THE EFFECTS OF ACUTE 17β-ESTRADIOL TREATMENT ON GENE EXPRESSION IN THE YOUNG FEMALE MOUSE HIPPOCAMPUS

Angela S. Pechenino[1] and **Karyn M. Frick**[1,2]

[1]*Department of Psychology, Yale University, New Haven, CT, 06520*

[2]*Interdepartmental Neuroscience Program, Yale University, New Haven, CT, 06520*

Abstract

Previous studies have demonstrated that treatment with 17β-estradiol (E_2) improves both spatial and nonspatial memory in young female mice. Still unclear, however, are the molecular mechanisms underlying the beneficial effects of E_2 on memory. We have previously demonstrated that a single post-training intraperitoneal (i.p.) injection of 0.2 mg/kg E_2 can enhance hippocampal-dependent spatial and object memory consolidation (e.g., Gresack and Frick, 2006b). Therefore, in the present study, we performed a microarray analysis on the dorsal hippocampi of 4 month-old female mice injected i.p. with vehicle or 0.2 mg/kg E_2. Genes were considered differentially expressed following E_2 treatment if they showed a greater than two-fold change in RNA expression levels compared to controls. Overall, out of a total of approximately 25,000 genes represented on the array, 204 genes showed altered mRNA expression levels upon E_2 treatment, with 111 up-regulated and 93 down-regulated. Of these, 17 of the up-regulated and 6 of the down-regulated genes are known to be involved in learning and memory. mRNA expression changes in 5 of the genes were confirmed by real-time quantitative PCR analysis, and protein changes in these same genes were confirmed by Western blot analysis: Hsp70, a heat shock protein known to be estrogen responsive; Igfbp2, an IGF-I binding protein; Actn4, an actin binding protein involved in protein trafficking; Tubb2a, the major component of microtubules; and Snap25, a synaptosome-specific protein required for neurotransmitter release. The types of genes altered indicate that E_2 may induce changes in the structural mechanics of cells within the dorsal hippocampus that could be conducive to promoting memory consolidation.

Keywords

Estrogen; microarray; memory; Igfbp2; Hsp70; Actn4; Tubb2a; Snap25

1. Introduction

The loss of estrogens at menopause has been associated with increases in dementia and age-related memory decline observed in aging women (Yaffe et al., 2000; Wolf and Kirschbaum,

To whom correspondence should be addressed. Karyn M. Frick, Ph.D., Department of Psychology, Yale University, P.O. Box 208205, New Haven, CT 06520, Phone: (203) 432-4673, Fax: (203) 432-7172, E-mail: karyn.frick@yale.edu.

2002). Despite the findings of the Women's Health Initiative Memory Study (WHIMS), which demonstrated that giving estrogens alone or in combination with progesterone failed to prevent dementia in postmenopausal women (Shumaker et al., 2003; Shumaker et al., 2004), other evidence has shown that estrogen administration can have beneficial effects on memory. For example, giving estrogen to healthy postmenopausal women can improve spatial working memory (Duff and Hampson, 2000), object memory (Duka et al., 2000), and verbal memory (Kampen and Sherwin, 1994). Research has also demonstrated the beneficial effects of the potent estrogen, 17β-estradiol (E_2), in rodent models. For example, E_2 administered intraperitoneally (i.p.) immediately post-training enhances both spatial reference memory in the Morris water maze (Rissanen et al., 1999; Heikkinen et al., 2002; Gresack and Frick, 2006a) and novel object recognition memory (Luine et al., 2003; Gresack and Frick, 2006b) in mice and rats. Spatial and object memory are also enhanced by pre-training E_2 treatments administered systemically by injection or silastic capsules (Daniel et al., 1997; Luine et al., 1998; Bimonte and Denenberg, 1999; Sandstrom and Williams, 2001; Vaucher et al., 2002; Sandstrom and Williams, 2004). However, systemic hormone administration in women can lead to a host of physiological problems, including an increased incidence of coronary artery disease, stroke, and invasive breast cancer (Mastorakos et al., 2006), calling into question whether the potential benefits of E_2 on cognition outweigh the risks associated with hormone therapy.

An alternative approach to systemic hormone administration may be the specific targeting of proteins within the brain that are modulated by E_2. For example, if the downstream effectors of E_2 could be elucidated, then therapies that directly target these proteins could be developed that would enhance memory without the side effects of systemic hormone administration. E_2 is known to increase dendritic spine density (Woolley and McEwen, 1993; Frick et al., 2004) and synaptic protein expression (Stone et al., 1998) in the CA1 region of the hippocampus and enhance neurogenesis in the hippocampal dentate gyrus (Tanapat et al., 1999; Galea et al., 2006). Additionally, acute E_2 administration can activate several intracellular kinase cascades, including phosphatidylinositol 3-kinase (PI3K; Cardona-Gomez et al., 2002; Yokomaku et al., 2003; Mannella and Brinton, 2006) and extracellular signal-regulated kinase (ERK; Fernandez et al., 2008; Kuroki et al., 2000; Fitzpatrick et al., 2002; Wade and Dorsa, 2003), both of which can phosphorylate and activate CREB, an important protein involved in memory consolidation (Bozon et al., 2003). Previous microarray studies report that E_2 alters expression of several genes in the hypothalamus (Malyala et al., 2004) and the hippocampus (Aenlle et al., 2007), but these studies have been conducted using chronic E_2 administration. No microarray study has yet examined the effects of a single, acute dose of E_2 on gene transcription in the hippocampus. Such information is important to the development of hormone-based drug treatments in order to more closely link E_2-induced changes in signal transduction to alterations in gene transcription.Therefore, the present study endeavored to identify genes whose mRNA and protein expression levels were altered by an acute dose of water-soluble E_2 known in young ovariectomized mice to enhance spatial and object memory (Gresack and Frick, 2006b) and activate the ERK cascade in the dorsal hippocampus (Fernandez et al., 2008; Lewis et al., 2008).

2. Methods

2.1. Subjects

Four month old female C57BL/6 mice were obtained from Taconic (Germantown, NY). Mice were bilaterally ovariectomized 1 week after arrival as per previously published methods (Fernandez and Frick, 2004), and housed up to 5/cage in a room with a 12:12 light/dark cycle (lights on at 07:00) for at least a week before treatments. Animals were handled 5 min per day over the course of 5 days, and had *ad libitum* access to food and water. All procedures were

approved by the Institutional Animal Care and Usage Committee of Yale University, and conformed to the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Mice were randomly assigned to groups receiving i.p. injections of either 0.2 mg/kg 17β-estradiol (E_2) conjugated to the solubility enhancer 2-hydroxypropyl-β-cyclodextrin (HBC) and dissolved in physiological saline (E_2 group, n=3) or HBC dissolved in an equal volume of physiological saline containing the same amount of cyclodextrin present in the HBC-E_2 solution (vehicle group, n=3). HBC is a solubility-enhancer for steroid hormones that does not alter the bioefficacy of the hormones (Pitha and Pitha, 1985), but allows them to successfully cross the blood-brain barrier and rapidly dissociate into the tissue while the HBC remains in the circulation (Taylor et al., 1989). This hormone preparation is metabolized within 24 hours (Pitha et al., 1986; Taylor et al., 1989) and our laboratory has shown that that a single i.p. injection of the 0.2 mg/kg dose given to young female mice immediately after training specifically enhances memory consolidation in both spatial Morris water maze and novel object recognition tasks (Gresack and Frick, 2006a). Previous studies have shown that a 1 μg dose of estradiol dissolved in oil produces levels similar to those seen in the estrus phase of the estrous cycle, and a 10 μg dose produces levels similar to those seen during the proestrus phase of the cycle (Akinci and Johnston, 1997). Given mean body weight for young ovariectomized females of 22 g, the approximate estradiol levels for the 0.2 mg/kg dose is 4.4 μg, and thus near the middle of the physiological range.

In the present study, injections of vehicle or 0.2 mg/kg E_2 were given 1 hour before sacrifice, as our previous work found an increase in dorsal hippocampal p42 ERK phosphorylation at this time point (Fernandez et al., 2008; Lewis et al., 2008). One hour (for RNA analyses) or 3 and 4 hours (for protein analyses) after injection, mice were cervically dislocated, and dorsal hippocampi were bilaterally dissected and stored at -80°C until use.

