.017	-1.15
GNG11	guanine nucleotide binding protein (G protein), gamma 11	0.001	-1.17	0.006	-1.15
GSTM2	glutathione S-transferase mu 2 (muscle)	0.016	-1.16	0.006	-1.22
HPCA	hippocalcin	0.017	-1.15	0.017	-1.18
HS1BP3	HCLS1 binding protein 3	0.023	-1.09	0.015	-1.14
HSPA8	heat shock 70kDa protein 8	0.002	-1.36	0.004	1.123
IFITM3	interferon induced transmembrane protein 3 (1-8U)	0.006	-1.30	0.019	-1.29
KCTD13	potassium channel tetramerisation domain containing 13	0.007	-1.14	0.020	-1.14
LUC7L2	LUC7-like 2 (S. cerevisiae)	0.023	-1.17	0.018	2.83
METTL2B	methyltransferase like 2B	0.016	-1.25	0.005	-1.35
MVD	mevalonate (diphospho) decarboxylase	0.014	-1.25	0.010	-1.29
NAT6	N-acetyltransferase 6 (GCN5-related)	0.009	-1.14	0.020	-1.15
NSD1	nuclear receptor binding SET domain protein 1	0.000	-1.19	0.019	-1.07
ORAI1	ORAI calcium release-activated calcium modulator 1	0.011	-1.23	0.001	-1.28
PDLIM7	PDZ and LIM domain 7 (enigma)	0.016	-1.32	0.013	-1.24
PLEKHG2	pleckstrin homology domain containing, family G (with RhoGef domain) member 2	0.022	-1.13	0.013	-1.13
POLD2	polymerase (DNA directed), delta 2, regulatory subunit 50kDa	0.007	-1.15	0.014	-1.16
PORCN	porcupine homolog (Drosophila)	0.019	-1.20	0.014	1.17
RELB	v-rel reticuloendotheliosis viral oncogene homolog B	0.015	-1.16	0.004	-1.24
RGS10	regulator of G-protein signaling 10	0.022	-1.09	0.011	-1.12
RPS17	ribosomal protein S17	0.023	-1.17	0.018	-1.24
SCAMP3	secretory carrier membrane protein 3	0.024	-1.10	0.022	-1.11

Table 4-4 Continued

		WT Oil vs. WT EB		WT Oil vs. ERβKO Oil	
Symbol	Description	p-value	FC	p-value	FC
TAGLN2	transgelin 2	0.005	-1.19	0.017	-1.13
SMYD2	SET and MYND domain containing 2	0.005	-1.14	0.018	-1.12
SORD	sorbitol dehydrogenase	0.006	-1.19	0.005	-1.19
TARBP2	TAR (HIV-1) RNA binding protein 2	0.005	-1.23	0.017	-1.22
THYN1	thymocyte nuclear protein 1	0.018	-1.12	0.006	-1.16
TNFAIP8L1	tumor necrosis factor, alpha-induced protein 8-like 1	0.004	-1.13	0.007	-1.13
ZFAND2B	zinc finger, AN1-type domain 2B	0.018	-1.13	0.017	-1.16
ABL2	v-abl Abelson murine leukemia viral oncogene homolog 2 (arg, Abelson-related gene)	0.016	1.09	0.020	2.162
ACSL3	acyl-CoA synthetase long-chain family member 3	0.006	1.29	0.002	1.34
AP2B1	adaptor-related protein complex 2, beta 1 subunit	0.022	1.52	0.005	1.52

APH1B	anterior pharynx defective 1 homolog B (C. elegans)	0.021	1.18	0.005	1.43
AR	androgen receptor	0.002	1.29	0.011	1.31
ARIH1	ariadne homolog, ubiquitin-conjugating enzyme E2 binding protein, 1 (Drosophila)	0.022	1.28	0.010	1.31
ASB7	ankyrin repeat and SOCS box-containing 7	0.003	1.19	0.017	1.43
ASH1L	ash1 (absent, small, or homeotic)-like (Drosophila)	0.005	1.14	0.005	1.36
ATP2B1	ATPase, Ca++ transporting, plasma membrane 1	0.024	1.34	0.011	3.73
C10ORF46	chromosome 10 open reading frame 46	0.021	1.10	0.019	1.27
CAND1	cullin-associated and neddylation-dissociated 1	0.011	1.15	0.017	1.17
CBX3	chromobox homolog 3 (HP1 gamma homolog, Drosophila)	0.017	1.18	0.006	1.24
CCAR1	cell division cycle and apoptosis regulator 1	0.008	1.63	0.016	1.87
CCNT1	cyclin T1	0.004	1.28	0.023	1.29
CLOCK	clock homolog (mouse)	0.002	1.33	0.019	1.28
CNKSR2	connector enhancer of kinase suppressor of Ras 2	0.008	1.21	0.001	1.33
CPEB3	cytoplasmic polyadenylation element binding protein 3	0.005	1.71	0.013	1.87
CREB1	cAMPresponsive element binding protein1	0.004	1.51	0.003	1.83
CREBBP	CREB binding protein	0.009	1.17	0.014	1.22
CTNND2	catenin (cadherin-associated protein), delta 2 (neural plakophilin-related arm-repeat protein)	0.017	1.13	0.023	1.18
DAB1	disabled homolog 1 (Drosophila)	0.024	1.10	0.012	1.85
DIO2	deiodinase, iodothyronine, type II	0.006	1.45	0.014	1.23
DKC1	dyskeratosis congenita 1, dyskerin	0.015	1.15	0.017	1.22
DLG2	discs, large homolog 2 (Drosophila)	0.015	1.31	0.011	1.35
DPH5	DPH5 homolog (S. cerevisiae)	0.020	1.26	0.003	1.41
DTNA	dystrobrevin, alpha	0.012	1.24	0.001	1.29

Table 4-4 Continued

		WT Oil vs. WT EB		WT Oil vs. ERβKO Oil	
Symbol	Description	p-value	FC	p-value	FC
EIF3A	eukaryotic translation initiation factor 3, subunit A	0.008	1.24	0.002	1.38
EIF2S1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	0.004	1.86	0.019	1.68
EIF2S3	eukaryotic translation initiation factor 2, subunit 3 gamma, 52kDa	0.011	1.15	0.022	1.15
EIF5	eukaryotic translation initiation factor 5	0.014	1.55	0.007	1.88
EPRS	glutamyl-prolyl-tRNA synthetase	0.015	1.58	0.011	1.87
ESF1	ESF1, nucleolar pre-rRNA processing protein, homolog (S. cerevisiae)	0.002	1.64	0.003	1.97
FAM107A	family with sequence similarity 107, member A	0.012	1.20	0.016	1.12
FRYL	FRY-like	0.009	1.40	0.021	1.51
FUT9	fucosyltransferase 9 (alpha (1,3) fucosyltransferase)	0.025	2.13	0.002	4.20

G3BP2	GTPase activating protein (SH3 domain) binding protein 2	0.018	1.06	0.010	2.36
GABRA4	gamma-aminobutyric acid (GABA) A receptor, alpha 4	0.007	1.73	0.005	2.04
GIGYF2	GRB10 interacting GYF protein 2	0.003	1.18	0.010	1.21
HCCS	holocytochrome c synthase (cytochrome c heme-lyase)	0.012	1.17	0.023	1.22
HEXIM1	hexamethylene bis-acetamide inducible 1	0.006	1.14	0.021	1.15
HIP1	huntingtin interacting protein 1	0.021	1.15	0.005	1.24
HMG1L1	high-mobility group box 1-like 1	0.005	1.08	0.018	1.55
HTRA1	HtrA serine peptidase 1	0.015	1.27	0.018	1.21
ICK	intestinal cell (MAK-like) kinase	0.009	1.32	0.011	1.41
IDE	insulin-degrading enzyme	0.024	1.33	0.011	1.56
IGF1R	insulin-like growth factor 1 receptor	0.001	1.45	0.012	1.56
ITFG1	integrin alpha FG-GAP repeat containing 1	0.023	1.14	0.024	1.16
JARID1B	jumonji, AT rich interactive domain 1B	0.018	1.51	0.004	1.95
KCNA1	potassium voltage-gated channel, shaker-related subfamily, member 1 (episodic ataxia with myokymia)	0.010	1.09	0.012	2.76
KHSRP	KH-type splicing regulatory protein	0.004	1.79	0.013	1.92
KIAA1128	KIAA1128	0.008	1.15	0.003	2.54
KIAA1881	KIAA1881	0.005	1.32	0.012	1.16
KLF9	Kruppel-like factor 9	0.001	1.17	0.024	1.12
KLHL9	kelch-like 9 (Drosophila)	0.005	1.18	0.018	1.18
LIMCH1	LIM and calponin homology domains 1	0.012	1.45	0.022	1.54
MAPRE1	microtubule-associated protein, RP/EB family, member 1	0.016	1.43	0.008	1.60
MARCH7	membrane-associated ring finger (C3HC4) 7	0.001	1.14	0.009	1.88
MARCKS	myristoylated alanine-rich protein kinase C substrate	0.013	1.61	0.014	1.66
MAT2A	methionine adenosyltransferase II, alpha	0.009	1.49	0.025	1.55

Table 4-4 Continued

		WT Oil vs. WT EB		WT Oil vs. ERβKO Oil	
Symbol	Description	p-value	FC	p-value	FC
MBOAT2	membrane bound O-acyltransferase domain containing 2	0.005	1.22	0.003	1.38
MERTK	c-mer proto-oncogene tyrosine kinase	0.006	1.48	0.003	1.41
MTPN	myotrophin	0.019	1.20	0.004	1.29
MYH9	myosin, heavy chain 9, non-muscle	0.009	1.16	0.008	1.25
NAPB	N-ethylmaleimide-sensitive factor attachment protein, beta	0.017	1.26	0.023	1.81
NAT13	N-acetyltransferase 13 (GCN5-related)	0.001	1.10	0.018	1.09
NCOR1	nuclear receptor co-repressor 1	0.021	1.42	0.014	2.77
NEK7	NIMA (never in mitosis gene a)-related kinase 7	0.010	1.43	0.018	1.47
NLK	nemo-like kinase	0.023	1.49	0.004	1.97
NRIP1	nuclear receptor interacting protein 1	0.017	1.19	0.006	2.11
NRXN3	neurexin 3	0.019	1.34	0.010	1.47

NUCKS1	nuclear casein kinase and cyclin-dependent kinase substrate 1	0.011	1.11	0.000	1.24
OPCML	opioid binding protein/cell adhesion molecule-like	0.016	1.15	0.006	1.18
PAQR5	progestin and adipoQ receptor family member V	0.001	1.26	0.016	1.24
PCDH17	protocadherin 17	0.014	1.33	0.004	1.79
PCNP	PEST proteolytic signal containing nuclear protein	0.006	1.27	0.008	1.35
PGM2L1	phosphoglucomutase 2-like 1	0.008	1.21	0.007	1.23
PHIP	pleckstrin homology domain interacting protein	0.024	2.39	0.004	3.95
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	0.020	1.26	0.003	1.69
PIP4K2A	phosphatidylinositol-5-phosphate 4-kinase, type II, alpha	0.001	1.19	0.013	1.16
PITPNC1	phosphatidylinositol transfer protein, cytoplasmic 1	0.024	1.22	0.014	1.41
PLXNA2	plexin A2	0.012	1.12	0.016	1.42
PNRC1	proline-rich nuclear receptor coactivator 1	0.020	1.18	0.005	1.26
PPARGC1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	0.025	1.22	0.010	1.35
PPAT	phosphoribosyl pyrophosphate amidotransferase	0.007	1.18	0.020	1.22
PRKG2	protein kinase, cGMP-dependent, type II	0.008	1.22	0.020	1.26
PRPF38A	PRP38 pre-mRNA processing factor 38 (yeast) domain containing A	0.008	1.82	0.002	2.1
PTGES3	prostaglandin E synthase 3 (cytosolic)	0.002	1.35	0.002	1.54
PTP4A1	protein tyrosine phosphatase type IVA, member 1	0.018	1.14	0.023	1.17
PUM2	pumilio homolog 2 (Drosophila)	0.001	1.13	0.004	1.11
RB1CC1	RB1-inducible coiled-coil 1	0.012	1.15	0.004	2.04
RHOU	ras homolog gene family, member U	0.016	1.37	0.015	1.45
RIN2	Ras and Rab interactor 2	0.006	1.47	0.005	1.34
ROCK2	Rho-associated, coiled-coil containing protein kinase 2	0.004	1.21	0.025	1.36

Tabe 4-4 Continued

		WT Oil vs. WT EB		WT Oil vs. ERβKO Oil	
Symbol	Description	p-value	FC	p-value	FC
RPGRIP1	retinitis pigmentosa GTPase regulator interacting protein 1	0.006	1.49	0.010	1.49
RPL34	ribosomal protein L34	0.001	1.11	0.011	1.08
RPL37A	ribosomal protein L37a	0.019	1.08	0.001	-1.21
RPS6KB1	ribosomal protein S6 kinase, 70kDa, polypeptide 1	0.011	1.33	0.020	1.42
SAP30	Sin3A-associated protein, 30kDa	0.020	1.23	0.005	1.21
SCHIP1	schwannomin interacting protein 1	0.001	1.12	0.024	1.13
SDC4	syndecan 4	0.015	1.21	0.003	1.53
SEPHS2	selenophosphate synthetase 2	0.007	1.41	0.005	1.43
SESN3	sestrin 3	0.019	1.81	0.008	4.01

SLC24A4	solute carrier family 24 (sodium/potassium/calcium exchanger), member 4	0.003	1.21	0.006	1.3
SLITRK4	SLIT and NTRK-like family, member 4	0.024	1.59	0.002	2.38
SMAD4	SMAD family member 4	0.012	1.24	0.015	1.42
SMC3	structural maintenance of chromosomes 3	0.002	1.15	0.022	1.13
SMEK2	SMEK homolog 2, suppressor of mek1 (Dictyostelium)	0.009	1.23	0.021	1.43
SNAP29	synaptosomal-associated protein, 29kDa	0.004	1.15	0.008	1.21
ST18	suppression of tumorigenicity 18 (breast carcinoma) (zinc finger protein)	0.014	1.24	0.003	1.23
SUZ12	suppressor of zeste 12 homolog (Drosophila)	0.011	1.27	0.020	1.34
SYNM	synemin, intermediate filament protein	0.020	1.82	0.011	2.06
TARDBP	TAR DNA binding protein	0.005	1.15	0.004	1.18
THOC2	THO complex 2	0.003	1.11	0.006	1.65
TMED9	transmembrane emp24 protein transport domain containing 9	0.007	1.13	0.014	1.17
TOP1	topoisomerase (DNA) I	0.003	1.18	0.015	1.19
TRIP12	thyroid hormone receptor interactor 12	0.022	1.09	0.021	1.13
TROVE2	TROVE domain family, member 2	0.022	1.61	0.004	2.14
TSHZ1	teashirt zinc finger homeobox 1	0.021	1.18	0.016	1.33
UBE2D3	ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	0.012	1.15	0.024	1.20
UBXN4	UBX domain protein 4	0.007	1.23	0.007	1.29
USP15	ubiquitin specific peptidase 15	0.008	1.49	0.003	1.74
VEZF1	vascular endothelial zinc finger 1	0.005	1.13	0.005	1.16
WAC	WW domain containing adaptor with coiled-coil	0.004	1.16	0.007	1.79
WASF3	WAS protein family, member 3	0.006	1.15	0.011	1.16
WDR12	WD repeat domain 12	0.008	1.25	0.000	1.56
ZC3H7B	zinc finger CCCH-type containing 7B	0.008	1.48	0.015	1.36
ZNF148	zinc finger protein 148	0.016	1.18	0.013	2.19
ZNF451	zinc finger protein 451	0.021	1.16	0.021	1.48

The gene symbol, gene name, p-value and fold change of genes that were significantly increasing an decreasing in WT EB vs WT Oil treated mice and ERβKO Oil and WT Oil treated mice.