2.2. RNA Isolation

RNA was isolated from hippocampal tissue using the Trizol reagent isolation protocol (Invitrogen, Carlsbad, CA). Briefly, 800 μL Trizol reagent was added to each tube, and the tissue was homogenized by 10 passes of a Dounce homogenizer (Kontes Glass Co, Vineland, NJ). The homogenate was incubated for 5 min at room temperature and subsequently extracted with 0.2 mL of chloroform to remove proteins. The aqueous phase was transferred to a fresh tube, and the RNA was precipitated using 0.5 mL of isopropanol and centrifuged to pellet the RNA. The RNA pellet was washed twice by resuspension in 1 mL of 75% ethanol. After the last wash, the RNA pellet was allowed to air dry and was then resuspended in DEPC-treated water. The RNA was further purified using the RNAeasy Mini Kit RNA Cleanup protocol (Qiagen, Valencia, CA) per manufacturer's instructions. The RNA concentration and purity were measured by reading the absorbance at 260 nm and 280 nm on a SmartSpec 3000 Spectrophotometer (BioRad, Hercules, CA). The quality of the RNA was further assessed using the Agilent Bioanalyser (Agilent, Santa Clara, CA).

2.3. Microarray Analysis

The microarrays used were OMM25K arrays developed by the Yale University WM Keck Foundation Biotechnology Resource Laboratory. The arrays were fabricated from a 70mer oligo set consisting of 16,463 oligos from the Operon Mouse Version 2.0 set and 8,097 oligos from the Operon Mouse Version 3.0 set, both designed from the publicly available Ensembl Mouse 14.30 database (http://www.ensembl.org/index.html) and the Mouse Genome Sequencing Project (http://www.hgsc.bcm.tmc.edu/projects/mouse). Isolated RNA was labeled by either Cy5 (RNA from vehicle-treated mice, n=3) or Cy3 (RNA from E_2-treated mice, n=3) dyes and hybridized onto the arrays using the Genisphere Array900 Expression

Array Detection kit (Genisphere, Hatfield, PA) according to the manufacturer's protocol. Hybridizations were performed on an Advalytix SlideBooster hybridization station (Olympus America Inc, Concord, MA), and slides were subsequently scanned on an Axon GenPix 4100 scanner and imaged using GenePix 5.0 software (Axon, Sunnyvale, CA). The data were then analyzed according to the Genespring program (Agilent), in collaboration with the Biostatistics Department of the Yale University WM Keck Foundation Biotechnology Resource Laboratory. The intensity values for each spot were averaged across the three arrays to give an average intensity of the spot for each group, and these values were normalized to background intensity. Only those spots whose intensity was twice the background value were considered in the analysis. The data were first subjected to multiple t-tests, and then to a false discovery rate (FDR) correction. Genes were considered to have an altered expression with E_2 treatment if they showed a greater than 2-fold change in expression level, and had an FDR-adjusted p-value of less than 0.05.

2.4. Quantitative real-time PCR

RNA was isolated from dorsal hippocampi according to the protocol described above. Total RNA (1.5 μg) was reverse transcribed in the presence of random hexamers using the SuperScript First-Strand Synthesis kit (Invitrogen) to synthesize cDNA. One ng of cDNA from each sample (vehicle-treated, n = 3; E_2-treated, n = 3) was used in analysis. Quantitative real-time PCR (QPCR) was performed using the QuantiTect SYBR Green PCR Kit, with the QuantiTect Primer Assay PCR primers (Qiagen). Quantitation of PCR products was performed using the relative standard curve method. The cDNA standards were prepared from the liver of a 4 month-old mouse (for all genes except Snap25) or mouse brain reference RNA (Applied Biosystems, Foster City, CA, for Snap25) in the concentrations of 100, 50, 25, 10, and 1 ng/ μL. Standards were loaded onto the reaction plate in each experimental run with each primer set. Standards were run in duplicate and samples in triplicate, with the housekeeping gene GAPDH run on each plate for internal normalization. The PCR reactions were performed on an ABI 7900 Sequence Detection System (Applied Biosystems) for 40 cycles (15 s at 95°C, 30 s at the annealing temperature, 30 s at 70°C), followed by a dissociation step at 60°C to visualize the melt curve and determine the purity of the product. The threshold cycle (Ct) value was calculated as the cycle number in which the SYBR green fluorescent signal crossed detection threshold limit set by the instrument, which is the midpoint of the log phase of the amplification reaction. A standard curve for each primer set was generated by plotting the concentration versus the Ct value for the reference samples. The concentrations of unknown samples were determined by substituting the Ct values for each sample into the best fit line where $y = mx + b$, and solving for the concentration x. The concentrations of each sample were reported as concentration equivalents of reference sample cDNA. Using SPSS 14.0 (SPSS Inc., Chicago, IL), separate independent samples *t*-tests were run for each gene comparing vehicle and E_2 groups.

Western blot analysis

Dorsal hippocampi were homogenized and Western blot analysis performed as previously described (Fernandez et al., 2008; Lewis et al., 2008). Briefly, six μg of protein from each homogenate (vehicle-treated, n = 3; E_2-treated, n = 3) were run on a 10% SDS-PAGE (BioRad, Hercules, CA), transferred to a PVDF membrane (Millipore, Temecula, CA), blocked in a 5% non-fat dry milk dissolved in Tris-buffered saline containing 0.1% Tween-20 (TTBS), and probed with Hsp70 (1:2000, #sc-33575, Santa Cruz, Santa Cruz, CA), IGFBP2 (1:1000, #06-107, Upstate, Lake Placid, NY), Actn 4 (1:2000, #05-384, Millipore), Tubb (1:4000, #3146, Cell Signaling, Danvers, MA), or SNAP-25 (1:1000, #610366, BD Biosciences, San Jose, CA) diluted in 5% bovine serum albumin (BSA) in TTBS. Secondary antibodies were either anti-rabbit horse radish peroxidase (HRP)-conjugated (1:2000, #7074, Cell Signaling, used for Hsp70, IGFBP2, and Tubb2a) or anti-mouse-HRP (1:2000, #7076, Cell Signaling,

used for Actn 4 and SNAP-25). Blots were stripped and then re-probed with GAPDH (1:2500, #ab9484, Abcam, Cambridge, MA) for normalization. Band intensity was measured by densitometry using Kodak 1D 3.6 software on the Kodak Image Station 440 CF. Data were analyzed as described above.

3. Results

In order to identify the effects of E_2 on gene expression in the hippocampus of young adult mice, the dorsal hippocampi of 4 month-old mice were subjected to microarray analyses using DNA oligo microarrays containing oligos from 25,000 genes. Overall, 73 genes were up-regulated and 53 genes were down-regulated by E_2 treatment ($p < 0.05$ by FDR multiple t-test correction). Of the genes up-regulated by E_2, 17 are known to be specifically involved in learning and memory. These genes were divided into functional categories by a PubMed search to elucidate their known activities *in vivo*. Two of the 17 genes encode for proteins that act as transcription factors, 3 encode proteins involved in metabolism, 6 encode structural proteins, 6 encode neuropeptides/growth factors/receptors, and 1 encodes a molecular chaperone (these 17 genes shown in bold and italics, Table 1). Functional categories for other up-regulated genes are also reported in Table 1. Of the genes down-regulated by E_2, 6 are known to be specifically involved in learning and memory. Of the 6 genes known to be involved in learning and memory, 4 encode transcription factors, 1 encodes a transport protein, and 1 encodes an anti-apoptotic protein (these genes shown in bold and italics, Table 2). Functional categories for other down-regulated genes are also reported in Table 2.

Of the genes whose expression was altered by E_2, 5 were selected for further analysis by quantitative real-time PCR (QPCR), followed by Western blot analysis based on reports in the literature strongly linking them with learning and memory. The first chosen was heat shock protein 70 (Hsp70-1), as it is not only associated with increased memory retention in the Morris water maze (Pizarro et al., 2003), but is also known to be up-regulated by E_2 (Olazabal et al., 1992). Hsp70-1 mRNA levels increased 3-fold by QPCR ($t(4) = 3.731$, $p = 0.02$; Figure 2A), matching the 2.1-fold increase seen on the microarray (Table 1). Additionally, when protein levels of Hsp70-1 were measured by Western blot analysis, there was a 1.4-fold increase at 4 hours after treatment with E_2 ($t(5) = 4.418$, $p = 0.007$; Figure 2A).

The next gene chosen was the transcription factor-associated gene insulin-like growth factor binding protein 2 (Igfbp2). IGFBP2 is a serum protein secreted by a variety of cells, and is a part of the insulin-like growth factor (IGF-I) signaling cascade. IGF-I is known to be involved in memory, as long-term IGF-I treatment in aged rats improves learning and memory (Sonntag et al., 2005), and is thought to exert its memory-enhancing affects by promoting adult neurogenesis (Aberg et al., 2000; Perez-Martin et al., 2003) or neuronal survival (Subramaniam et al., 2005). IGFBP2 binds IGF-I in the serum and prevents it from binding its receptor and activating the cellular signaling cascade (Chesik et al., 2007); additionally, the serum levels of IGFBP2 are down- regulated in humans after conjugated equine estrogen treatment (Heald et al., 2005). The mRNA levels of Igfbp2 were down-regulated 2.4-fold on the microarrays (Table 2), a finding which was verified by QPCR, with a significant decrease of 1.5-fold relative to vehicle ($t(4) = 3.90$, $p = 0.18$; Figure 2B). Additionally, protein levels of IGFBP2 showed a 2-fold decrease 4 hours after E_2 treatment ($t(2.145) = 5.402$, $p = 0.028$; Figure 2B), further confirming the mRNA data and indicating that IGFBP2 levels were decreased by E_2 treatment.

The last 3 genes analyzed by QPCR were all structural proteins: α-actinin 4 (Actn 4), tubulin β2 (Tubb), and synaptosomal-associated protein 25 (Snap-25). Because estradiol is known to increase dendritic spine density (Woolley and McEwen, 1993) and enhance neurogenesis (Tanapat et al., 1999; Galea et al., 2006), these genes were predicted to be up-regulated by E_2 treatment. Actn 4, an actin binding protein that regulates hippocampus spine morphology

and density (Nakagawa et al., 2004), showed a 2.6-fold increase in mRNA levels upon microarray analysis (Table 1), a smaller but significant 0.5-fold increase relative to vehicle with QPCR ($t(4) = 3.562$, $p = 0.024$; Figure 2C), and 1.3-fold increase in protein levels 3 hours after treatment ($t(5) = 3.603$, $p = 0.015$; Figure 2C). Additionally, Tubb, an isoform of the microtubule-forming protein tubulin that is used as a marker for neuronal differentiation and whose expression increases with isoflavone treatment (Bu and Lephart, 2005), showed a 2.7-fold increase in mRNA levels on the microarrays (Table 1), a significant 2-fold increase relative to vehicle upon QPCR analysis ($t(4) = 3.069$, $p = 0.037$; Figure 2C), and a 1.7–fold increase in protein levels 4 hours after E_2 treatment ($t(5) = 2.568$, $p = 0.049$; Figure 2C). Lastly, SNAP-25, a protein that localizes to synapses and may be required for long-term memory formation (Hou et al., 2006), showed a 2.8-fold increase in expression on the microarrays (Table 1), a significant 1.5-fold increase relative to vehicle upon QPCR analysis ($t(4) = 4.623$, $p = 0.01$; Figure 2C), and a 1.9–fold increase in protein levels 4 hours after E_2 treatment ($t(5) = 2.887$, $p = 0.034$; Figure 2C).

4. Discussion

E_2 administration led to increased RNA levels of 73 genes and decreased RNA levels of 53 genes. Of these, 17 of the up-regulated and 6 of the down-regulated genes have been previously associated with learning and memory as described above. Five of these genes were chosen for verification of RNA expression level changes by QPCR based on the availability of information in the literature relating to their involvement in learning and memory. The changes in mRNA and protein expression levels of all 5 of the genes were confirmed by QPCR and Western blot analysis: Hsp70-1, Actn 4, Tubb, and Snap-25 were up-regulated with E_2 treatment, and Igfbp2 was down-regulated with E_2 treatment. The increase in Hsp70 mRNA and protein levels was consistent with previous work, as estradiol has been shown to enhance Hsp70 expression (Olazabal et al., 1992). The decrease in IGFBP2 mRNA and protein levels may suggest a role for the IGF-I signaling pathway in mediating the mnemonic response to E_2, whereas the increases in mRNA and protein levels of Actn 4, Tubb, and Snap-25 suggest that E_2-induced alterations in the expression of these structural genes may play a role in the beneficial effects of E_2 on memory consolidation in the dorsal hippocampus. Interestingly, of the 5 genes examined, only one, Hsp70, contains an estrogen response element in its promoter (Hamilton et al., 2004), which suggests that most of the gene expression changes observed were likely mediated primarily by mechanisms other than the classical estrogen receptor binding. Although the present analyses cannot directly link E_2-induced alterations in gene and protein expression to those in learning and memory, the fact that a behaviorally effective dose of E_2 was used in this study lends strength to the correlative evidence linking E_2-induced changes in the hippocampus to memory. More study will be needed to determine the extent to which the molecular alterations observed in the present study are necessary for E_2 to modulate hippocampal memory function.

Hsp70-1 belongs to the extensive heat shock protein family, and is responsible for assisting in protein folding (Burston and Clarke, 1995). Hsp70 is also an integral part of the cytosolic estrogen receptor (ER) protein complex that keeps ER in an inactive state until it binds its estrogens and translocates to the nucleus (Whitesell and Lindquist, 2005). Hsp70 expression has been well-documented to increase with E_2 treatment in a wide variety of cell types, such as breast cancer cells (Takahashi et al., 1994), heart (Hamilton et al., 2004), and brain, where it may show differential expression based on sex (Olazabal et al., 1992). Hsp70 isoforms have also been implicated in learning. Hsp70-1 mRNA and protein levels are increased in the hippocampus of rats trained in the spatial Morris water maze (Pizarro et al., 2003), and the inducible form of Hsp70, Hsp72, is increased in the cerebellum during acquisition of a two-way avoidance task in rats (Ambrosini et al., 1999). Further, when Hsp72 is knocked out in mice, this deletion prevents spatial memory acquisition in the radial arm maze (Ambrosini et

al., 2005), suggesting an active and potentially critical role for Hsp70 isoforms in learning and memory beyond their traditional function of simply complexing with ER in the cytoplasm and maintaining it in the inactive state.

Insulin-like growth factor binding protein 2 (Igfbp2) is the predominant IGF-I binding protein secreted by neuronal and glial cells in the brain (Bezchlibnyk et al., 2006). Igfbp2 binds IGF-I in the serum and prevents it from activating the IGF-I receptor (IGF-IR) and initiating intracellular signaling cascades such as PI3K and ERK (Aberg et al., 2006; Chesik et al., 2007). Thus, an E_2-induced down-regulation of Igfbp2 may lead to greater IGF-I availability, and subsequently to increased activation of the PI3K and ERK cascades. Links between the IGF-I signaling cascade and E_2 are well-established. For example, treatment with E_2 can activate IGF-IR signaling, and IGF-I has been shown to regulate the transcriptional activation of the classical estrogen receptors (ER) alpha and beta (Garcia-Segura et al., 2006). Administration of the ER antagonist ICI 182,780 blocks IGF-I-induced neurogenesis in female rats (Perez-Martin et al., 2003), suggesting that interactions with classical ERs are important in mediating the effects of IGF-I on hippocampal plasticity. Previous microarray analyses have shown a 1.7-fold increase in mRNA levels of another IGF-I binding protein, Igfbp6, in the hippocampus of male intact middle-aged rats relative to young rats (Blalock et al., 2003), and a decrease in the expression of Igfbp2 mRNA in the dentate gyrus of rats that have undergone passive avoidance training relative to untrained controls (O'Sullivan et al., 2007). An age-related increase in Igfbp6 mRNA could suggest that less IGF-I is available for binding to its receptor, which would block downstream signaling cascades. In contrast, the decrease in Igfbp2 mRNA observed after passive avoidance and in the present study could suggest more IGF-I available for binding, and may indicate an activity- or E_2-induced increase in the activity of the downstream signaling cascades associated with IGF-I, as we have previously observed with ERK after 0.2 mg/kg E_2 treatment (Fernandez et al., 2008).

Cytoskeletal structural genes are involved in several aspects of cellular function in the brain, including transport of proteins to various cellular locations (Antar et al., 2005), endocytosis and exocytosis of neurotransmitters (Chin et al., 2000), cell division during neurogenesis (German and Eisch, 2004), neuronal plasticity (Bianchi et al., 2005), and dendritic spine morphogenesis (Sekino et al., 2007). Several microarray studies have found alterations in the expression patterns of structural genes with learning. For example, synaptojanin II, a protein required for synaptic vesicle recycling, is up-regulated in aged rats who were classified as superior learners in the spatial Morris water maze task as compared to age-matched control learners (Burger et al., 2007). In addition, the mRNA levels of procollagen-type I, collagen type III and coronin, an actin binding protein, were down-regulated with age in the rat hippocampus, although vimentin and α-tubulin were both up-regulated with age (Blalock et al., 2003). Fewer structural genes appear to be influenced by E_2 alone in array studies. The vesicle-associated membrane protein 2 and synaptogyrin were up-regulated in the basal hypothalamus of female guinea pigs following E_2 treatment (Malyala et al., 2004). In non-array based studies, however, α-actinin 4 (Actn 4), a structural protein that binds to the actin cytoskeleton and is involved in protein trafficking within the cell, was reportedly up-regulated by the synthetic estrogen clomiphene citrate in rat uteri (Hosie et al., 2008) and was up-regulated by E_2 treatment in the present study as well. Actn 4 mRNA levels are also increased in the amygdala during fear conditioning (Ressler et al., 2002), suggesting a role for this structural protein both in response to E_2 and in learning and memory.