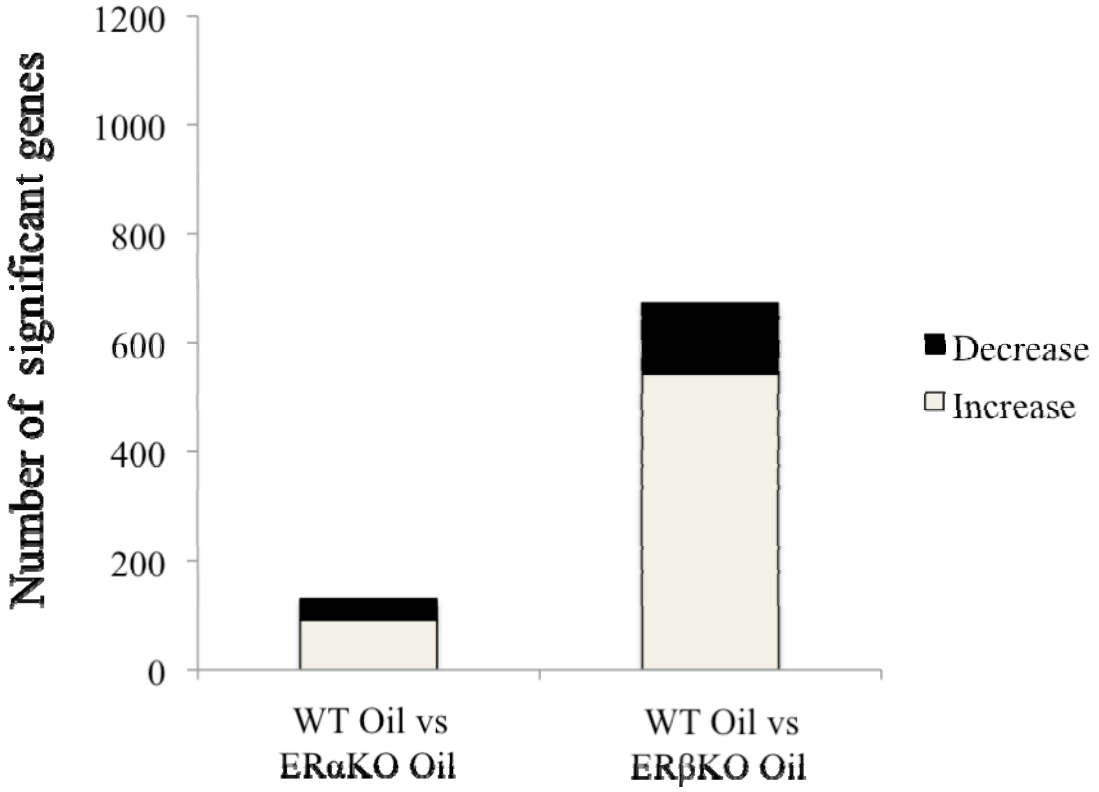

Figure 4-1. Hippocampal gene expression is sensitive to the loss of ERβ. Illustration of the total number of genes significantly ($p<0.025$) increasing and decreasing in ERαKO Oil treated and ERβKO Oil treated mice compared to WT Oil treated. ERβKO Oil treated mice exhibited a number of probes above chance, with the majority (80%) decreasing.

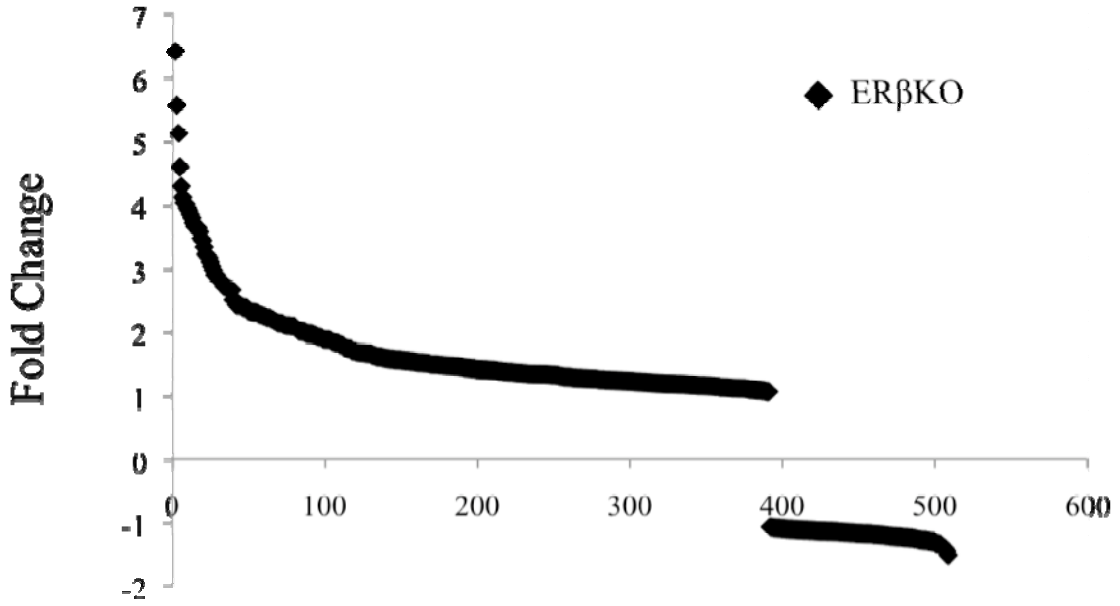

Figure 4-2. Fold change of the 674 genes significantly increasing and decreasing in ERβKO Oil treated mice compared to WT Oil treated mice.

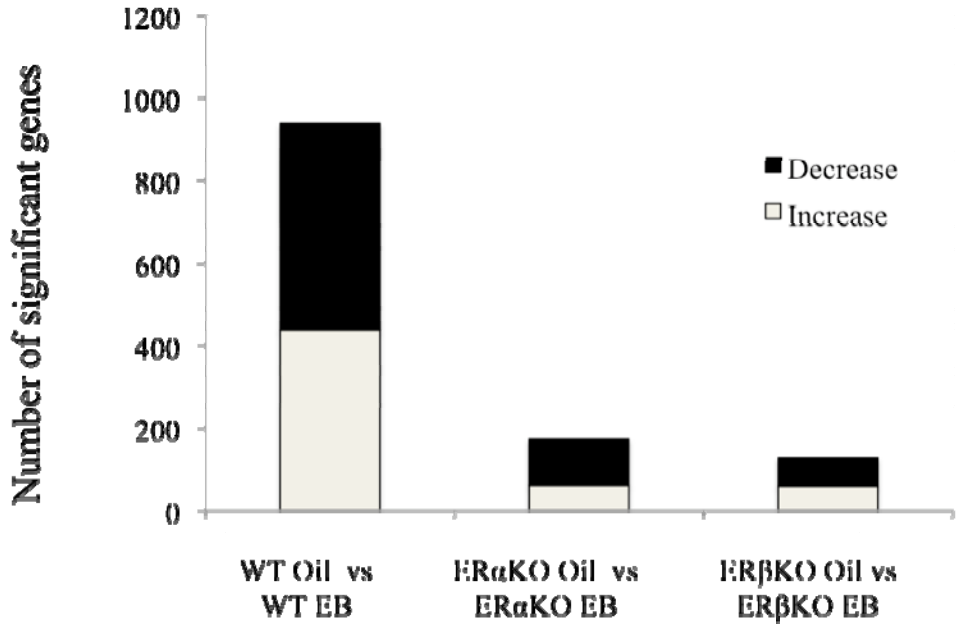

Figure 4-3. Differential effect of EB treatment in WT and ERKO mice. Illustration of the total number of genes significantly ($p<0.025$) increasing and decreasing in WT EB, ERαKO EB and ERβKO EB treated mice compared to Oil treated counterparts.

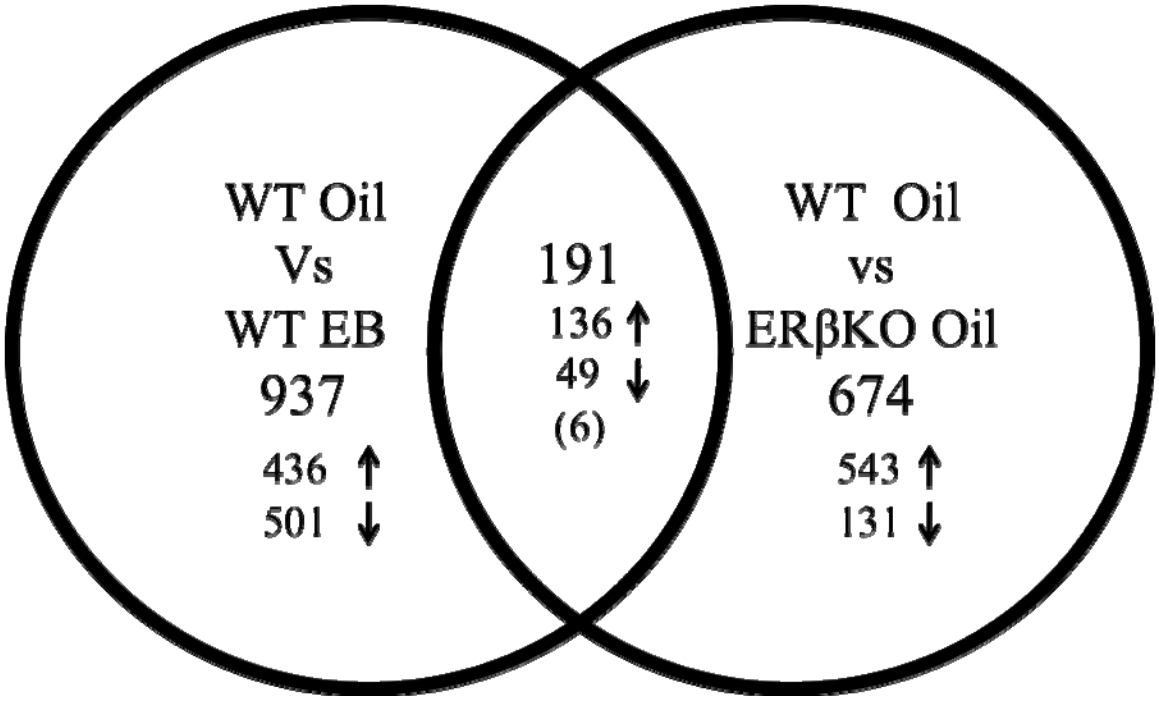

Figure 4-4. Venn Diagram of genes differentially expressed in WT Oil vs WT EB and WT Oil vs ERβKO Oil. The number in parentheses represents genes with expression levels in opposite directions.

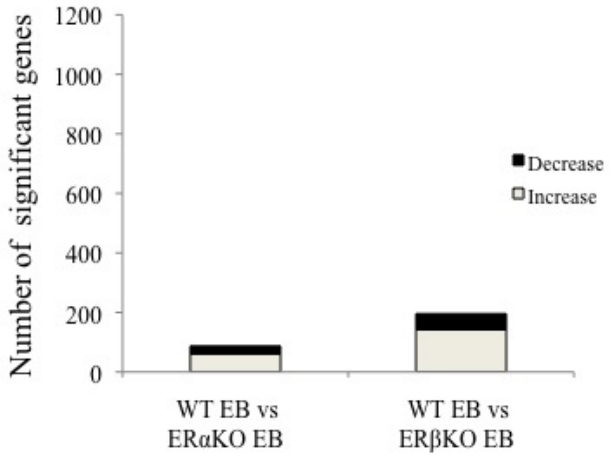

Figure 4-5. ERKO mice display similar gene expression levels after an acute EB treatment as WT mice. Illustration of the total number of genes significantly ($p<0.025$) increasing and decreasing in ERαKO EB and ERβKO EB treated mice compared to WT EB treated. The number of genes significantly different between ERKO EB treated and WT EB treated is below chance.

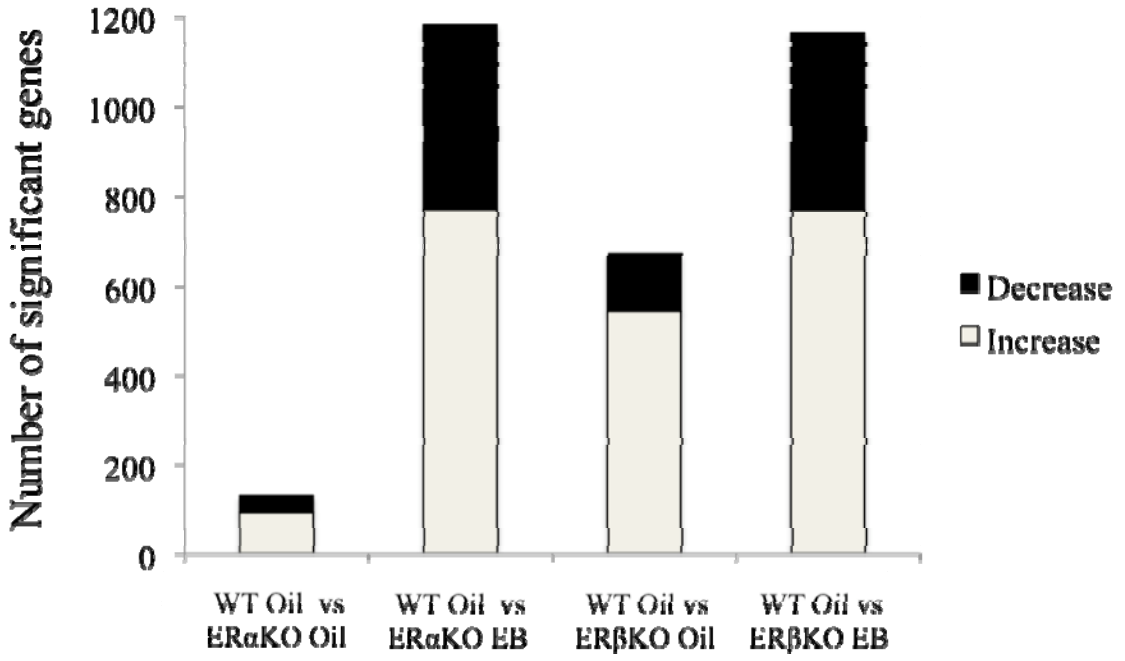

Figure 4-6. EB treatment enhances basal activity in ERKO mice. Comparison of genes significantly differentially expressed in ERKO mice treated with Oil and EB compared to WT Oil treated mice. Illustration of the total number of genes significantly ($p<0.025$) increasing and decreasing in ERαKO EB and ERβKO EB treated mice compared to WT Oil treated.