Tubulin-β (Tubb) is the major structural component of the microtubule network inside of cells. Column retention studies using hippocampal cell lysate have shown that tubulin-β binds directly to E_2 (Ramirez et al., 2001). Additionally, yeast two-hybrid screens using MCF-7 cells indicate that microtubules may mediate the response of E_2 by binding to and helping to shuttle the ERs to various locations within the cell (Manavathi et al., 2006). On the other hand, studies

in breast cancer cells show that E_2 actually disrupts microtubule formation (Aizu-Yokota et al., 1994), casting some doubt on whether there is a positive interaction between Tubb and E_2. Tubulin-β is often used as a marker for neurogenesis (Rai et al., 2007), indicating that the increase in Tubb mRNA levels seen in this study may be due to cells preparing for future cell division and neurogenesis. Given that E_2 has been shown to enhance neurogenesis in the hippocampal dentate gyrus (Tanapat et al., 1999; Galea et al., 2006), the increase in Tubb may not reflect a direct interaction between E_2 and tubulin-β, but rather be an indirect result of E_2-induced neurogenesis.

The final gene examined that was up-regulated by the microarray and QPCR analysis, as well as the Western blot analysis, was the synaptosomal-associated protein of 25 kD (Snap-25). SNAP-25 is a part of the synaptic vesicle docking and fusion process and is essential for neurotransmitter release (Wang and Tang, 2006). Interestingly, previous *in situ* hybridization data indicated that E_2 treatment of ovariectomized rats decreased Snap-25 mRNA levels in the pituitary (Jacobsson et al., 1998), and another microarray study indicated that Snap-25 mRNA levels decrease after passive avoidance learning in the dentate gyrus of male rats (O'Sullivan et al., 2007). Although both of these findings conflict with the present data, other studies have shown that SNAP-25 is required for memory formation. For example, granule cells of the dentate gyrus show an increase in Snap-25 mRNA expression 2 hours after LTP stimulation (Roberts et al., 1998), and a Snap-25 antisense oligonucleotide added to either rat cortical neurons or PC12 cells impairs axon growth (Osen-Sand et al., 1993). Male intact rats infused with a SNAP-25 antisense oligonucleotide directly into either CA1 (Hou et al., 2004) or CA3 (Hou et al., 2006) exhibited impaired consolidation of contextual fear memory and spatial memory, suggesting that SNAP-25 is involved in memory consolidation in the hippocampus. As such, the increase in Snap-25 mRNA levels observed in our study indicate that treatment with a dose of E_2 that enhances hippocampal memory consolidation (Gresack and Frick, 2006b) has a beneficial effect on the expression of this important gene, perhaps by influencing an increase in neurotransmitter release.

In conclusion, this study demonstrates that a single i.p. injection of a dose of E_2 that enhances spatial and object memory consolidation (Gresack and Frick, 2006b) can influence the mRNA and protein expression levels of genes involved in learning and memory within 60 minutes of injection. We have found that E_2 can enhance the expression of genes that act as chaperone proteins, such as Hsp70; transcription factor-related genes such as Igfbp2; genes involved in intracellular trafficking pathways, such as α-actinin 4 and tubulin-β; and synaptosome-associated proteins, such as Snap-25. These expression changes demonstrate that E_2 may exert its effects on a wide variety of intracellular mechanisms and render cells within the hippocampus more amenable to the physical changes necessary to consolidate memories. More work is needed to understand if these gene expression changes specifically affect memory consolidation within the hippocampus. Nevertheless, the present data provide information that will be critical to understanding how E_2 and similar hormones modulate memory.

Acknowledgements

References

Aberg MA, Aberg ND, Hedbacker H, Oscarsson J, Eriksson PS. Peripheral infusion of IGF-I selectively induces neurogenesis in the adult rat hippocampus. Journal of Neuroscience 2000;20:2896–2903. [PubMed: 10751442]

Aberg ND, Brywe KG, Isgaard J. Aspects of growth hormone and insulin-like growth factor-I related to neuroprotection, regeneration, and functional plasticity in the adult brain. ScientificWorldJournal 2006;18:53–80. [PubMed: 16432628]

Aenlle KK, Kumar A, Cui L, Jackson TC, Foster TC. Estrogen effects on cognition and hippocampal transcription in middle-aged mice. Neurobiology of Aging. 200710.1016/j.neurobiolaging. 2007.09.004Epub ahead of print

Aizu-Yokota E, Ichinoseki K, Sato Y. Microtubule disruption induced by estradiol in estrogen receptor-positive and -negative human breast cancer cell lines. Carcinogenesis 1994;15:1875–1879. [PubMed: 7923580]

Akinci MK, Johnston GA. Sex differences in the effects of gonadectomy and acute swim stress on GABAA receptor binding in mouse forebrain membranes. Neurochemistry International 1997;31:1–10. [PubMed: 9185158]

Ambrosini MV, Mariucci G, Tantucci M, Brushchelli G, Giuditta A. Induction of cerebellar hsp72 in rats learning a two-way active avoidance task. Molecular Brain Research 1999;70:164–166. [PubMed: 10381556]

Ambrosini MV, Mariucci G, Tantucci M, Van Hooijdonk L, Ammassari-Teule M. Hippocampal 72-kD heat shock protein expression varies according to mice learning performance independently from chronic exposure to stress. Hippocampus 2005;15:413–417. [PubMed: 15719414]

Antar LN, Dictenburg JB, Plociniak M, Afroz R, Bassell GJ. Localization of FMRP-associated mRNA granules and requirement of microtubules for activity-dependent trafficking in hippocampal neurons. Genes, Brain and Behavior 2005;4:350–359.

Bezchlibnyk YB, Wang J-F, Shao L, Young LT. Insulin-like growth factor binding protein-2 expression is decreased by lithium. Molecular Neuroscience 2006;17:897–901.

Bianchi M, Hagan JJ, Heidbreder CA. Neuronal plasticity, stress and depression: involvement of the cytoskeletal microtubular system? Current Drug Targets CNS Neurologic Disorders 2005;4:597–611.

Bimonte HA, Denenberg VH. Estradiol facilitates performance as working memory load increases. Psychoneuroendocrinology 1999;24:161–173. [PubMed: 10101725]

Blalock EM, Chen K-C, Sharrow K, Herman JP, Porter NM, Foster TC, Landfield PW. Gene microarrays in hippocampal aging: Statistical profiling identifies novel processes correlated with cognitive impairment. Journal of Neuroscience 2003;23:3807–3819. [PubMed: 12736351]

Bozon B, Kelly A, Josselyn SA, Silva AJ, Davis S, Laroche S. MAPK, CREB, and zif268 are all required for the consolidation of recognition memory. Phil Trans R Soc Lond 2003;358:805–814. [PubMed: 12740127]

Bu L, Lephart ED. Soy isoflavones modulate the expression of BAD and neuron-specific beta III tubulin in male rat brain. Neuroscience Letters 2005;385:153–157. [PubMed: 15951108]

Burger C, Lopez MC, Feller JA, Baker HV, Muzyczka N, Mandel RJ. Changes in transcription within the CA1 field of the hippocampus are associated with age-related spatial learning impairments. Neurobiology of Learning and Memory 2007;87:21–41. [PubMed: 16829144]

Burston SG, Clarke AR. Molecular chaperones: physical and mechanistic properties. Essays Biochemistry 1995;29:125–136.

Cardona-Gomez GP, Mendez P, Garcia-Segura LM. Synergistic interaction of estradiol and insulin-like growth factor-I in the activation of PI3K/Akt signaling in the adult rat hypothalamus. Molecular Brain Research 2002;107:80–88. [PubMed: 12414126]

Chesik D, De Keyser J, Wilczak N. Insulin-like growth factor binding protein-2 as a regulator of IGF actions in CNS: Implications in multiple sclerosis. Cytokine and Growth Factor Reviews 2007;18:267–278. [PubMed: 17485236]

Chin L-S, Nugent RD, Raynor MC, Vavalle JP, Li L. SNIP, a novel SNAP-25-interacting protein implicated in regulated exocytosis. Journal of Biological Chemistry 2000;275:1191–1200. [PubMed: 10625663]

Daniel JM, Fader AJ, Spencer AL, Dohanich GP. Estrogen enhances performance of female rats during acquisition of a radial arm maze. Hormones and Behavior 1997;32:217–225. [PubMed: 9454673]

Duff SJ, Hampson E. A beneficial effect of estrogen on working memory in postmenopausal women taking hormone replacement therapy. Hormones and Behavior 2000;38:262–276. [PubMed: 11104644]

Duka T, Tasker R, McGowan JF. The effect of 3-week estrogen hormone replacement on cognition in elderly healthy females. Psychopharmacology (Berl) 2000;149:129–139. [PubMed: 10805607]

Fernandez SM, Frick KM. Chronic oral estrogen affects memory and neurochemistry in middle-aged female mice. Behavioral Neuroscience 2004;118:1340–1351. [PubMed: 15598143]

Fernandez SM, Lewis MC, Pechenino AS, Orr PT, Gresack JE, Harburger LL, Schafe GE, Frick KM. Estradiol-induced enhancement of object memory consolidation involves hippocampal ERK and membrane-bound estrogen receptors. Journal of Neuroscience. 2008In press

Fitzpatrick JL, Mize AL, Wade CB, Harris JA, Shapiro RA, Dorsa DM. Estrogen-mediated neuroprotection against beta-amyloid toxicity requires expression of estrogen receptor alpha or beta and activation of the MAPK pathway. Journal of Neurochemistry 2002;82:674–682. [PubMed: 12153491]

Frick KM, Fernandez SM, Bennett JC, Prange-Kiel J, MacLusky NJ, Leranth C. Behavioral training interferes with the ability of gonadal hormones to increase CA1 spine density in ovariectomized female rats. Behavior and Brain Research 2004;19:3026–3032.

Galea LAM, Spritzer MD, Barker JM, Pawluski JL. Gonadal hormone modulation of hippocampal neurogenesis in the adult. Hippocampus 2006;16:225–232. [PubMed: 16411182]

Garcia-Segura LM, Sanz A, Mendez P. Cross-talk between IGF-1 and estradiol in the brain: focus on neuroprotection. Neurosteroids and Neuroprotection 2006;84:275–279.

German DC, Eisch AJ. Mouse models of Alzheimer's disease: insight into treatment. Reviews in Neuroscience 2004;15:353–369.

Gresack JE, Frick KM. Effects of continuous and intermittent estrogen treatments on memory in aging female mice. Brain Research 2006a;1115:135–147. [PubMed: 16920082]

Gresack JE, Frick KM. Post-training estrogen enhances spatial and object memory consolidation in female mice. Pharmacology, Biochemistry and Behavior 2006b;84:112–119.

Hamilton KL, Gupta L, Knowlton A. Estrogen and regulation of heat shock protein expression in female cardiomyocytes: cross-talk with NFkappaB signaling. Journal of Molecular and Cellular Cardiology 2004;36:577–584. [PubMed: 15081317]

Heald A, Kaushal K, Anderson S, Redpath M, Durrington PN, Selby PL, Gibson MJ. Effects of hormone replacement therapy on insulin-like growth factor (IGF)-1, IGF-II and IGF binding protein (IGFBP)-1 to IGFBP-4: Implications for cardiovascular risk. Gynecological Endocrinology 2005;20:176–182. [PubMed: 16019358]

Heikkinen T, Puolivali J, Liu L, Rissanen A, Tanila H. Effects of ovariectomy and estrogen treatment on learning and hippocampal neurotransmitters in mice. Hormones and Behavior 2002;41:22–32. [PubMed: 11863380]

Hosie M, Adamson M, Penny C. Actin binding protein expression is altered in uterine luminal epithelium by clomiphene citrate, a synthetic estrogen receptor modulator. Theriogenology 2008;69:700–713. [PubMed: 18258291]

Hou Q-L, Gao X, Zhang X-H, Kong LW, Wang X, Bian W, Tu YY, Jin ML, Zhao G-P, Li B-M, Jing N-H, Yu L. SNAP-25 in hippocampal CA1 region is involved in memory consolidation. European Journal of Neuroscience 2004;20:1593–1603. [PubMed: 15355326]

Hou Q-L, Gao X, Lu Q, Zhang X-H, Tu Y-Y, Jin M-L, Zhao G-P, Yu L, Jing N-H, Li B-M. SNAP-25 in hippocampal CA3 region is required for long-term memory formation. Biochemical and Biophysical Research Communications 2006;347:955–962. [PubMed: 16870134]

Jacobsson G, Razani H, Ogren SO, Meister B. Estrogen down-regulates mRNA encoding the exocytotic protein SNAP-25 in the rat pituitary gland. Journal of Neuroendocrinology 1998;10:157–163. [PubMed: 9576603]

Kampen DL, Sherwin BB. Estrogen and verbal memory in healthy postmenopausal women. Obstetrics and Gynocology 1994;83:979–983.

Kuroki Y, Fukushima K, Kanda Y, Mizuno K, Watanabe Y. Putative membrane-bound estrogen receptors possibly stimulate mitogen-activated protein kinase in the rat hippocampus. European Journal of Pharmacology 2000;400:205–209. [PubMed: 10988335]

Lewis MC, Kerr KM, Orr PT, Frick KM. Estradiol-induced enhancement of object memory consolidation involves NMDA receptors and protein kinase A in the dorsal hippocampus of female C57BL/6 mice. Behavioral Neuroscience 2008;122:716–721. [PubMed: 18513142]

Luine VN, Richards ST, Wu VY, Beck KD. Estradiol enhances learning and memory in a spatial memory task and effects levels of monoaminergic neurotransmitters. Hormones and Behavior 1998;34:149–162. [PubMed: 9799625]

Luine VN, Jacome LF, MacClusky NJ. Rapid enhancement of visual and place memory by estrogens in rats. Endocrinology 2003;144:2836–44. [PubMed: 12810538]

Malyala A, Pattee P, Nagalla SR, Kelly MJ, Ronnekleiv OK. Suppression subtractive hybridization and microarray identification of estrogen-regulated hypothalamic genes. Neurochemical Research 2004;29:1189–1200. [PubMed: 15176476]

Manavathi B, Acconcia F, Rayala SK, Kumar R. An inherent role of microtubule network in the action of nuclear receptor. Proceedings of the National Academy of Sciences of the United States of America 2006;103:15981–15986. [PubMed: 17043237]

Mannella P, Brinton RK. Estrogen receptor protein interaction with phosphatidylinositol 3-kinase leads to activation of phosphorylated Akt and extracellular signal-regulated kinase 1/2 in the same population of cortical neurons: a unified mechanism of estrogen action. Journal of Neuroscience 2006;26:9439–9447. [PubMed: 16971528]

Mastorakos G, Sakkas EGR, Xydakis AM, Creatsas G. Pitfalls of the WHIs Women's Health Initiative. Annals of the New York Acadamy of Sciences 2006;1092:331–340.

Nakagawa T, Engler JA, Sheng M. The dynamic turnover and functional roles of alpha-actinin in dendritic spines. Neuropharmacology 2004;47:734–745. [PubMed: 15458845]

O'Sullivan NC, McGettigan PA, Sheridan GK, Pickering M, Conboy L, O'Connor JJ, Moynagh PN, Higgins DG, Regan CM, Murphy KJ. Temporal changes in gene expression in the rat dentate gyrus following passive avoidance learning. Journal of Neurochemistry 2007;101:1085–1098. [PubMed: 17298388]

Olazabal UE, Pfaff DW, Mobbs CV. Sex differences in the regulation of heat shock protein 70 kDa and 90 kDa in the rat ventromedial hypothalamus by estrogen. Brain Research 1992;596:311–314. [PubMed: 1467994]

Osen-Sand A, Catsicas M, Staple JK, Jones KA, Ayala G, Knowles J, Grenningloh G, Stephan C. Inhibition of axonal growth by SNAP-25 antisense oligonucleotides in vitro and in vivo. Nature 1993;364:445–448. [PubMed: 8332215]

Perez-Martin M, Azcoitia I, Trejo JL, Sierra A, Garcia-Segura LM. An antagonist of estrogen receptors blocks the induction of adult neurogenesis by insulin-like growth factor-1 in the dentate gyrus of adult female rat. European Journal of Neuroscience 2003;18:923–930. [PubMed: 12925018]

Pitha J, Pitha J. Amorphous water soluble derivatives of cyclodextrins: Nontoxic dissolution enhancing excipients. Journal of Pharmaceutical Sciences 1985;74:987–990. [PubMed: 4067854]

Pitha J, Harman SM, Michel ME. Hydrophilic cyclodextrin derivatives enable effective oral administration of steroidal hormones. Journal of Pharmaceutical Sciences 1986;75:165–167. [PubMed: 3958926]

Pizarro JM, Haro LS, Barea-Rodriguez EJ. Learning associated increase in heat shock cognate 70 mRNA and protein expression. Neurobiology of Learning and Memory 2003;79:142–151. [PubMed: 12591223]

Rai KS, Hattiangady B, Shetty AK. Enhanced production and dendritic growth of new dentate granule cells in the middle-aged hippocampus following intracerebroventricular FGF-2 infusions. European Journal of Neuroscience 2007;26:1765–1779. [PubMed: 17883411]

Ramirez VD, Kipp JL, Joe I. Estradiol, in the CNS, targets several physiologically relevant membrane-associated proteins. Brain Research Reviews 2001;37:141–152. [PubMed: 11744082]

Ressler KJ, Paschall G, Zhou X-l, Davis M. Regulation of synaptic plasticity genes during consolidation of fear conditioning. Journal of Neuroscience 2002;22:7892–7902. [PubMed: 12223542]

Rissanen A, Puolivali J, van Groen T, Riekkinen PJ. In mice tonic estrogen replacement therapy improves non-spatial and spatial memory in a water maze task. NeuroReport 1999;10:1369–1372. [PubMed: 10363955]

Roberts LA, Morris BJ, O'Shaughnessy CT. Involvement of two isoforms of SNAP-25 in the expression of long-term potentiation in the rat hippocampus. NeuroReport 1998;9:33–36. [PubMed: 9592043]

Sandstrom NJ, Williams CL. Memory retention is modulated by acute estradiol and progesterone replacement. Behavioral Neuroscience 2001;115:383–393.