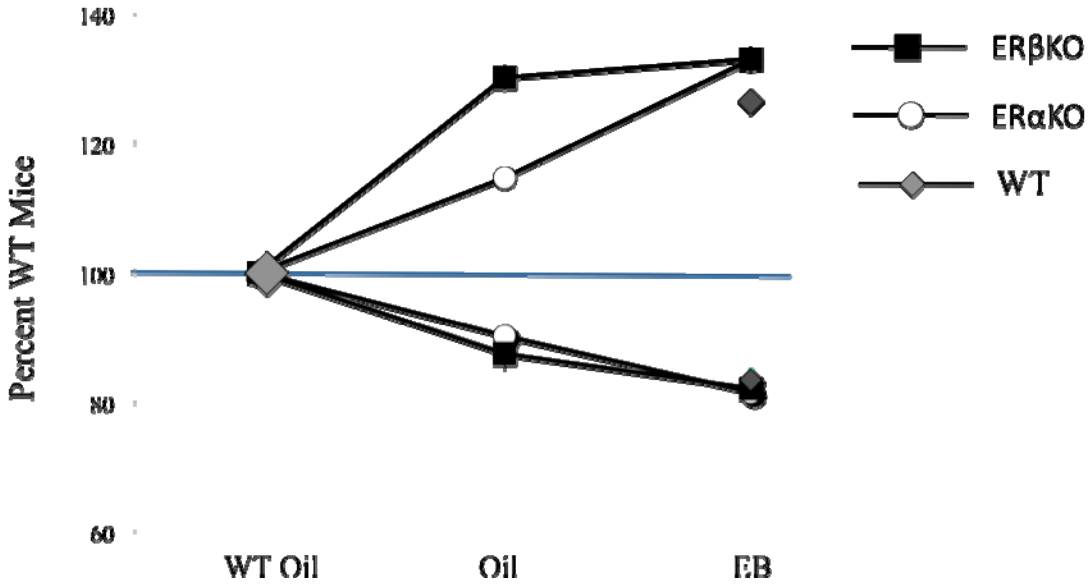

Figure 4-7. ERKO mice display monotonic response to EB treatment. The average percent change in gene expression of ERαKO and ERβKO relative to WT Oil treated mice across treatment groups. While ERβKO mice show an intial change in gene expression without EB treatment, ERαKO display an increase in gene expression after EB treatment. Additionaly, ERβKO and ERαKO mice regardless of treatment display similar gene expression levels when those the level of gene expression decreases. Error bars represent standard error of the mean.

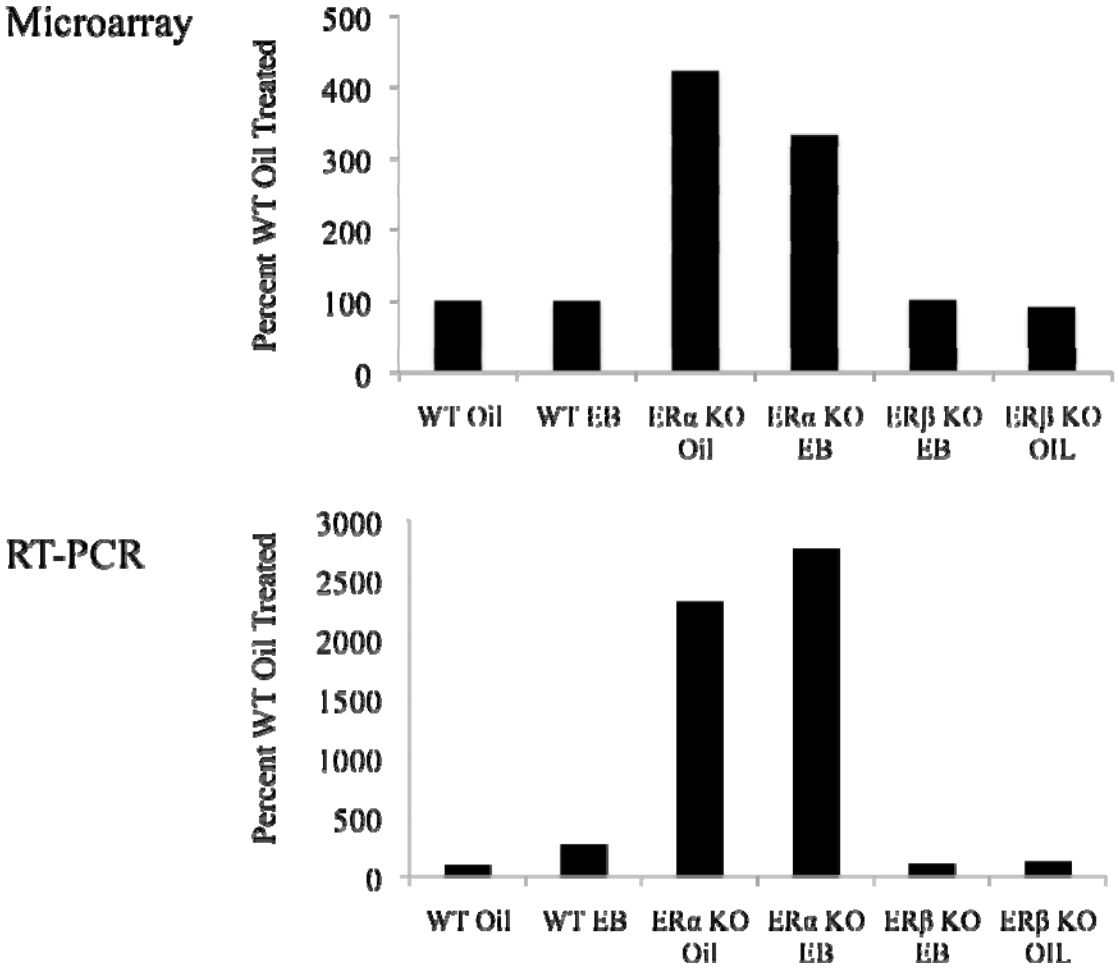

Figure 4-8. ERα mRNA is upregulated in ERαKO mice. Microarray expression levels and Real time (RT)-PCR analysis of ERα mRNA relative to WT Oil treated mice.

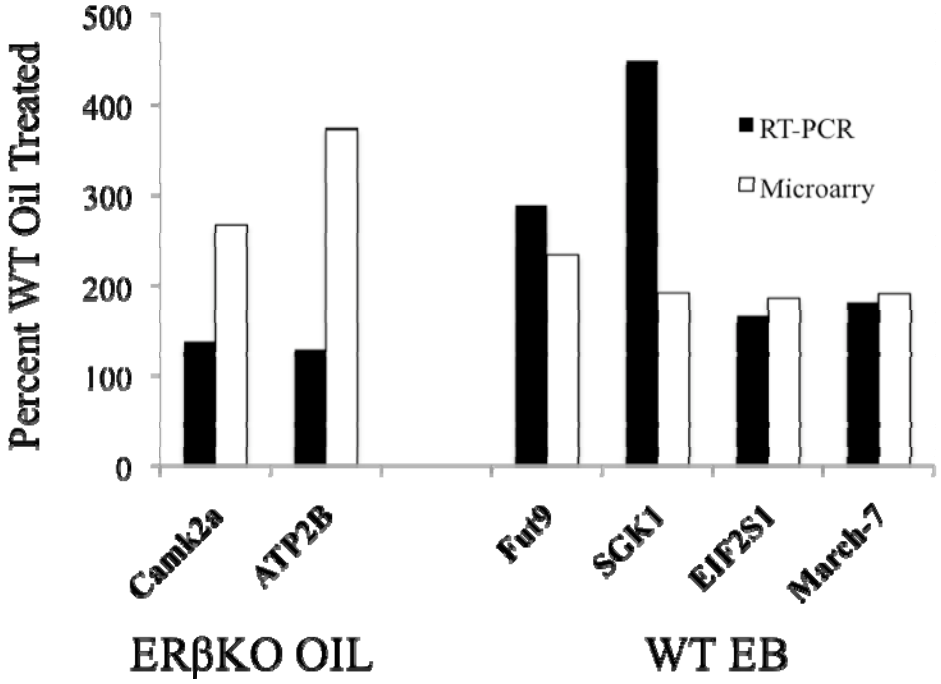

Figure 4-9. Validation of microarray results for 7 genes using RT-PCR. The bars represent the mean fold change in the expression for ERβKO Oil treated and WT EB treated compared to mean of WT Oil treated animals using RT-PCR (filled bars) and microarray analysis (open bars).

CHAPTER 5
CONCLUSION

Summary and Discussion

Together classic and non-classic mechanisms contribute to estrogen's trophic influence within the hippocampus; for example, estrogen has been shown to promote proliferation of neurons in the adult dentate gyrus, block apoptotic signal cascades to encourage neuron survival, and induce dendritic growth in the hippocampus (Jover et al., 2002; Tanapat et al., 1999). An influential discovery was the finding that hippocampal CA1 cells display a fluctuating spine density coupled to the estrus cycle with the spine density peaking at proestrus, when estrogen levels have peaked (Woolley et al., 1990). Gould et al. (1990) showed that ovariectomy for 6 days results in decrease of dendritic spine density of hippocamapal CA1 pyramidal cells. At the same time it was shown that estrogen can prevent ovariectomy induced decrease in spine density (Gould et al., 1990). Woolley and McEwen (1993) further demonstrated that estrogen can reverse the ovariectomy induced decrease in spine density 48-72 hours after treatment. This work suggests that estrogen has long-term, possibly genomic influences that out lasts the presence of estrogen in the plasma.

Furthermore, estrogen's trophic responses have been shown to contribute to synaptic growth and strength. For example, GAP-43 (also known as B-50), a presynaptic protein associated with growth and regeneration of axons, is increased by estrogen in preoptic area (Shughrue and Dorsa, 1993). Estrogen increases the expression of insulin like growth factor-1 (IGF-1) and interacts with IGF-1 to induce dendritic growth and synaptic plasticity (Cardona-Gomez et al., 2000). Toran-Allerand et al. (1996) reported that estrogen receptors colocalize with neurotrophin receptors; p75, trkA, and trkB in the basal forebrain. However, the direction in which estrogen influences nerve growth factor (NGF) is still unclear (Gibbs, 1998; Gibbs et al.,

1994; Scully and Otten, 1995). While, estrogen has also been shown to increase the expression of NGF in the hippocampus (Simpkins et al., 1997), Gibbs (1998) found no changes in NGF mRNA in the hippocampus after estrogen treatment. These conflicting results may be due to the system being used (in vitro versus in vivo), time point (20 minutes versus 24 hours), or product being examined (protein versus mRNA) and may also represent a homeostatic control over certain signaling cascades by estrogen. The ability to turn "on" or turn "off" particular genes/protein and cascades may help regulate the system.

Together these results indicate that estrogen, possibly through transcription, has trophic influences involved in the regulation of the growth of dendritic spines, synapse number, and cell survival. The trophic influences are likely important for understanding estrogens effects in delaying brain aging; unfortunately, much of the research has been conducted in young animals and cell cultures. Therefore, it was estentially to determine how estrogen effects middle-aged and aged animals, the functional relevance of ERα and ERβ, and to determine the potential mechanism mediating estrogen's ability to reverse age related changes particularly in areas of the brain sensitive to aging processes, such as the hippocampus

In this study we used a combination of behavioral testing and microarray analysis to determine age-related alteration in hippocampal gene expression in young, middle-aged, and aged animals. It was our hypothesis that EB treatment initiated at middle-age will maintain hippocampal function and gene expression. Moreover, we hypothesized that the alteration of receptor ratio is contributing to reduced responses in aged animals; this theory was tested in ERKO mice.

Middle-age is a time of vulnerability, when the hippocampus begins to undergo age-related alteration in gene expression, CA^{2+} homeostasis, decrease in synaptic proteins, shift in

phosphate/kinase activity (Adams et al., 2008; Blalock et al., 2003) (Foster and Kumar, 2002). It has been postulated that middle-age is a time when treatment to fight age-related changes in the hippocampus should be initiated (Maki, 2006; Sherwin, 2007). Accordingly, when we initiated EB treatment in middle-age animals they maintained cognitive function after long-term EB treatment by performing better on MWM than middle-aged oil treated counterparts (Chapter 2). In addition, we found that long-term cyclic treatment of EB could reverse these age related changes in hippocampal transcription; including the expression of genes involved in transcription, apoptosis/cell health, receptor/cell signaling, cell growth/structural organization among others. The results suggest that EB treatment initiated in middle-aged mice prevents cognitive decline and maintains youthful hippocampal transcriptional regulation.

Similarly, microarray analyses reveal that after acute EB treatment middle-age animals maintain youthful hippocampal gene expression. However, it is unclear how long treatment would need to be continued to maintain the cognitive benefits seen in middle-age and therefore important to determine if long-term cyclic treatment initiated at middle-aged and continued into the later part of life will remain neuroprotective. Thus, we will need to maintain middle-aged mice on EB treatments through late life and test their cognitive function (MWM) and hippocampal gene expression (microarray analysis).

Given recent reports that EB treatment initiated in postmenopausal women will not improve cognition (Espeland et al., 2004), or increase dendritic spine density (a marker of learning and memory) (Adams et al., 2001b), it may be surprising that aged female mice maintained a transcriptional response to a single injection of EB 12hr after treatment. However, the aged hippocampus has been found to respond to EB treatment by reversing age-related increase in L-type voltage gated calcium channels (Brewer et al., 2009) and increasing synaptic

NMDAR 2B levels (Adams et al., 2004). These processes play an important role in synaptic plasticity but the effects are still not enough to reverse age-related cognitive decline. Therefore, EB treatment alone is not enough to restore hippocampal function and results suggest other mechanisms contributing to the age-related decrease in gene expression regulation by EB.

Our results found that aged animals display a response to EB treatment by an increase of genes involved in rapid estrogen response pathways, while the effects of EB on young and MA increased genes involved in synapses. This shift away from an increase genes involved in synapse may explain why aged animals are unable to increase dendritic spine density. Moreover, differential gene expression in aged mice may be a compensatory mechanism for changes in estrogen signaling needed to prime the system for subsequent responses to EB (Yun et al., 2007), suggesting the aged animals do not have the necessary kinases, phosphatases, scaffolding proteins, coregulators or receptors ready for signaling (Foster, 2005). Notably, the reduced response in gene expression and alteration in synaptic responses to EB in aged mice coincides with age-related decrease in ERs (Mehra et al., 2005; Thakur and Sharma, 2007). This led us to hypothesize that the decrease of ERs is contributing to the truncated response to EB in the aged hippocampus.

To test the theory that decrease of ERs will affect hippocampal gene expression we used ERKO mice treated with and without EB. Chapter 4 provided evidence that the receptor ratio impacts hippocampal gene expression even with out circulating EB. More specifically, ERβKO Oil treated mice displayed an increase of genes involved in neuroprotection, while ERαKO Oil treated mice showed little change in gene expression compared to WT Oil treated. Moreover, ERβKO mice displayed an increase in the expression of genes involved in synapse suggesting that unregulated ERα is influencing synaptic function and supporting reports that ERα is

involved in synaptic plasticity(Adams and Morrison, 2003). Therefore, an alteration of receptor ratio where the levels of ERα are higher than ERβ promotes the expression of genes involved in synapse, suggesting that altering the ratio of ERα and ERβ my be a compensatory mechanism to help maintain EB's neuroprotective properties.

Similarly, neuroprotective effects have been seen ERβKO mice following middle cerebral artery occlusion (MCAO) by displaying less total infarct volume. Moreover, increased levels of ERα in cortex immediately following MCAO correlate with smaller infarct volume (Dubal et al., 2006). These results suggest that the levels of ERs are altered in response to injury in the cortex and increasing the level of ERα compared to ERβ will promote neuroprotective pathways.

An increase in the level of either ERα or ERβ can impact hippocampal function by focusing the estrogen response on different signaling pathway. For example, ERα has been found to solely interact with metabotropic glutamate receptor (mGLuR) 1a but both ERs interact with mGlur2/3 receptors. Therefore an increase in ERα could lead to an increase in mGLuR1a receptor activation and subsequent activation of CREB. Conversely, an age-related decrease of ERα could promote the activation of mGlur2/3 and lead to a decrease in CREB phosphorylation. Additionally, age-related decrease in NMDARs and AMPARs can also be contributing to reduced response to EB treatment in aged animals. Finally, our results found that young ERαKO and ERβKO EB treated mice displayed similar gene expression levels as WT EB treated mice, suggesting other mechanisms (ERX, mER, GPR30) are contributing to estrogen signaling and the impact these receptors have in the aging hippocampus still needs to be determined. Therefore, age-related decrease in an estrogen receptor combined with age-related decrease in synaptic proteins is likely contributing to reduced and shifted response in aged animals.

The data presented in this dissertation used gene expression analysis as a novel approach to compare EB effects in the aging hippocampus. The results reveal EB increases the expression of genes involved in neuroprotection through middle-age. However, the regulation of gene expression in aged animals is reduced and shifted toward rapid signaling pathways, which may represent compensation mechanism due to the disrupted estrogen receptor signaling. Together, these results suggest that age-related decrease of estrogen receptors are contributing to the gene expression changes found in aged mice. Therefore, more research is needed to determine how the regualtion of gene expression effects hippocampal function and how the results can be translated into improved treatment strategies for women.

Microarray Relaiblity and Scientific Significance

The use of microarrays has grown over the past 20 years and with it comes improved technology. Microarrays enable a researcher to scan thousands of gene expression levels simultaneously and can result in important biological findings. However, microarray data can be limited by poor study design and interpretation; therefore, microarrays are not the end all experiment but are just the beginning observation. Moreover, key steps can be taken to improve microarray reliability and scientific integrity. These key steps include; a clear objective hypothesis, large sample size, and appropriate interpretation. I will address these key steps and discuss how I implemented them into my own experiments to improve the reliability of my microarray studies.

A scientific experiment begins by producing a clear and concise hypothesis. Microarray experiments are often criticized for not being hypothesis driven experiments but rather discovery driven. I argue that gene expression analysis can be hypothesis driven. A scientific experiment asks a question, tests that question and determines if your question was true or false. In our first microarray experiment we asked the simply question, “Does estrogen treatment reverse age-

related changes in gene expression?" We compared the gene expression in young and middle-aged mice and found that estrogen treatment did reverse age-related changes in transcription. That leads us to the why? Even though we have proved our hypothesis, answering the why is still difficult and needs further testing, beyond RT-PCR validation. Therefore, microarray analysis is the starting point to future more in depth studies.

Another serious problem with microarrays can be sample size. The initial microarray experiments were plagued with many problems, the biggest was the small sample size used in the analysis. Using a small sample size will increase the likelihood of biological variablilty and outliers due to experimental error. Due to the large expense of microarray analysis, it is difficult to run large studies. However, appropriate sample numbers are necessary to obtain reliable scientific data.

Once the data has been collected accordingly, the results of the data must be appropriately interpreted. It is important to remind the reader what you are dealing with. Microarrays indirectly measure the levels of thousands of RNA levels and with the measure of thousands of genes will also come error. One will control for error but with any scientific test error is possible and in testing tens of thousands of genes a few thousand genes may be false positives (type II error). Furthermore, microarrays need to be validated and for a publication or within one lab it is not feasible to validate thousands of genes. Therefore the vast majority of data will not be validated. Due to limited validation, the reader should understand that gene X has been found to increase or decrease in your test but further examination in its role in a particular process is needed. Often researches find a significant change in Gene X and concluded that it is involve in memory. This interpretation is premature. While microarray data gleams interesting insight on the

potential role of a particular gene it cannot conclusively say that it is involved in anything. Additional tests and screening need to be done to conclusively state the role of Gene X.

I have attempted to reduce microarray variability by using larger sample size (n>5) and performing the isolation and processing of samples myself, to reduce processing error. Also, I have focused our results on clustering of gene pathways that are significantly altered rather than changes in the expression of particular genes. Given the potential error, 10% of my genes are likely false positives, if I had a pathway with 10 genes significantly altered by my treatment, one gene may be a false positive. However, one gene may be significantly altered but 9 genes are still significant in that pathway and that pathway is still significantly influenced by my treatment. Focusing my results and interpretation on pathway analysis has yielded useful biological information and confers additional reliability in the statistical analysis.

Future Directions

Microarray studies enable a researcher to scan thousands of potential estrogen responsive genes in a single test and pave the way for more in depth research but can also leave the researcher with more questions. The same is true of the current data. For example, by analyzing the entire mouse hippocampus we were unable to determine regional specificity of the gene expression changes. A recent microarray study in the mouse found 2% of the genes tested were differentially expressed between CA1 and CA3, 1% between CA1 and DG, and 1% between CA3 and DG (Zhao et al., 2001). These result suggest that there is differential gene expression between hippocampal regions; therefore, future studies will employ *in situ* hybridization techniques to examine regional changes in gene expression. In this regard, I have compiled a list of genes that may be of interest to the lab for further exploration of the regional response to EB

in the hippocampus (Table 5.1). These genes were chosen because they were found significantly altered between young oil treated and young EB treated (6hr) mice in Chapter 3 and Chapter 4.

Utilizing KO mice is a valuable tool in estrogen research and has given us insight into the mechanisms of estrogen signaling; however, it is difficult to determine if the effects seen are due to treatment in our animals or from developmental changes that occurred due to the lack of an ER. Our lab has shown that by viral mediated delivery of ERα into ERαKO mice restores cognitive function, suggesting the developmental loss of ERα is not completely contributing to the behavior deficits seen grown mice. Therefore, the results from the current KO study are probably not due to developmental loss of ERs. However, this issue can be avoided by blocking ERs in adult animals using antagonists. Additionally, it is unclear if the results from KO study are from one receptor taking over the transcriptional activity of the other, another type of estrogen receptor or a non-estrogen receptor-signaling pathway influencing gene expression changes. The results presented in Chapter 4, ERKO EB treated mice display similar gene expression to WT EB treated, suggests a non-estrogen receptor mechanism contributing to the transcriptional response to EB treatment. To help determine other potential mechanisms contributing EB mediated gene expression we can use ERα, ERβ, and GPR30 agonists and antagonist.

Using microarray technology in combination with these pharmacological measures to compare gene expression activity in young, middle-age and aged animals will help delineate changes in estrogen signaling pathways during aging. Moreover, to determine if the alteration of estrogen receptors are contributing to reduced response to EB in aged animals we need to alter the level of estrogen receptor in aged animals. This can be done using viral vector mediated delivery of ERα and ERβ into the hippocampus of aged animals. Therefore, we can determine if

maintaining the levels of ERα, ERβ, or both will improve estrogen responsiveness in aged animals and protect against age-related cognitive decline. Also, it would be interesting to use RNA interference to reduce the levels of ERs in the entire hippocampus or specific regions of the hippocampus in young animals and see if they display similar reduced responsiveness to EB as aged animals.

Table 5-1 Genes significant after 6hr of EB treatment in young animals

			Chapter 3		Chapter 4	
			Y Oil vs Y EB 6hr		WT Oil vs WT EB 6hr	
Affy ID	Symbol	Description	p-value	FC	p-value	FC
1436918_at	4932442E05RIK	RIKEN cDNA 1810038L18 gene	0.00	1.31	0.00	1.27
1453529_at	6330418B08RIK	RIKEN cDNA 6330418B08 gene	0.01	-1.13	0.01	-1.41
1452599_s_at	AI413582	expressed sequence AI413582	0.01	-1.30	0.01	-1.15
1435768_at	ARID4B	AT rich interactive domain 4B (Rbp1 like)	0.01	1.11	0.02	1.15
1449622_s_at	ATP6AP1	ATPase, H+ transporting, lysosomal accessory protein 1	0.00	-1.20	0.01	-1.08
1435397_at	BC038156	cDNA sequence BC038156	0.02	1.24	0.02	1.19
1418660_at	CLOCK	circadian locomoter output cycles kaput	0.01	1.18	0.00	1.33
1428738_a_at	D14ERTD449E	DNA segment, Chr 14, ERATO Doi 449, expressed	0.00	-1.15	0.01	-1.21
1439986_at	DGKI	diacylglycerol kinase, iota	0.01	-1.19	0.00	-1.17
1421882_a_at	ELAVL2	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)	0.01	1.23	0.01	1.35
1427329_a_at	IGH-6	immunoglobulin heavy chain 6 (heavy chain of IgM)	0.01	-1.13	0.01	-1.21
1429431_at	IKZF5	zinc finger protein, subfamily 1A, 5	0.00	1.26	0.00	1.36
1451047_at	ITM2A	integral membrane protein 2A	0.02	-1.52	0.01	-1.32
1420276_x_at	MMRN2	multimerin 2	0.01	-1.12	0.02	-1.12
1419077_at	MPP3	membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3)	0.01	1.08	0.01	1.09
1452670_at	MYL9	myosin, light polypeptide 9, regulatory	0.00	-1.26	0.00	-1.46
1415966_a_at	NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1	0.01	-1.10	0.01	-1.12
1419127_at	NPY	neuropeptide Y	0.00	-1.15	0.01	-1.26
1424413_at	OGFRL1	opioid growth factor receptor-like 1	0.00	1.23	0.00	1.19
1435486_at	PAK3	P21 (CDKN1A)-activated kinase 3	0.02	1.15	0.01	1.14

Table 5-1. Continued

Affy ID	Gene Symbol	Description	Y Oil vs Y EB 6hr		WT Oil vs WT EB 6hr	
			p-value	FC	p-value	FC
1426658_x_at	PHGDH	3-phosphoglycerate dehydrogenase	0.02	-1.23	0.01	-1.16
1423771_at	PRKCDBP	protein kinase C, delta binding protein	0.02	-1.15	0.01	-1.14
1437697_at	RAD23A	RAD23a homolog (S. cerevisiae)	0.01	-1.08	0.02	-1.23
1441737_s_at	RASSF1	Ras association (RalGDS/AF-6) domain family 1	0.01	-1.21	0.02	-1.11
1416882_at	RGS10	regulator of G-protein signalling 10	0.02	-1.11	0.02	-1.09
1418318_at	RNF128	ring finger protein 128	0.00	1.20	0.01	1.28
1417508_at	RNF19A	ring finger protein (C3HC4 type) 19	0.01	1.15	0.00	1.21
1448845_at	RPP25	ribonuclease P 25 subunit (human)	0.00	-1.25	0.01	-1.20
1417719_at	SAP30	sin3 associated polypeptide	0.00	1.13	0.02	1.23
1457118_at	SHC4	RIKEN cDNA 6230417E10 gene	0.00	1.62	0.01	1.16
1417954_at	SST	somatostatin	0.01	-1.39	0.01	-1.12
1456147_at	ST8SIA6	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 6	0.00	1.25	0.01	1.14
1420895_at	TGFBR1	transforming growth factor, beta receptor I	0.02	1.17	0.01	1.29
1416890_at	WDR74	WD repeat domain 74	0.00	-1.19	0.01	-1.11
1453156_s_at	ZADH1	zinc binding alcohol dehydrogenase, domain containing 1	0.00	3.05	0.02	1.14
1437556_at	ZFHX4	zinc finger homeodomain 4	0.02	1.27	0.02	1.18
1435188_at		similar to chromosome 1 open reading frame 51	0.02	-1.22	0.00	-1.19

LIST OF REFERENCES

Adams MM, Fink SE, Janssen WG, Shah RA, Morrison JH. 2004. Estrogen modulates synaptic N-methyl-D-aspartate receptor subunit distribution in the aged hippocampus. J Comp Neurol 474(3):419-26.

Adams MM, Fink SE, Shah RA, Janssen WG, Hayashi S, Milner TA, McEwen BS, Morrison JH. 2002. Estrogen and aging affect the subcellular distribution of estrogen receptor-alpha in the hippocampus of female rats. J Neurosci 22(9):3608-14.

Adams MM, Morrison JH. 2003. Estrogen and the aging hippocampal synapse. Cereb Cortex 13(12):1271-5.

Adams MM, Oung T, Morrison JH, Gore AC. 2001a. Length of postovariectomy interval and age, but not estrogen replacement, regulate N-methyl-D-aspartate receptor mRNA levels in the hippocampus of female rats. Exp Neurol 170(2):345-56.