Sandstrom NJ, Williams CL. Spatial memory retention is enhanced by acute and continuous estradiol replacement. Hormones and Behavior 2004;45:128–135. [PubMed: 15019800]

Sekino Y, Kojima N, Shirao T. Role of actin cytoskeleton in dendritic spine morphogenesis. Neurochemistry International 2007;51:92–104. [PubMed: 17590478]

Shumaker SA, Legault C, Rapp SR, Thal L, Wallace RB, Ockene JK, Hendrix SL, Jones BN III, Assaf AR, Jackson RD, Kotchen JM, Wassertheil-Smoller S, Wactawski-Wende J. Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women. Journal of the American Medical Association 2003;289:2651–2662. [PubMed: 12771112]

Shumaker SA, Legault C, Kuller L, Rapp SR, Thal L, Lane DS, Fillit H, Stefanick ML, Hendrix SL, Lewis CE, Masaki K, Coker LH. Conjugated equine estrogens and incidence of probable dementia and mild cognitive impairment in postmenopausal women. Journal of the American Medical Association 2004;291:2947–2958. [PubMed: 15213206]

Sonntag WE, Ramsey M, Carter CS. Growth hormone and insulin-like growth factor-1 (IGF-1) and their influence on cognitive aging. Ageing Research Reviews 2005;4:195–212. [PubMed: 16024298]

Stone DJ, Rozovsky I, Morgan TE, Anderson CP, Finch CE. Increased synaptic sprouting in response to estrogen via an apolipoprotein E-dependent mechanism: implications for Alzheimer's disease. Journal of Neuroscience 1998;18:3180–3185. [PubMed: 9547226]

Subramaniam S, Shahani N, Strelau J, Laliberte C, Brandt R, Kaplan D, Unsicker K. Insulin-like growth factor 1 inhibits extracellular signal-regulated kinase to promote neuronal survival via the phosphatidylinositol 3-kinase/protein kinase A/c-Raf pathway. Journal of Neuroscience 2005;25:2838–2852. [PubMed: 15772344]

Takahashi S, Mikami T, Watanabe Y, Okazaki M, Okazaki Y, Okazaki A, Sato T, Asaishi K, Hirata K, Narimatsu E. Correlation of heat shock protein 70 expression with estrogen receptor levels in invasive breast cancer. American Journal of Clinical Pathology 1994;101:519–525. [PubMed: 7909191]

Tanapat P, Hastings NB, Reeves AJ, Gould E. Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. Journal of Neuroscience 1999;19:5792–5801. [PubMed: 10407020]

Taylor GT, Weiss J, Pitha J. Testosterone in a cyclodextrin-containing formulation: Behavioral and physiological effects of episode-like pulses in rats. Pharmaceutical Research 1989;6:641–646. [PubMed: 2798316]

Vaucher E, Reymond I, Najaffe R, Kar S, Quirion R, Miller M, Franklin K. Estrogen effects on object memory and cholinergic receptors in young and old female mice. Neurobiology of Aging 2002;23:87–95. [PubMed: 11755023]

Wade CB, Dorsa DM. Estrogen activation of cyclic adenosine 5'-monophosphate response element-mediated transcription requires the extracellularly regulated kinase/mitogen-activated protein kinase pathway. Endocrinology 2003;144:832–838. [PubMed: 12586759]

Wang Y, Tang BL. SNAREs in neurons-beyond vesicle exocytosis (review). Molecular Membrane Biology 2006;23:377–384. [PubMed: 17060155]

Whitesell L, Lindquist SL. Hsp90 and the chaperoning of cancer. Nature Reviews 2005;5:761–772.

Wolf OT, Kirschbaum C. Endogenous estradiol and testosterone levels are associated with cognitive performance in older women and men. Hormones and Behavior 2002;41:259–266. [PubMed: 11971659]

Woolley CS, McEwen BS. Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. Journal of Comparative Neurology 1993;8:293–306. [PubMed: 8245220]

Yaffe K, Lui LY, Grady D, Cauley J, Kramer J, Cummings SR. Cognitive decline in women in relation to non-protein-bound oestradiol concentrations. Lancet 2000;356:708–712. [PubMed: 11085689]

Yokomaku D, Numakawa T, Numakawa Y, Suzuki S, Matsumoto T, Adachi N, Nishio C, Taguchi T, Hatanaka H. Estrogen enhances depolarization-induced glutamate release through activation of phosphatidylinositol 3-kinase and moitogen-activated protein kinase in cultured hippocampal neurons. Molecular Endocrinology 2003;17:831–844. [PubMed: 12554763]
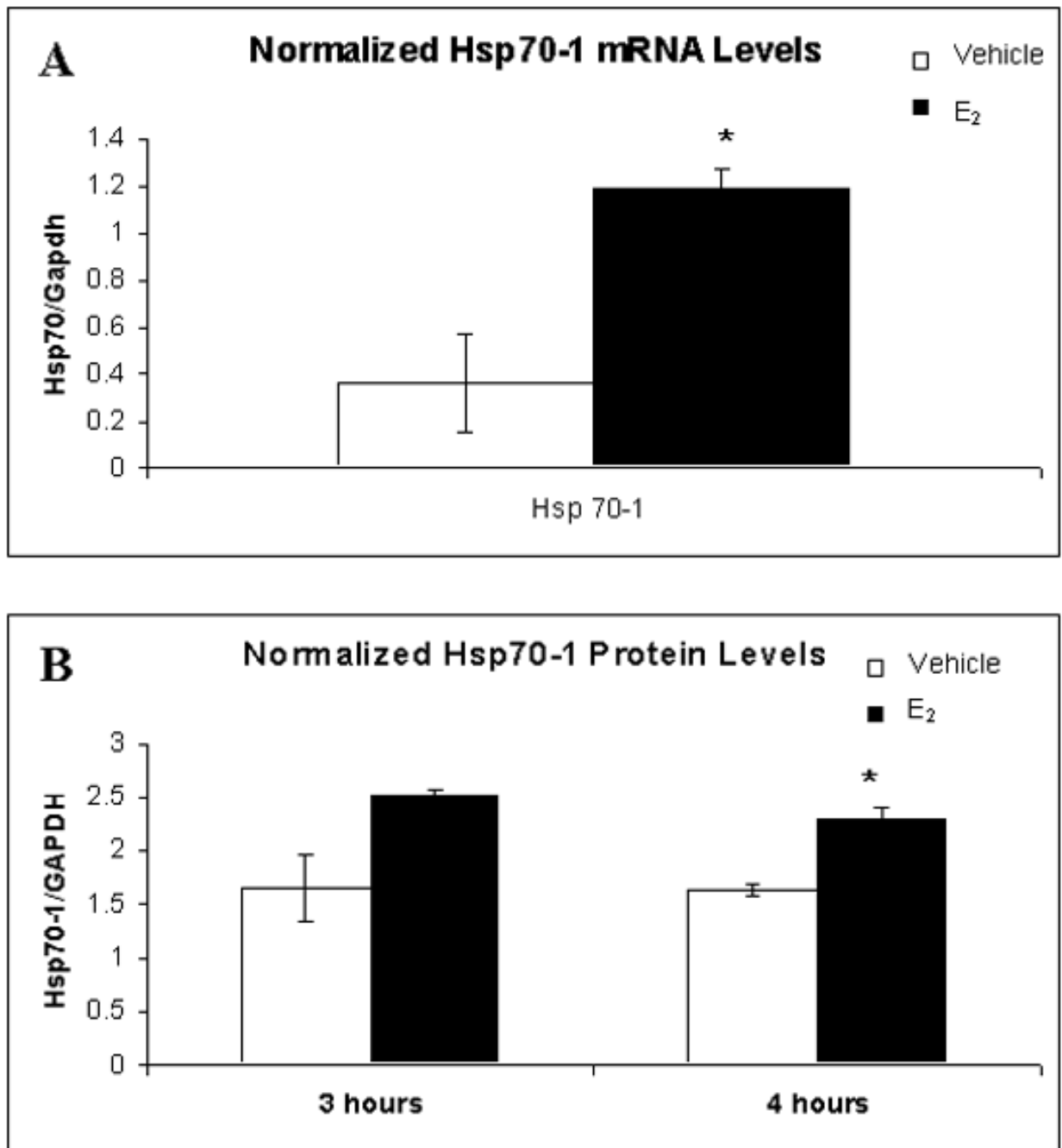
Figure 1.
Heat-shock protein 70-1 (Hsp70-1) gene and protein expression levels following E_2 treatment. A. Hsp70-1 mRNA expression levels significantly increased 1 hour after E_2 treatment. B. Hsp70-1 protein levels were also significantly higher than vehicle 4 hours after E_2 treatment. *$p < 0.05$ relative to vehicle.
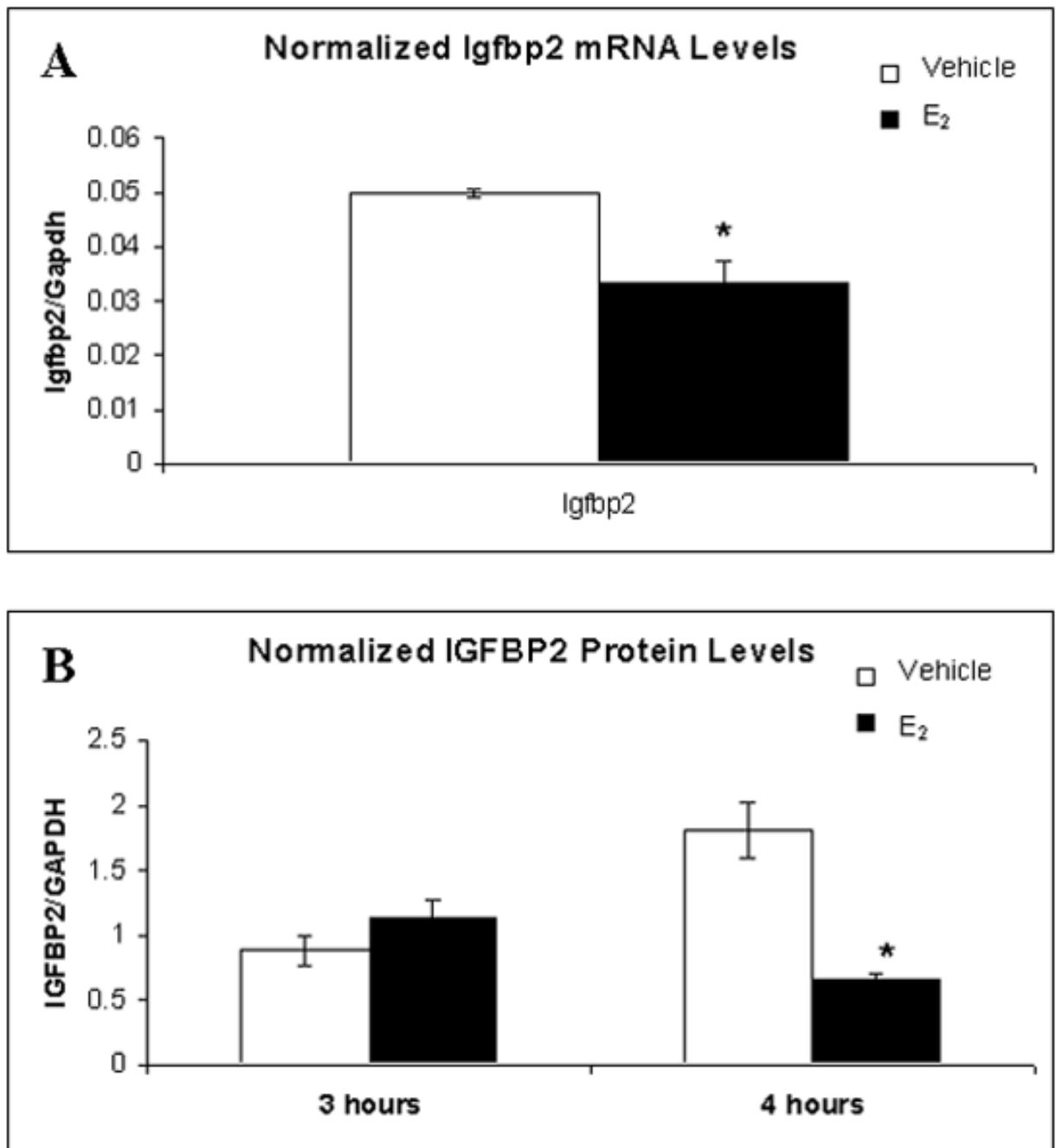
Figure 2.
IGFBP2 gene and protein expression levels following E_2 treatment. A. Igfbp2 mRNA expression levels significantly decreased 1 hour after E_2 treatment. B. IGFBP2 protein levels were also significantly lower than vehicle 4 hours after E_2 treatment. *$p < 0.05$ relative to vehicle.

Figure 3.
Structural protein gene and protein expression levels following E_2 treatment. A. Actn4, Tubb, and Snap-25 mRNA expression levels were all significantly increased 1 hour after E_2 treatment. B. Actn4 protein levels were increased 3 hours after E_2 treatment, whereas, SNAP-25 and Tubb protein levels were significantly higher than vehicle 4 hours after treatment. *$p < 0.05$ relative to vehicle.

Table 1

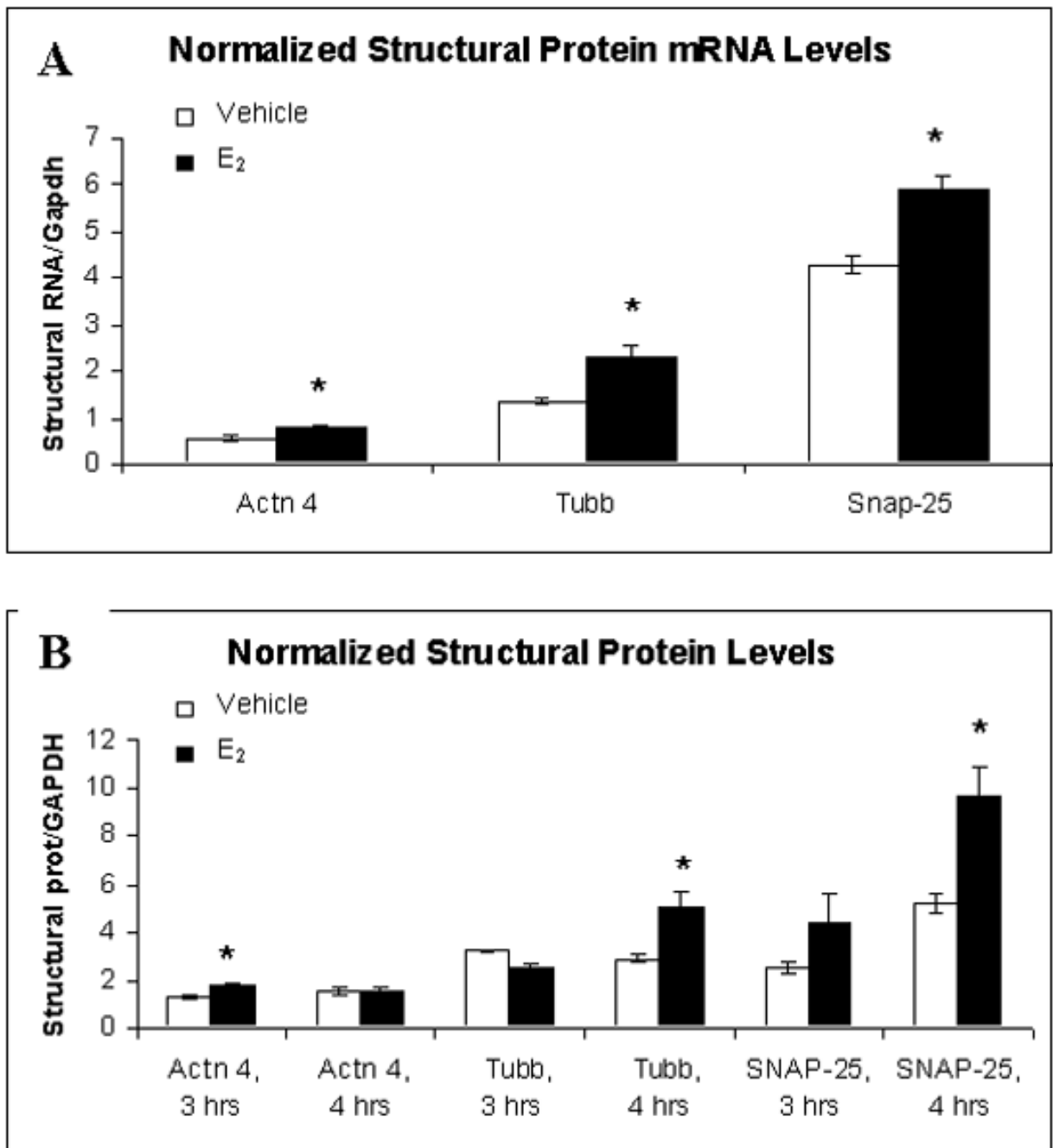**Genes whose expression was significantly up-regulated by E$_2$ treatment**

The *p*-values listed in the table represent significance reported after the false discovery rate (FDR) analysis. Genes in bold and italics indicate those that are known to be involved in learning and memory based on a PubMed search. The genes marked with an asterisk indicate those that were chosen for further study by real-time PCR and Western blotting.