Adams MM, Shah RA, Janssen WG, Morrison JH. 2001b. Different modes of hippocampal plasticity in response to estrogen in young and aged female rats. Proc Natl Acad Sci U S A 98(14):8071-6.

Adams MM, Shi L, Linville MC, Forbes ME, Long AB, Bennett C, Newton IG, Carter CS, Sonntag WE, Riddle DR and others. 2008. Caloric restriction and age affect synaptic proteins in hippocampal CA3 and spatial learning ability. Exp Neurol 211(1):141-9.

Aenlle KK, Foster TC. 2009. Aging alters the expression of genes for neuroprotection and synaptic function following acute estradiol treatment. Hippocampus In Press.

Aenlle KK, Kumar A, Cui L, Jackson TC, Foster TC. 2009. Estrogen effects on cognition and hippocampal transcription in middle-aged mice. Neurobiol Aging 30(6):932-45.

Akama KT, McEwen BS. 2003. Estrogen stimulates postsynaptic density-95 rapid protein synthesis via the Akt/protein kinase B pathway. J Neurosci 23(6):2333-9.

Aydin M, Yilmaz B, Alcin E, Nedzvetsky VS, Sahin Z, Tuzcu M. 2008. Effects of letrozole on hippocampal and cortical catecholaminergic neurotransmitter levels, neural cell adhesion molecule expression and spatial learning and memory in female rats. Neuroscience 151(1):186-94.

Azcoitia I, Sierra A, Garcia-Segura LM. 1999. Neuroprotective effects of estradiol in the adult rat hippocampus: interaction with insulin-like growth factor-I signalling. J Neurosci Res 58(6):815-22.

Bading H, Ginty DD, Greenberg ME. 1993. Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. Science 260(5105):181-6.

Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J, Nilsson S. 1998. Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. Mol Pharmacol 54(1):105-12.

Bednarski E, Ribak CE, Lynch G. 1997. Suppression of cathepsins B and L causes a proliferation of lysosomes and the formation of meganeurites in hippocampus. J Neurosci 17(11):4006-21.

Berno V, Amazit L, Hinojos C, Zhong J, Mancini MG, Sharp ZD, Mancini MA. 2008. Activation of estrogen receptor-alpha by E2 or EGF induces temporally distinct patterns of large-scale chromatin modification and mRNA transcription. PLoS ONE 3(5):e2286.

Bi R, Foy MR, Thompson RF, Baudry M. 2003. Effects of estrogen, age, and calpain on MAP kinase and NMDA receptors in female rat brain. Neurobiol Aging 24(7):977-83.

Bimonte-Nelson HA, Singleton RS, Williams BJ, Granholm AC. 2004. Ovarian hormones and cognition in the aged female rat: II. progesterone supplementation reverses the cognitive enhancing effects of ovariectomy. Behav Neurosci 118(4):707-14.

Bjornstrom L, Sjoberg M. 2005. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Mol Endocrinol 19(4):833-42.

Blalock EM, Chen KC, Sharrow K, Herman JP, Porter NM, Foster TC, Landfield PW. 2003. Gene microarrays in hippocampal aging: statistical profiling identifies novel processes correlated with cognitive impairment. J Neurosci 23(9):3807-19.

Boulware MI, Weick JP, Becklund BR, Kuo SP, Groth RD, Mermelstein PG. 2005. Estradiol activates group I and II metabotropic glutamate receptor signaling, leading to opposing influences on cAMP response element-binding protein. J Neurosci 25(20):5066-78.

Bowman RE, Ferguson D, Luine VN. 2002. Effects of chronic restraint stress and estradiol on open field activity, spatial memory, and monoaminergic neurotransmitters in ovariectomized rats. Neuroscience 113(2):401-10.

Brewer GJ, Reichensperger JD, Brinton RD. 2006. Prevention of age-related dysregulation of calcium dynamics by estrogen in neurons. Neurobiol Aging 27(2):306-17.

Brewer LD, Dowling AL, Curran-Rauhut MA, Landfield PW, Porter NM, Blalock EM. 2009. Estradiol reverses a calcium-related biomarker of brain aging in female rats. J Neurosci 29(19):6058-67.

Brinton RD. 2008. The healthy cell bias of estrogen action: mitochondrial bioenergetics and neurological implications. Trends Neurosci 31(10):529-37.

Brookmeyer R, Gray S, Kawas C. 1998. Projections of Alzheimer's disease in the United States and the public health impact of delaying disease onset. Am J Public Health 88(9):1337-42.

Burger C, Lopez MC, Feller JA, Baker HV, Muzyczka N, Mandel RJ. 2007. Changes in transcription within the CA1 field of the hippocampus are associated with age-related spatial learning impairments. Neurobiol Learn Mem 87(1):21-41.

Cardona-Gomez GP, DonCarlos L, Garcia-Segura LM. 2000. Insulin-like growth factor I receptors and estrogen receptors colocalize in female rat brain. Neuroscience 99(4):751-60.

Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoute J, Brodsky AS, Keeton EK, Fertuck KC, Hall GF and others. 2006. Genome-wide analysis of estrogen receptor binding sites. Nat Genet 38(11):1289-97.

Cavallaro S, Dagata V, Alkon DL. 2002. Programs of gene expression during the laying down of memory formation as revealed by DNA microarrays. Neurochem Res 27(10):1201-7.

Cavallaro S, Schreurs BG, Zhao W, D'Agata V, Alkon DL. 2001. Gene expression profiles during long-term memory consolidation. Eur J Neurosci 13(9):1809-15.

Chelvarajan RL, Liu Y, Popa D, Getchell ML, Getchell TV, Stromberg AJ, Bondada S. 2006. Molecular basis of age-associated cytokine dysregulation in LPS-stimulated macrophages. J Leukoc Biol 79(6):1314-27.

Cheng RY, Birely LA, Lum NL, Perella CM, Cherry JM, Bhat NK, Kasprzak KS, Powell DA, Alvord WG, Anderson LM. 2004. Expressions of hepatic genes, especially IGF-binding protein-1, correlating with serum corticosterone in microarray analysis. J Mol Endocrinol 32(1):257-78.

Cheng XR, Zhou WX, Zhang YX, Zhou DS, Yang RF, Chen LF. 2007. Differential gene expression profiles in the hippocampus of senescence-accelerated mouse. Neurobiol Aging 28(4):497-506.

Choi JM, Romeo RD, Brake WG, Bethea CL, Rosenwaks Z, McEwen BS. 2003. Estradiol increases pre- and post-synaptic proteins in the CA1 region of the hippocampus in female rhesus macaques (Macaca mulatta). Endocrinology 144(11):4734-8.

Corbo RM, Gambina G, Ruggeri M, Scacchi R. 2006. Association of estrogen receptor alpha (ESR1) PvuII and XbaI polymorphisms with sporadic Alzheimer's disease and their effect on apolipoprotein E concentrations. Dement Geriatr Cogn Disord 22(1):67-72.

Cordoba Montoya DA, Carrer HF. 1997. Estrogen facilitates induction of long term potentiation in the hippocampus of awake rats. Brain Res 778(2):430-8.

Craig MC, Murphy DG. 2008. Alzheimer's disease in women. Best Pract Res Clin Obstet Gynaecol.

D'Agata V, Cavallaro S. 2003. Hippocampal gene expression profiles in passive avoidance conditioning. Eur J Neurosci 18(10):2835-41.

Dai X, Chen L, Sokabe M. 2007. Neurosteroid estradiol rescues ischemia-induced deficit in the long-term potentiation of rat hippocampal CA1 neurons. Neuropharmacology 52(4):1124-38.

Daniel JM, Hulst JL, Berbling JL. 2006. Estradiol replacement enhances working memory in middle-aged rats when initiated immediately after ovariectomy but not after a long-term period of ovarian hormone deprivation. Endocrinology 147(1):607-14.

Day JR, Laping NJ, Lampert-Etchells M, Brown SA, O'Callaghan JP, McNeill TH, Finch CE. 1993. Gonadal steroids regulate the expression of glial fibrillary acidic protein in the adult male rat hippocampus. Neuroscience 55(2):435-43.

Day M, Sung A, Logue S, Bowlby M, Arias R. 2005. Beta estrogen receptor knockout (BERKO) mice present attenuated hippocampal CA1 long-term potentiation and related memory deficits in contextual fear conditioning. Behav Brain Res 164(1):128-31.

Delaunay F, Pettersson K, Tujague M, Gustafsson JA. 2000. Functional differences between the amino-terminal domains of estrogen receptors alpha and beta. Mol Pharmacol 58(3):584-90.

Donahue CP, Kosik KS, Shors TJ. 2006. Growth hormone is produced within the hippocampus where it responds to age, sex, and stress. Proc Natl Acad Sci U S A 103(15):6031-6.

Du B, Ohmichi M, Takahashi K, Kawagoe J, Ohshima C, Igarashi H, Mori-Abe A, Saitoh M, Ohta T, Ohishi A and others. 2004. Both estrogen and raloxifene protect against beta-amyloid-induced neurotoxicity in estrogen receptor alpha-transfected PC12 cells by activation of telomerase activity via Akt cascade. J Endocrinol 183(3):605-15.

Dubal DB, Rau SW, Shughrue PJ, Zhu H, Yu J, Cashion AB, Suzuki S, Gerhold LM, Bottner MB, Dubal SB and others. 2006. Differential modulation of estrogen receptors (ERs) in ischemic brain injury: a role for ERalpha in estradiol-mediated protection against delayed cell death. Endocrinology 147(6):3076-84.

Dubal DB, Zhu H, Yu J, Rau SW, Shughrue PJ, Merchenthaler I, Kindy MS, Wise PM. 2001. Estrogen receptor alpha, not beta, is a critical link in estradiol-mediated protection against brain injury. Proc Natl Acad Sci U S A 98(4):1952-7.

Dunah AW, Hueske E, Wyszynski M, Hoogenraad CC, Jaworski J, Pak DT, Simonetta A, Liu G, Sheng M. 2005. LAR receptor protein tyrosine phosphatases in the development and maintenance of excitatory synapses. Nat Neurosci 8(4):458-67.

Dupuy AM, Mas E, Ritchie K, Descomps B, Badiou S, Cristol JP, Touchon J. 2001. The relationship between apolipoprotein E4 and lipid metabolism is impaired in Alzheimer's disease. Gerontology 47(4):213-8.

El-Bakri NK, Islam A, Zhu S, Elhassan A, Mohammed A, Winblad B, Adem A. 2004. Effects of estrogen and progesterone treatment on rat hippocampal NMDA receptors: relationship to Morris water maze performance. J Cell Mol Med 8(4):537-44.

Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M, Gustafsson JA. 1997. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab 82(12):4258-65.

Espeland MA, Rapp SR, Shumaker SA, Brunner R, Manson JE, Sherwin BB, Hsia J, Margolis KL, Hogan PE, Wallace R and others. 2004. Conjugated equine estrogens and global cognitive function in postmenopausal women: Women's Health Initiative Memory Study. JAMA 291(24):2959-68.

Ewens WJ, Grant GR. 2005. Statistical methods in bioinformatics : an introduction. New York, N.Y.: Springer. xx, 597 p.

Fan QW, Yuasa S, Kuno N, Senda T, Kobayashi M, Muramatsu T, Kadomatsu K. 1998. Expression of basigin, a member of the immunoglobulin superfamily, in the mouse central nervous system. Neurosci Res 30(1):53-63.

Favit A, Fiore L, Nicoletti F, Canonico PL. 1991. Estrogen modulates stimulation of inositol phospholipid hydrolysis by norepinephrine in rat brain slices. Brain Res 555(1):65-9.

Ferri CP, Prince M, Brayne C, Brodaty H, Fratiglioni L, Ganguli M, Hall K, Hasegawa K, Hendrie H, Huang Y and others. 2005. Global prevalence of dementia: a Delphi consensus study. Lancet 366(9503):2112-7.

Fertuck KC, Eckel JE, Gennings C, Zacharewski TR. 2003. Identification of temporal patterns of gene expression in the uteri of immature, ovariectomized mice following exposure to ethynylestradiol. Physiol Genomics 15(2):127-41.

Filardo E, Quinn J, Pang Y, Graeber C, Shaw S, Dong J, Thomas P. 2007. Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. Endocrinology 148(7):3236-45.

Foster TC. 1999. Involvement of hippocampal synaptic plasticity in age-related memory decline. Brain Res Brain Res Rev 30(3):236-49.

Foster TC. 2005. Interaction of rapid signal transduction cascades and gene expression in mediating estrogen effects on memory over the life span. Front Neuroendocrinol 26(2):51-64.

Foster TC. 2006. Biological markers of age-related memory deficits: treatment of senescent physiology. CNS Drugs 20(2):153-66.

Foster TC. 2007. Calcium homeostasis and modulation of synaptic plasticity in the aged brain. Aging Cell 6(3):319-25.

Foster TC, Kumar A. 2002. Calcium dysregulation in the aging brain. Neuroscientist 8(4):297-301.

Foster TC, Rani A, Kumar A, Cui L, Semple-Rowland SL. 2008. Viral vector-mediated delivery of estrogen receptor-alpha to the hippocampus improves spatial learning in estrogen receptor-alpha knockout mice. Mol Ther 16(9):1587-93.

Foster TC, Sharrow KM, Kumar A, Masse J. 2003. Interaction of age and chronic estradiol replacement on memory and markers of brain aging. Neurobiol Aging 24(6):839-52.

Foy MR, Baudry M, Diaz Brinton R, Thompson RF. 2008a. Estrogen and hippocampal plasticity in rodent models. J Alzheimers Dis 15(4):589-603.

Foy MR, Baudry M, Foy JG, Thompson RF. 2008b. 17beta-estradiol modifies stress-induced and age-related changes in hippocampal synaptic plasticity. Behav Neurosci 122(2):301-9.

Frantz GD, Bohner AP, Akers RM, McConnell SK. 1994. Regulation of the POU domain gene SCIP during cerebral cortical development. J Neurosci 14(2):472-85.

Frick KM. 2009. Estrogens and age-related memory decline in rodents: what have we learned and where do we go from here? Horm Behav 55(1):2-23.

Frick KM, Fernandez SM, Bulinski SC. 2002. Estrogen replacement improves spatial reference memory and increases hippocampal synaptophysin in aged female mice. Neuroscience 115(2):547-58.

Fugger HN, Foster TC, Gustafsson J, Rissman EF. 2000. Novel effects of estradiol and estrogen receptor alpha and beta on cognitive function. Brain Res 883(2):258-64.

Funakoshi T, Yanai A, Shinoda K, Kawano MM, Mizukami Y. 2006. G protein-coupled receptor 30 is an estrogen receptor in the plasma membrane. Biochem Biophys Res Commun 346(3):904-10.