		Common Name	Fold change	p-value	PubMed Number
Transcription		*Junb*	2.35	0.0465	NM_008416
		Pik3cb	2.13	0.0368	AK003230
		Tcf4	2.24	0.0368	NM_013685
		Eef1d	2.07	0.0491	NM_023240
Metabolism		*Odc*	2.42	0.0487	NM_013614
		Got2	2.35	0.0368	NM_010325
		Atp1b3	2.28	0.0463	NM_007502
		Sult4a1	2.16	0.0368	NM_013873
		ATP F1	3.38	0.0203	BC013607
		Dlat	2.03	0.0203	BC003202
		Nec1	2.01	0.0368	AK018159
		Atp6b2	2.07	0.0435	BC012497
		Pmca1b	2.63	0.0398	AK013291
		E2RG	10.42	0.0368	AK019895
		Agk	2.05	0.0368	BC019145
		Hatpaseb1	2.06	0.0464	BC017127
Structure		*Myo6*	2.13	0.0435	NM_008662
		Tubb2a*	2.67	0.042	M28739
		Actn4*	2.63	0.0368	NM_021895
		Tmsb4x	2.29	0.0368	NM_021278
		Dctn4	2.4	0.0491	BC006677
		Snap25*	2.84	0.0487	BC018249
		Myh2	9.73	0.0368	BC008538
		Esm1	12.28	0.0435	BC020038
		Arfl10C	2.66	0.0465	BC013719
		Tpk2	2.45	0.0464	BC019149
Structure		Mtrp6	2.3	0.0368	BC020019
		Cct6a	2.26	0.0203	NM_009838
		Ccol	2.28	0.0368	NM_177177
NP/GF/Rec		*Rfrp*	2.03	0.0465	NM_021892
		Hgfl	3.69	0.0471	NM_008243
		Erbb4	2.32	0.0402	AF059177
		Neurod2	2.54	0.0368	NM_010895
		Pafah1b1	2.4	0.0368	NM_013625
		Kcna1	2.04	0.0471	NM_010595
		Olfr, put	2.01	0.0368	X89678

	Common Name	Fold change	p-value	PubMed Number
	Olfr67	2.78	0.0491	NM_013619
	Gpr124	2.05	0.0368	NM_054044
	lgsf1	2.35	0.0464	AY227771
	Scube1	2.34	0.046	NM_022723
	Eva	5 09	0.0368	BC015076
	Cd209e	2.39	0.0368	AF373412
	Neph	2.36	0.0471	NM_025684
Chaperone	HSP70-1*	2.11	0.0368	M12573
Differentiation	Morf	2.04	0.0464	NM_017479
	Cd81	2.21	0.049	BC011433
	Gcm2	2.5	0.0368	NM_008104
	Ctf1	2.84	0.0368	NM_007795
	Alcam	2.07	0.0435	U95030
	Mmd	2.37	0.0368	NM_026178
	Nuf2r	8.42	0.0368	NM_023284
	Ash1l	2.11	0.0389	AF247132
Oxidative Stress	Pxf	2.56	0.0368	AK012785
Inflammation	Tcra	2.49	0.0368	X72904
	Aldh1a7	2	0.0465	NM_011921
	Egln1	2.07	0.0386	BC006903
	CS1	2.23	0.0368	AK014517
Protein deg	Rbx1	2.05	0.0435	AK005127
	Rnf5	2.1	0.0398	NM_019403
	Bean	21.34	0.0368	AF240460
	Pep4	6.54	0.0368	NM_008820
	Serp23	2.18	0.0311	BC018517
	Psmd1	2.1	0.0368	AK010596
	Psmb2	2.06	0.0498	NM_011970
Transport	SV2	2.13	0.0399	AK013742
	Rab1b	2.54	0.0368	BC016408
	MLC1	2.11	0.0466	AF449425
	Snta1	2.17	0.0465	NM_009228
	Slc14a1	2.08	0.0389	NM_028122
	Rcc1	2.36	0.0465	BC019807
	Itgb4bp	2.22	0.0368	BC015274
	Ergic1	2.33	0.0464	NM_026170
	Ssra	2.07	0.0386	NM_025965

Abbreviations: Transcription, transcription factor; NP/GF/Rec, neuropeptides, growth factors, or receptors; Protein deg, involved in protein degradation.

Table 2

Genes whose expression was significantly down-regulated by E$_2$ treatment

The p-values listed in the table represent significance reported after the false discovery rate (FDR) analysis. Genes in bold and italics indicate those that are known to be involved in learning and memory based on a PubMed search. The genes marked with an asterisk indicate those that were chosen for further study by real-time PCR and Western blotting.

	Common Name	Fold change	p-value	PubMed ID
Transcription	*Plc*	16.67	0.0466	AK011892
	lnpp5d	3.57	0.0468	NM_010566
	Pla2g1b	3.85	0.0465	NM_011107
	*lgfbp2**	2.38	0.0389	NM_008342
	AF-9	20	0.0368	AK008707
	Brd7	25	0.0465	NM_012047
	Ikbkb	10	0.0363	AF088910
	Rbm8	3.23	0.0368	NM_025875
	Smarca3	6.67	0.0368	AF165911
	Lztfl1	2.13	0.0466	NM_033322
Transport	*Konj8*	2.13	0.0389	NM_008428
Apoptosis	*Birc4*	7.14	0.0368	NM_009688
Ca2+-assoc	Calb2	2.5	0.0368	BC017646
	Cacng3	2.56	0.0491	NM_019430
	Calmbp1	5	0.0402	NM_009791
	Cacybp	11.11	0.0368	U97327
	Trpv6	2.17	0.0368	NM_022413
	Pln	3.85	0.0466	AK002622
Metabolism	Anpep	2.38	0.0368	NM_008486
	Lrp6	2.17	0.0368	NM_008514
	Atic	2.56	0.0465	AK010611
	Fabp4	11.11	0.0465	BC002148
	Cyp40	50	0.0368	AB006034
	Nit1	3.03	0.0465	AK004988
	Ethi6	20	0.0435	AK010551
	Pit1	50	0.0389	AF196476
Structure	Lox	11.11	0.0368	NM_010728
	Siat8d	2.17	0.0368	NM_009183
	Dpt	100	0.0368	NM_019759
	Mtap4	50	0.0435	AK019611
	Pxn10	2.17	0.0203	-
	Cypt6	2.22	0.0368	NM_025738
	Adam18	2.63	0.049	NM_010084
	Adam rep	2.17	0.0491	AB112362
Differentiation	Rho	7.14	0.0368	BC013125
	Wnt4	3.85	0.0368	NM_009523

	Common Name	Fold change	p-value	PubMed ID
	Gli5	3.33	0.0368	BC021517
	Pcdh15	2.86	0.0435	NM_023115
	Pax5	8.33	0.0464	NM_008782
	Pax9	2.78	0.0468	NM_011041
	Ccna1	4.35	0.0203	NM_007628
	Pol theta	2.7	0.0368	AK020790
	Cdc2l2	2.22	0.0368	NM_007661
GF/Rec	Pglp	50	0.0368	AJ293619
	Cpr2	6.67	0.0401	BC016483
	Gadd45a	20	0.0464	NM_007836
Protein deg	Arih2	2.86	0.0495	NM_011790
	Psg4-1	2.08	0.0464	AK017550
Immune sys	Cd2	2.38	0.0384	NM_013486
	Itgae	4.17	0.0435	NM_008399
	Tslp	2.22	0.0471	NM_021367
	Fcnb	3.7	0.0368	AK010913
	BCA3	2.13	0.0465	NM_020616

Abbreviations: Transcription, transcription factor; Ca2+-assoc, involved in calcium signaling or processing; GF/Rec, growth factors or receptors; Protein deg, involved in protein degradation; Immune sys, genes related to the immune system.