Gabrieli JD, Cohen NJ, Corkin S. 1988. The impaired learning of semantic knowledge following bilateral medial temporal-lobe resection. Brain Cogn 7(2):157-77.

Galea LA, Spritzer MD, Barker JM, Pawluski JL. 2006. Gonadal hormone modulation of hippocampal neurogenesis in the adult. Hippocampus 16(3):225-32.

Garcia-Segura LM, Diz-Chaves Y, Perez-Martin M, Darnaudery M. 2007. Estradiol, insulin-like growth factor-I and brain aging. Psychoneuroendocrinology 32 Suppl 1:S57-61.

Garcia-Segura LM, Naftolin F, Hutchison JB, Azcoitia I, Chowen JA. 1999. Role of astroglia in estrogen regulation of synaptic plasticity and brain repair. J Neurobiol 40(4):574-84.

Garcia-Segura LM, Sanz A, Mendez P. 2006. Cross-talk between IGF-I and estradiol in the brain: focus on neuroprotection. Neuroendocrinology 84(4):275-9.

Gibbs RB. 1998. Levels of trkA and BDNF mRNA, but not NGF mRNA, fluctuate across the estrous cycle and increase in response to acute hormone replacement. Brain Res 787(2):259-68.

Gibbs RB. 2000. Long-term treatment with estrogen and progesterone enhances acquisition of a spatial memory task by ovariectomized aged rats. Neurobiol Aging 21(1):107-16.

Gibbs RB, Aggarwal P. 1998. Estrogen and basal forebrain cholinergic neurons: implications for brain aging and Alzheimer's disease-related cognitive decline. Horm Behav 34(2):98-111.

Gibbs RB, Wu D, Hersh LB, Pfaff DW. 1994. Effects of estrogen replacement on the relative levels of choline acetyltransferase, trkA, and nerve growth factor messenger RNAs in the basal forebrain and hippocampal formation of adult rats. Exp Neurol 129(1):70-80.

Gilardi KV, Shideler SE, Valverde CR, Roberts JA, Lasley BL. 1997. Characterization of the onset of menopause in the rhesus macaque. Biol Reprod 57(2):335-40.

Giusto NM, Salvador GA, Castagnet PI, Pasquare SJ, Ilincheta de Boschero MG. 2002. Age-associated changes in central nervous system glycerolipid composition and metabolism. Neurochem Res 27(11):1513-23.

Gonzalez M, Cabrera-Socorro A, Perez-Garcia CG, Fraser JD, Lopez FJ, Alonso R, Meyer G. 2007. Distribution patterns of estrogen receptor alpha and beta in the human cortex and hippocampus during development and adulthood. J Comp Neurol 503(6):790-802.

Goodman AL, Descalzi CD, Johnson DK, Hodgen GD. 1977. Composite pattern of circulating LH, FSH, estradiol, and progesterone during the menstrual cycle in cynomolgus monkeys. Proc Soc Exp Biol Med 155(4):479-81.

Gottfried-Blackmore A, Croft G, McEwen BS, Bulloch K. 2007. Transcriptional activity of estrogen receptors ERalpha and ERbeta in the EtC.1 cerebellar granule cell line. Brain Res 1186:41-7.

Gould E, Woolley CS, Frankfurt M, McEwen BS. 1990. Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood. J Neurosci 10(4):1286-91.

Gu Q, Moss RL. 1996. 17 beta-Estradiol potentiates kainate-induced currents via activation of the cAMP cascade. J Neurosci 16(11):3620-9.

Guerra B, Diaz M, Alonso R, Marin R. 2004. Plasma membrane oestrogen receptor mediates neuroprotection against beta-amyloid toxicity through activation of Raf-1/MEK/ERK cascade in septal-derived cholinergic SN56 cells. J Neurochem 91(1):99-109.

Hall JM, McDonnell DP. 1999. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 140(12):5566-78.

Healy SD, Braham SR, Braithwaite VA. 1999. Spatial working memory in rats: no differences between the sexes. Proc Biol Sci 266(1435):2303-8.

Heikkinen T, Puolivali J, Liu L, Rissanen A, Tanila H. 2002. Effects of ovariectomy and estrogen treatment on learning and hippocampal neurotransmitters in mice. Horm Behav 41(1):22-32.

Heikkinen T, Puolivali J, Tanila H. 2004. Effects of long-term ovariectomy and estrogen treatment on maze learning in aged mice. Exp Gerontol 39(9):1277-83.

Henderson VW. 2004. Hormone therapy and Alzheimer's disease: benefit or harm? Expert Opin Pharmacother 5(2):389-406.

Henderson VW, Paganini-Hill A, Miller BL, Elble RJ, Reyes PF, Shoupe D, McCleary CA, Klein RA, Hake AM, Farlow MR. 2000. Estrogen for Alzheimer's disease in women: randomized, double-blind, placebo-controlled trial. Neurology 54(2):295-301.

Himeda T, Mizuno K, Kato H, Araki T. 2005. Effects of age on immunohistochemical changes in the mouse hippocampus. Mech Ageing Dev 126(6-7):673-7.

Hosack DA, Dennis G, Jr., Sherman BT, Lane HC, Lempicki RA. 2003. Identifying biological themes within lists of genes with EASE. Genome Biol 4(10):R70.

Irwin LN. 2001. Gene expression in the hippocampus of behaviorally stimulated rats: analysis by DNA microarray. Brain Res Mol Brain Res 96(1-2):163-9.

Irwin RW, Yao J, Hamilton RT, Cadenas E, Brinton RD, Nilsen J. 2008. Progesterone and estrogen regulate oxidative metabolism in brain mitochondria. Endocrinology 149(6):3167-75.

Ishunina TA, Swaab DF. 2007. Alterations in the human brain in menopause. Maturitas 57(1):20-2.

Jakacka M, Ito M, Martinson F, Ishikawa T, Lee EJ, Jameson JL. 2002. An estrogen receptor (ER)alpha deoxyribonucleic acid-binding domain knock-in mutation provides evidence for nonclassical ER pathway signaling in vivo. Mol Endocrinol 16(10):2188-201.

Jelks KB, Wylie R, Floyd CL, McAllister AK, Wise P. 2007. Estradiol targets synaptic proteins to induce glutamatergic synapse formation in cultured hippocampal neurons: critical role of estrogen receptor-alpha. J Neurosci 27(26):6903-13.

Ji Y, Urakami K, Wada-Isoe K, Adachi Y, Nakashima K. 2000. Estrogen receptor gene polymorphisms in patients with Alzheimer's disease, vascular dementia and alcohol-associated dementia. Dement Geriatr Cogn Disord 11(3):119-22.

Jover T, Tanaka H, Calderone A, Oguro K, Bennett MV, Etgen AM, Zukin RS. 2002. Estrogen protects against global ischemia-induced neuronal death and prevents activation of apoptotic signaling cascades in the hippocampal CA1. J Neurosci 22(6):2115-24.

Jover-Mengual T, Zukin RS, Etgen AM. 2007. MAPK signaling is critical to estradiol protection of CA1 neurons in global ischemia. Endocrinology 148(3):1131-43.

Kalita K, Szymczak S, Kaczmarek L. 2005. Non-nuclear estrogen receptor beta and alpha in the hippocampus of male and female rats. Hippocampus 15(3):404-12.

Kapadia R, Yi JH, Vemuganti R. 2008. Mechanisms of anti-inflammatory and neuroprotective actions of PPAR-gamma agonists. Front Biosci 13:1813-26.

Kawa S, Fujimoto J, Tezuka T, Nakazawa T, Yamamoto T. 2004. Involvement of BREK, a serine/threonine kinase enriched in brain, in NGF signalling. Genes Cells 9(3):219-32.

Kawas C, Resnick S, Morrison A, Brookmeyer R, Corrada M, Zonderman A, Bacal C, Lingle DD, Metter E. 1997. A prospective study of estrogen replacement therapy and the risk of developing Alzheimer's disease: the Baltimore Longitudinal Study of Aging. Neurology 48(6):1517-21.

Kelly MJ, Levin ER. 2001. Rapid actions of plasma membrane estrogen receptors. Trends Endocrinol Metab 12(4):152-6.

Kempermann G, Wiskott L, Gage FH. 2004. Functional significance of adult neurogenesis. Curr Opin Neurobiol 14(2):186-91.

King DL, Arendash GW. 2002. Maintained synaptophysin immunoreactivity in Tg2576 transgenic mice during aging: correlations with cognitive impairment. Brain Res 926(1-2):58-68.

Ko J, Na M, Kim S, Lee JR, Kim E. 2003. Interaction of the ERC family of RIM-binding proteins with the liprin-alpha family of multidomain proteins. J Biol Chem 278(43):42377-85.

Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O. 1998. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc Natl Acad Sci U S A 95(26):15677-82.

Kretz O, Fester L, Wehrenberg U, Zhou L, Brauckmann S, Zhao S, Prange-Kiel J, Naumann T, Jarry H, Frotscher M and others. 2004. Hippocampal synapses depend on hippocampal estrogen synthesis. J Neurosci 24(26):5913-21.

Krezel W, Dupont S, Krust A, Chambon P, Chapman PF. 2001. Increased anxiety and synaptic plasticity in estrogen receptor beta -deficient mice. Proc Natl Acad Sci U S A 98(21):12278-82.

Kumar A, Foster TC. 2002. 17beta-estradiol benzoate decreases the AHP amplitude in CA1 pyramidal neurons. J Neurophysiol 88(2):621-6.

Kurisaki T, Wakatsuki S, Sehara-Fujisawa A. 2002. Meltrin beta mini, a new ADAM19 isoform lacking metalloprotease and disintegrin domains, induces morphological changes in neuronal cells. FEBS Lett 532(3):419-22.

Kuroki Y, Fukushima K, Kanda Y, Mizuno K, Watanabe Y. 2000. Putative membrane-bound estrogen receptors possibly stimulate mitogen-activated protein kinase in the rat hippocampus. Eur J Pharmacol 400(2-3):205-9.

Kuroki Y, Fukushima K, Kanda Y, Mizuno K, Watanabe Y. 2001. Neuroprotection by estrogen via extracellular signal-regulated kinase against quinolinic acid-induced cell death in the rat hippocampus. Eur J Neurosci 13(3):472-6.

Kyng KJ, May A, Kolvraa S, Bohr VA. 2003. Gene expression profiling in Werner syndrome closely resembles that of normal aging. Proc Natl Acad Sci U S A 100(21):12259-64.

Lacreuse A, Verreault M, Herndon JG. 2001. Fluctuations in spatial recognition memory across the menstrual cycle in female rhesus monkeys. Psychoneuroendocrinology 26(6):623-39.

Lacreuse A, Wilson ME, Herndon JG. 2002. Estradiol, but not raloxifene, improves aspects of spatial working memory in aged ovariectomized rhesus monkeys. Neurobiol Aging 23(4):589-600.

Lauren J, Airaksinen MS, Saarma M, Timmusk T. 2003. A novel gene family encoding leucine-rich repeat transmembrane proteins differentially expressed in the nervous system. Genomics 81(4):411-21.

Lee SJ, Campomanes CR, Sikat PT, Greenfield AT, Allen PB, McEwen BS. 2004. Estrogen induces phosphorylation of cyclic AMP response element binding (pCREB) in primary hippocampal cells in a time-dependent manner. Neuroscience 124(3):549-60.

Leil TA, Ossadtchi A, Nichols TE, Leahy RM, Smith DJ. 2003. Genes regulated by learning in the hippocampus. J Neurosci Res 71(6):763-8.

Leong H, Riby JE, Firestone GL, Bjeldanes LF. 2004. Potent ligand-independent estrogen receptor activation by 3,3'-diindolylmethane is mediated by cross talk between the protein kinase A and mitogen-activated protein kinase signaling pathways. Mol Endocrinol 18(2):291-302.

Levin ER. 2002. Cellular functions of plasma membrane estrogen receptors. Steroids 67(6):471-5.

Levin ER. 2009. G protein-coupled receptor 30: estrogen receptor or collaborator? Endocrinology 150(4):1563-5.

Li C, Brake WG, Romeo RD, Dunlop JC, Gordon M, Buzescu R, Magarinos AM, Allen PB, Greengard P, Luine V and others. 2004. Estrogen alters hippocampal dendritic spine shape and enhances synaptic protein immunoreactivity and spatial memory in female mice. Proc Natl Acad Sci U S A 101(7):2185-90.

Li C, Wong WH. 2001. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc Natl Acad Sci U S A 98(1):31-6.

Lindberg MK, Moverare S, Skrtic S, Gao H, Dahlman-Wright K, Gustafsson JA, Ohlsson C. 2003. Estrogen receptor (ER)-beta reduces ERalpha-regulated gene transcription, supporting a "ying yang" relationship between ERalpha and ERbeta in mice. Mol Endocrinol 17(2):203-8.

Liu F, Day M, Muniz LC, Bitran D, Arias R, Revilla-Sanchez R, Grauer S, Zhang G, Kelley C, Pulito V and others. 2008. Activation of estrogen receptor-beta regulates hippocampal synaptic plasticity and improves memory. Nat Neurosci 11(3):334-43.

Liu MM, Albanese C, Anderson CM, Hilty K, Webb P, Uht RM, Price RH, Jr., Pestell RG, Kushner PJ. 2002. Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression. J Biol Chem 277(27):24353-60.

Lu Q, Surks HK, Ebling H, Baur WE, Brown D, Pallas DC, Karas RH. 2003. Regulation of estrogen receptor alpha-mediated transcription by a direct interaction with protein phosphatase 2A. J Biol Chem 278(7):4639-45.

Lu T, Pan Y, Kao SY, Li C, Kohane I, Chan J, Yankner BA. 2004. Gene regulation and DNA damage in the ageing human brain. Nature 429(6994):883-91.

Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. 1993. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci U S A 90(23):11162-6.

Luo Y, Long JM, Spangler EL, Longo DL, Ingram DK, Weng NP. 2001. Identification of maze learning-associated genes in rat hippocampus by cDNA microarray. J Mol Neurosci 17(3):397-404.

Lynch MA. 1998. Age-related impairment in long-term potentiation in hippocampus: a role for the cytokine, interleukin-1 beta? Prog Neurobiol 56(5):571-89.

MacLennan AH, Henderson VW, Paine BJ, Mathias J, Ramsay EN, Ryan P, Stocks NP, Taylor AW. 2006. Hormone therapy, timing of initiation, and cognition in women aged older than 60 years: the REMEMBER pilot study. Menopause 13(1):28-36.

Maki PM. 2006. Hormone therapy and cognitive function: is there a critical period for benefit? Neuroscience 138(3):1027-30.

Markham JA, Pych JC, Juraska JM. 2002. Ovarian hormone replacement to aged ovariectomized female rats benefits acquisition of the morris water maze. Horm Behav 42(3):284-93.

Markowska AL, Savonenko AV. 2002. Effectiveness of estrogen replacement in restoration of cognitive function after long-term estrogen withdrawal in aging rats. J Neurosci 22(24):10985-95.

Martin B, Mattson MP, Maudsley S. 2006. Caloric restriction and intermittent fasting: two potential diets for successful brain aging. Ageing Res Rev 5(3):332-53.

Massart F, Paolini S, Piscitelli E, Brandi ML, Solaini G. 2002. Dose-dependent inhibition of mitochondrial ATP synthase by 17 beta-estradiol. Gynecol Endocrinol 16(5):373-7.

McEwen BS, Alves SE. 1999. Estrogen actions in the central nervous system. Endocr Rev 20(3):279-307.

McEwen BS, Alves SE, Bulloch K, Weiland NG. 1997. Ovarian steroids and the brain: implications for cognition and aging. Neurology 48(5 Suppl 7):S8-15.

Mehra RD, Sharma K, Nyakas C, Vij U. 2005. Estrogen receptor alpha and beta immunoreactive neurons in normal adult and aged female rat hippocampus: a qualitative and quantitative study. Brain Res 1056(1):22-35.

Mendez P, Cardona-Gomez GP, Garcia-Segura LM. 2005. Interactions of insulin-like growth factor-I and estrogen in the brain. Adv Exp Med Biol 567:285-303.

Meng X, Lu X, Li Z, Green ED, Massa H, Trask BJ, Morris CA, Keating MT. 1998. Complete physical map of the common deletion region in Williams syndrome and identification and characterization of three novel genes. Hum Genet 103(5):590-9.

Milner TA, Ayoola K, Drake CT, Herrick SP, Tabori NE, McEwen BS, Warrier S, Alves SE. 2005. Ultrastructural localization of estrogen receptor beta immunoreactivity in the rat hippocampal formation. J Comp Neurol 491(2):81-95.

Milner TA, McEwen BS, Hayashi S, Li CJ, Reagan LP, Alves SE. 2001. Ultrastructural evidence that hippocampal alpha estrogen receptors are located at extranuclear sites. J Comp Neurol 429(3):355-71.

Minano A, Xifro X, Perez V, Barneda-Zahonero B, Saura CA, Rodriguez-Alvarez J. 2008. Estradiol facilitates neurite maintenance by a Src/Ras/ERK signalling pathway. Mol Cell Neurosci.

Miranda P, Williams CL, Einstein G. 1999. Granule cells in aging rats are sexually dimorphic in their response to estradiol. J Neurosci 19(9):3316-25.

Morissette M, Le Saux M, Di Paolo T. 2008a. Effect of oestrogen receptor alpha and beta agonists on brain N-methyl-D-aspartate receptors. J Neuroendocrinol 20(8):1006-14.

Morissette M, Le Saux M, Di Paolo T. 2008b. Effect of Oestrogen Receptor Alpha and Beta Agonists on Brain Nmda Receptors. J Neuroendocrinol.

Morris R. 1984. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11(1):47-60.

Morris RG, Schenk F, Tweedie F, Jarrard LE. 1990. Ibotenate Lesions of Hippocampus and/or Subiculum: Dissociating Components of Allocentric Spatial Learning. Eur J Neurosci 2(12):1016-1028.

Mukai H, Tsurugizawa T, Murakami G, Kominami S, Ishii H, Ogiue-Ikeda M, Takata N, Tanabe N, Furukawa A, Hojo Y and others. 2007. Rapid modulation of long-term depression and spinogenesis via synaptic estrogen receptors in hippocampal principal neurons. J Neurochem 100(4):950-67.

Mukai H, Tsurugizawa T, Ogiue-Ikeda M, Murakami G, Hojo Y, Ishii H, Kimoto T, Kawato S. 2006. Local neurosteroid production in the hippocampus: influence on synaptic plasticity of memory. Neuroendocrinology 84(4):255-63.

Murakami G, Tsurugizawa T, Hatanaka Y, Komatsuzaki Y, Tanabe N, Mukai H, Hojo Y, Kominami S, Yamazaki T, Kimoto T and others. 2006. Comparison between basal and apical dendritic spines in estrogen-induced rapid spinogenesis of CA1 principal neurons in the adult hippocampus. Biochem Biophys Res Commun 351(2):553-8.

Murphy DD, Segal M. 1996. Regulation of dendritic spine density in cultured rat hippocampal neurons by steroid hormones. J Neurosci 16(13):4059-68.

Murphy DD, Segal M. 1997. Morphological plasticity of dendritic spines in central neurons is mediated by activation of cAMP response element binding protein. Proc Natl Acad Sci U S A 94(4):1482-7.

Naciff JM, Overmann GJ, Torontali SM, Carr GJ, Khambatta ZS, Tiesman JP, Richardson BD, Daston GP. 2007. Uterine temporal response to acute exposure to 17alpha-ethinyl estradiol in the immature rat. Toxicol Sci 97(2):467-90.

Naruhashi K, Kadomatsu K, Igakura T, Fan QW, Kuno N, Muramatsu H, Miyauchi T, Hasegawa T, Itoh A, Muramatsu T and others. 1997. Abnormalities of sensory and memory functions in mice lacking Bsg gene. Biochem Biophys Res Commun 236(3):733-7.

Nilsen J, Brinton RD. 2002. Impact of progestins on estradiol potentiation of the glutamate calcium response. Neuroreport 13(6):825-30.

Nilsen J, Irwin RW, Gallaher TK, Brinton RD. 2007. Estradiol in vivo regulation of brain mitochondrial proteome. J Neurosci 27(51):14069-77.

Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA. 2001. Mechanisms of estrogen action. Physiol Rev 81(4):1535-65.

Niu S, Renfro A, Quattrocchi CC, Sheldon M, D'Arcangelo G. 2004. Reelin promotes hippocampal dendrite development through the VLDLR/ApoER2-Dab1 pathway. Neuron 41(1):71-84.

O'Lone R, Frith MC, Karlsson EK, Hansen U. 2004. Genomic targets of nuclear estrogen receptors. Mol Endocrinol 18(8):1859-75.

O'Lone R, Knorr K, Jaffe IZ, Schaffer ME, Martini PG, Karas RH, Bienkowska J, Mendelsohn ME, Hansen U. 2007. Estrogen receptors alpha and beta mediate distinct pathways of vascular gene expression, including genes involved in mitochondrial electron transport and generation of reactive oxygen species. Mol Endocrinol 21(6):1281-96.

Obeid LM, Hannun YA. 2003. Ceramide, stress, and a "LAG" in aging. Sci Aging Knowledge Environ 2003(39):PE27.

Olsen L, Rasmussen HB, Hansen T, Bagger YZ, Tanko LB, Qin G, Christiansen C, Werge T. 2006. Estrogen receptor alpha and risk for cognitive impairment in postmenopausal women. Psychiatr Genet 16(2):85-8.

Olton DS. 1977. The function of septo-hippocampal connections in spatially organized behaviour. Ciba Found Symp(58):327-49.

Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS. 1997. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science 277(5331):1508-10.

Patrone C, Pollio G, Vegeto E, Enmark E, de Curtis I, Gustafsson JA, Maggi A. 2000. Estradiol induces differential neuronal phenotypes by activating estrogen receptor alpha or beta. Endocrinology 141(5):1839-45.

Pechenino AS, Frick KM. 2009. The effects of acute 17beta-estradiol treatment on gene expression in the young female mouse hippocampus. Neurobiol Learn Mem 91(3):315-22.

Pedram A, Razandi M, Levin ER. 2006. Nature of functional estrogen receptors at the plasma membrane. Mol Endocrinol 20(9):1996-2009.

Perez-Martin M, Azcoitia I, Trejo JL, Sierra A, Garcia-Segura LM. 2003. An antagonist of estrogen receptors blocks the induction of adult neurogenesis by insulin-like growth factor-I in the dentate gyrus of adult female rat. Eur J Neurosci 18(4):923-30.

Pettersson K, Delaunay F, Gustafsson JA. 2000. Estrogen receptor beta acts as a dominant regulator of estrogen signaling. Oncogene 19(43):4970-8.

Poon HF, Shepherd HM, Reed TT, Calabrese V, Stella AM, Pennisi G, Cai J, Pierce WM, Klein JB, Butterfield DA. 2006. Proteomics analysis provides insight into caloric restriction mediated oxidation and expression of brain proteins associated with age-related impaired cellular processes: Mitochondrial dysfunction, glutamate dysregulation and impaired protein synthesis. Neurobiol Aging 27(7):1020-34.

Pozas E, Pascual M, Nguyen Ba-Charvet KT, Guijarro P, Sotelo C, Chedotal A, Del Rio JA, Soriano E. 2001. Age-dependent effects of secreted Semaphorins 3A, 3F, and 3E on developing hippocampal axons: in vitro effects and phenotype of Semaphorin 3A (-/-) mice. Mol Cell Neurosci 18(1):26-43.

Prolla TA. 2002. DNA microarray analysis of the aging brain. Chem Senses 27(3):299-306.

Ramirez VD, Zheng J, Siddique KM. 1996. Membrane receptors for estrogen, progesterone, and testosterone in the rat brain: fantasy or reality. Cell Mol Neurobiol 16(2):175-98.

Rapp PR, Morrison JH, Roberts JA. 2003. Cyclic estrogen replacement improves cognitive function in aged ovariectomized rhesus monkeys. J Neurosci 23(13):5708-14.

Rapp PR, Rosenberg RA, Gallagher M. 1987. An evaluation of spatial information processing in aged rats. Behav Neurosci 101(1):3-12.

Raz L, Khan MM, Mahesh VB, Vadlamudi RK, Brann DW. 2008. Rapid estrogen signaling in the brain. Neurosignals 16(2-3):140-53.

Rissman EF. 2008. Roles of oestrogen receptors alpha and beta in behavioural neuroendocrinology: beyond Yin/Yang. J Neuroendocrinol 20(6):873-9.

Rissman EF, Heck AL, Leonard JE, Shupnik MA, Gustafsson JA. 2002. Disruption of estrogen receptor beta gene impairs spatial learning in female mice. Proc Natl Acad Sci U S A 99(6):3996-4001.

Roberts JA, Gilardi KV, Lasley B, Rapp PR. 1997. Reproductive senescence predicts cognitive decline in aged female monkeys. Neuroreport 8(8):2047-51.

Romeo RD, McCarthy JB, Wang A, Milner TA, McEwen BS. 2005. Sex differences in hippocampal estradiol-induced N-methyl-D-aspartic acid binding and ultrastructural localization of estrogen receptor-alpha. Neuroendocrinology 81(6):391-9.

Rosa AO, Egea J, Martinez A, Garcia AG, Lopez MG. 2008. Neuroprotective effect of the new thiadiazolidinone NP00111 against oxygen-glucose deprivation in rat hippocampal slices: implication of ERK1/2 and PPARgamma receptors. Exp Neurol 212(1):93-9.

Rosenzweig ES, Barnes CA. 2003. Impact of aging on hippocampal function: plasticity, network dynamics, and cognition. Prog Neurobiol 69(3):143-79.

Rune GM, Frotscher M. 2005. Neurosteroid synthesis in the hippocampus: role in synaptic plasticity. Neuroscience 136(3):833-42.

Safe S. 2001. Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. Vitam Horm 62:231-52.

Sandstrom NJ, Williams CL. 2001. Memory retention is modulated by acute estradiol and progesterone replacement. Behav Neurosci 115(2):384-93.

Sanguino E, Roglans N, Rodriguez-Calvo R, Alegret M, Sanchez RM, Vazquez-Carrera M, Laguna JC. 2006. Ageing introduces a complex pattern of changes in several rat brain transcription factors depending on gender and anatomical localization. Exp Gerontol 41(4):372-9.

Santagati S, Melcangi RC, Celotti F, Martini L, Maggi A. 1994. Estrogen receptor is expressed in different types of glial cells in culture. J Neurochem 63(6):2058-64.

Santos MJ, Quintanilla RA, Toro A, Grandy R, Dinamarca MC, Godoy JA, Inestrosa NC. 2005. Peroxisomal proliferation protects from beta-amyloid neurodegeneration. J Biol Chem 280(49):41057-68.

Saravia F, Beauquis J, Pietranera L, De Nicola AF. 2007. Neuroprotective effects of estradiol in hippocampal neurons and glia of middle age mice. Psychoneuroendocrinology 32(5):480-92.

Sarkar SN, Huang RQ, Logan SM, Yi KD, Dillon GH, Simpkins JW. 2008. Estrogens directly potentiate neuronal L-type Ca2+ channels. Proc Natl Acad Sci U S A 105(39):15148-53.

Savonenko AV, Markowska AL. 2003. The cognitive effects of ovariectomy and estrogen replacement are modulated by aging. Neuroscience 119(3):821-30.

Sawai T, Bernier F, Fukushima T, Hashimoto T, Ogura H, Nishizawa Y. 2002. Estrogen induces a rapid increase of calcium-calmodulin-dependent protein kinase II activity in the hippocampus. Brain Res 950(1-2):308-11.

Schnoes KK, Jaffe IZ, Iyer L, Dabreo A, Aronovitz M, Newfell B, Hansen U, Rosano G, Mendelsohn ME. 2008. Rapid recruitment of temporally distinct vascular gene sets by estrogen. Mol Endocrinol 22(11):2544-56.

Scully JL, Otten U. 1995. Neurotrophin expression modulated by glucocorticoids and oestrogen in immortalized hippocampal neurons. Brain Res Mol Brain Res 31(1-2):158-64.

Serra-Pages C, Medley QG, Tang M, Hart A, Streuli M. 1998. Liprins, a family of LAR transmembrane protein-tyrosine phosphatase-interacting proteins. J Biol Chem 273(25):15611-20.

Setalo G, Jr., Singh M, Guan X, Toran-Allerand CD. 2002. Estradiol-induced phosphorylation of ERK1/2 in explants of the mouse cerebral cortex: the roles of heat shock protein 90 (Hsp90) and MEK2. J Neurobiol 50(1):1-12.

Sharrow KM, Kumar A, Foster TC. 2002. Calcineurin as a potential contributor in estradiol regulation of hippocampal synaptic function. Neuroscience 113(1):89-97.

Sheen VL, Feng Y, Graham D, Takafuta T, Shapiro SS, Walsh CA. 2002. Filamin A and Filamin B are co-expressed within neurons during periods of neuronal migration and can physically interact. Hum Mol Genet 11(23):2845-54.

Sherwin BB. 2005. Estrogen and memory in women: how can we reconcile the findings? Horm Behav 47(3):371-5.

Sherwin BB. 2006. Estrogen and cognitive aging in women. Neuroscience 138(3):1021-6.

Sherwin BB. 2007. The critical period hypothesis: can it explain discrepancies in the oestrogen-cognition literature? J Neuroendocrinol 19(2):77-81.

Sherwin BB, Henry JF. 2008. Brain aging modulates the neuroprotective effects of estrogen on selective aspects of cognition in women: a critical review. Front Neuroendocrinol 29(1):88-113.

Shingo AS, Kito S. 2002. Estrogen induces elevation of cAMP-dependent protein kinase activity in immortalized hippocampal neurons: imaging in living cells. J Neural Transm 109(2):171-4.

Shughrue PJ, Dorsa DM. 1993. Estrogen modulates the growth-associated protein GAP-43 (Neuromodulin) mRNA in the rat preoptic area and basal hypothalamus. Neuroendocrinology 57(3):439-47.

Shughrue PJ, Lane MV, Merchenthaler I. 1999. Biologically active estrogen receptor-beta: evidence from in vivo autoradiographic studies with estrogen receptor alpha-knockout mice. Endocrinology 140(6):2613-20.

Shughrue PJ, Merchenthaler I. 2001. Distribution of estrogen receptor beta immunoreactivity in the rat central nervous system. J Comp Neurol 436(1):64-81.

Shumaker SA, Legault C, Kuller L, Rapp SR, Thal L, Lane DS, Fillit H, Stefanick ML, Hendrix SL, Lewis CE and others. 2004. Conjugated equine estrogens and incidence of probable dementia and mild cognitive impairment in postmenopausal women: Women's Health Initiative Memory Study. JAMA 291(24):2947-58.

Simpkins JW, Dykens JA. 2008. Mitochondrial mechanisms of estrogen neuroprotection. Brain Res Rev 57(2):421-30.

Simpkins JW, Green PS, Gridley KE, Singh M, de Fiebre NC, Rajakumar G. 1997. Role of estrogen replacement therapy in memory enhancement and the prevention of neuronal loss associated with Alzheimer's disease. Am J Med 103(3A):19S-25S.

Sinagra M, Verrier D, Frankova D, Korwek KM, Blahos J, Weeber EJ, Manzoni OJ, Chavis P. 2005. Reelin, very-low-density lipoprotein receptor, and apolipoprotein E receptor 2 control somatic NMDA receptor composition during hippocampal maturation in vitro. J Neurosci 25(26):6127-36.

Singh M, Dykens JA, Simpkins JW. 2006. Novel mechanisms for estrogen-induced neuroprotection. Exp Biol Med (Maywood) 231(5):514-21.

Singh M, Setalo G, Jr., Guan X, Warren M, Toran-Allerand CD. 1999. Estrogen-induced activation of mitogen-activated protein kinase in cerebral cortical explants: convergence of estrogen and neurotrophin signaling pathways. J Neurosci 19(4):1179-88.

Small SA, Stern Y, Tang M, Mayeux R. 1999. Selective decline in memory function among healthy elderly. Neurology 52(7):1392-6.

Smith CC, McMahon LL. 2005. Estrogen-induced increase in the magnitude of long-term potentiation occurs only when the ratio of NMDA transmission to AMPA transmission is increased. J Neurosci 25(34):7780-91.

Spencer JL, Waters EM, Milner TA, McEwen BS. 2008a. Estrous cycle regulates activation of hippocampal Akt, LIM kinase, and neurotrophin receptors in C57BL/6 mice. Neuroscience 155(4):1106-19.

Spencer JL, Waters EM, Romeo RD, Wood GE, Milner TA, McEwen BS. 2008b. Uncovering the mechanisms of estrogen effects on hippocampal function. Front Neuroendocrinol 29(2):219-37.

Stefanick ML. 2005. Estrogens and progestins: background and history, trends in use, and guidelines and regimens approved by the US Food and Drug Administration. Am J Med 118 Suppl 12B:64-73.

Stirone C, Duckles SP, Krause DN, Procaccio V. 2005. Estrogen increases mitochondrial efficiency and reduces oxidative stress in cerebral blood vessels. Mol Pharmacol 68(4):959-65.

Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA. 2004. Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. Proc Natl Acad Sci U S A 101(6):1566-71.

Suzuki S, Handa RJ. 2004. Regulation of estrogen receptor-beta expression in the female rat hypothalamus: differential effects of dexamethasone and estradiol. Endocrinology 145(8):3658-70.

Sze CI, Troncoso JC, Kawas C, Mouton P, Price DL, Martin LJ. 1997. Loss of the presynaptic vesicle protein synaptophysin in hippocampus correlates with cognitive decline in Alzheimer disease. J Neuropathol Exp Neurol 56(8):933-44.

Talboom JS, Williams BJ, Baxley ER, West SG, Bimonte-Nelson HA. 2008. Higher levels of estradiol replacement correlate with better spatial memory in surgically menopausal young and middle-aged rats. Neurobiol Learn Mem 90(1):155-63.

Tanapat P, Hastings NB, Reeves AJ, Gould E. 1999. Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. J Neurosci 19(14):5792-801.

Terman JR, Mao T, Pasterkamp RJ, Yu HH, Kolodkin AL. 2002. MICALs, a family of conserved flavoprotein oxidoreductases, function in plexin-mediated axonal repulsion. Cell 109(7):887-900.

Thakur MK, Sharma PK. 2007. Transcription of estrogen receptor alpha and beta in mouse cerebral cortex: effect of age, sex, 17beta-estradiol and testosterone. Neurochem Int 50(2):314-21.

Toran-Allerand CD. 2004. Estrogen and the brain: beyond ER-alpha and ER-beta. Exp Gerontol 39(11-12):1579-86.

Toran-Allerand CD, Guan X, MacLusky NJ, Horvath TL, Diano S, Singh M, Connolly ES, Jr., Nethrapalli IS, Tinnikov AA. 2002. ER-X: a novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. J Neurosci 22(19):8391-401.

Uittenbogaard M, Chiaramello A. 2002. Expression of the bHLH transcription factor Tcf12 (ME1) gene is linked to the expansion of precursor cell populations during neurogenesis. Brain Res Gene Expr Patterns 1(2):115-21.

Vaucher E, Reymond I, Najaffe R, Kar S, Quirion R, Miller MM, Franklin KB. 2002. Estrogen effects on object memory and cholinergic receptors in young and old female mice. Neurobiol Aging 23(1):87-95.

Vegeto E, Benedusi V, Maggi A. 2008. Estrogen anti-inflammatory activity in brain: a therapeutic opportunity for menopause and neurodegenerative diseases. Front Neuroendocrinol 29(4):507-19.

Verbitsky M, Yonan AL, Malleret G, Kandel ER, Gilliam TC, Pavlidis P. 2004. Altered hippocampal transcript profile accompanies an age-related spatial memory deficit in mice. Learn Mem 11(3):253-60.

von Bohlen und Halbach O, Zacher C, Gass P, Unsicker K. 2006. Age-related alterations in hippocampal spines and deficiencies in spatial memory in mice. J Neurosci Res 83(4):525-31.

Wang JM, Irwin RW, Brinton RD. 2006. Activation of estrogen receptor alpha increases and estrogen receptor beta decreases apolipoprotein E expression in hippocampus in vitro and in vivo. Proc Natl Acad Sci U S A 103(45):16983-8.

Wang PN, Liao SQ, Liu RS, Liu CY, Chao HT, Lu SR, Yu HY, Wang SJ, Liu HC. 2000. Effects of estrogen on cognition, mood, and cerebral blood flow in AD: a controlled study. Neurology 54(11):2061-6.

Warren SG, Humphreys AG, Juraska JM, Greenough WT. 1995. LTP varies across the estrous cycle: enhanced synaptic plasticity in proestrus rats. Brain Res 703(1-2):26-30.

Weiland NG, Orikasa C, Hayashi S, McEwen BS. 1997. Distribution and hormone regulation of estrogen receptor immunoreactive cells in the hippocampus of male and female rats. J Comp Neurol 388(4):603-12.

West MJ, Kawas CH, Martin LJ, Troncoso JC. 2000. The CA1 region of the human hippocampus is a hot spot in Alzheimer's disease. Ann N Y Acad Sci 908:255-9.

Williams C, Edvardsson K, Lewandowski SA, Strom A, Gustafsson JA. 2008. A genome-wide study of the repressive effects of estrogen receptor beta on estrogen receptor alpha signaling in breast cancer cells. Oncogene 27(7):1019-32.

Wilson ME, Rosewell KL, Kashon ML, Shughrue PJ, Merchenthaler I, Wise PM. 2002. Age differentially influences estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) gene expression in specific regions of the rat brain. Mech Ageing Dev 123(6):593-601.

Woolley CS. 2007. Acute effects of estrogen on neuronal physiology. Annu Rev Pharmacol Toxicol 47:657-80.

Woolley CS, Gould E, Frankfurt M, McEwen BS. 1990. Naturally occurring fluctuation in dendritic spine density on adult hippocampal pyramidal neurons. J Neurosci 10(12):4035-9.

Woolley CS, McEwen BS. 1992. Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. J Neurosci 12(7):2549-54.

Woolley CS, McEwen BS. 1993. Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. J Comp Neurol 336(2):293-306.

Woolley CS, Wenzel HJ, Schwartzkroin PA. 1996. Estradiol increases the frequency of multiple synapse boutons in the hippocampal CA1 region of the adult female rat. J Comp Neurol 373(1):108-17.

Wu Q, Maniatis T. 1999. A striking organization of a large family of human neural cadherin-like cell adhesion genes. Cell 97(6):779-90.

Wu TW, Wang JM, Chen S, Brinton RD. 2005. 17Beta-estradiol induced Ca2+ influx via L-type calcium channels activates the Src/ERK/cyclic-AMP response element binding protein signal pathway and BCL-2 expression in rat hippocampal neurons: a potential initiation mechanism for estrogen-induced neuroprotection. Neuroscience 135(1):59-72.

Wu X, Li H, Chen JD. 2001. The human homologue of the yeast DNA repair and TFIIH regulator MMS19 is an AF-1-specific coactivator of estrogen receptor. J Biol Chem 276(26):23962-8.

Wulf P, Suter U. 1999. Embryonic expression of epithelial membrane protein 1 in early neurons. Brain Res Dev Brain Res 116(2):169-80.

Yaffe K, Lindquist K, Sen S, Cauley J, Ferrell R, Penninx B, Harris T, Li R, Cummings SR. 2009. Estrogen receptor genotype and risk of cognitive impairment in elders: findings from the Health ABC study. Neurobiol Aging 30(4):607-14.

Yildirim M, Janssen WG, Tabori NE, Adams MM, Yuen GS, Akama KT, McEwen BS, Milner TA, Morrison JH. 2008. Estrogen and aging affect synaptic distribution of phosphorylated LIM kinase (pLIMK) in CA1 region of female rat hippocampus. Neuroscience 152(2):360-370.

Yun SH, Park KA, Kwon S, Woolley CS, Sullivan PM, Pasternak JF, Trommer BL. 2007. Estradiol enhances long term potentiation in hippocampal slices from aged apoE4-TR mice. Hippocampus 17(12):1153-7.

Zhang QG, Wang R, Khan M, Mahesh V, Brann DW. 2008. Role of Dickkopf-1, an antagonist of the Wnt/beta-catenin signaling pathway, in estrogen-induced neuroprotection and attenuation of tau phosphorylation. J Neurosci 28(34):8430-41.

Zhang QH, Huang YH, Hu YZ, Wei GZ, Han XF, Lu SY, Zhao YF. 2004. Disruption of estrogen receptor beta in mice brain results in pathological alterations resembling Alzheimer disease. Acta Pharmacol Sin 25(4):452-7.

Zhang W, Han SW, McKeel DW, Goate A, Wu JY. 1998. Interaction of presenilins with the filamin family of actin-binding proteins. J Neurosci 18(3):914-22.

Zhao C, Matthews J, Tujague M, Wan J, Strom A, Toresson G, Lam EW, Cheng G, Gustafsson JA, Dahlman-Wright K. 2007. Estrogen receptor beta2 negatively regulates the transactivation of estrogen receptor alpha in human breast cancer cells. Cancer Res 67(8):3955-62.

Zhao L, Brinton RD. 2007. Estrogen receptor alpha and beta differentially regulate intracellular Ca(2+) dynamics leading to ERK phosphorylation and estrogen neuroprotection in hippocampal neurons. Brain Res 1172:48-59.

Zhao X, Lein ES, He A, Smith SC, Aston C, Gage FH. 2001. Transcriptional profiling reveals strict boundaries between hippocampal subregions. J Comp Neurol 441(3):187-96.

Zheng J, Ramirez VD. 1999. Rapid inhibition of rat brain mitochondrial proton F0F1-ATPase activity by estrogens: comparison with Na+, K+ -ATPase of porcine cortex. Eur J Pharmacol 368(1):95-102.

Ziegler DR, Gallagher M. 2005. Spatial memory in middle-aged female rats: assessment of estrogen replacement after ovariectomy. Brain Res 1052(2):163-73.

Znamensky V, Akama KT, McEwen BS, Milner TA. 2003. Estrogen levels regulate the subcellular distribution of phosphorylated Akt in hippocampal CA1 dendrites. J Neurosci 23(6):2340-7.

BIOGRAPHICAL SKETCH

Kristina Katharine Aenlle was born in Niles, Michigan, to Marianne and Thomas Davis, in July of 1978. Along with her older sister (Elizabeth) and younger brother (Steven), she attended Niles community schools, graduating with honors in 1996. She then attended Michigan State University, in East Lansing, Michigan, where she received her B.S. in Psychology. During her time at MSU, Kristina had the pleasure if working in the labs of Dr. Sheryl Sisk and Dr. John I Johnson. Dr. Sisk, a leading neuroendocrinologist introduced Kristina to hormonal regulation of behavior. Dr. John Johnson, a renowned comparative neurobiologist, taught Kristina the intricacies of brain function. It was the amazing experience and guidance at MSU that her love for research grew and in August of 2003 she enrolled in the University of Florida's Interdisciplinary Program in Biomedical Science. The interdisciplinary program allowed her to explorer many fields of research and expands her scientific research experience. In the spring of 2004, Kristina joined the laboratory of Dr. Thomas C. Foster, where she conducted experiments examining the role of estrogen on cognitive function and hippocampal gene expression during aging. Using microarrays analysis of hippocampal tissue, she has found that estrogen can reverse age-related changes in the expression of genes of the hippocampus during middle-age but the response to estrogen treatment in aged animals is attenuated and an alteration of estrogen receptors in contributing to the truncated response to estrogen treatment. Kristina's dissertation work will be published in three first author publications. It is her hope that the results of her work will contribute to improved and safer therapeutic strategies to prevent age-related cognitive decline.

With her husband, Jeff, and two sons, Dante and Dominic, she enjoys spending time at the beach and traveling.