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The effects of acute 17β-estradiol treatment on gene expression in the young female mouse hippocampus

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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 & 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 2-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.

1. Introduction

The loss of estrogens at menopause has been associated with increases in dementia and age-related memory decline observed in aging women (Wolf & Kirschbaum, 2002; Yaffe et al., 2000). 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 & Hampson, 2000), object memory (Duka, Tasker, & McGowan, 2000), and verbal memory (Kampen & 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 (Gresack & Frick, 2006a; Heikkinen, Puolivali, Liu, Rissanen, & Tanila, 2002; Rissanen, Puolivali, van Groen, & Riekkinen, 1999) and novel object recognition memory (Gresack & Frick, 2006b; Luine, Jacome, & MacClusky, 2003) in mice and rats. Spatial and object memory are also enhanced by pre-training E_2 treatments administered systemically by injection or silastic capsules (Bimonte & Denenberg, 1999; Daniel, Fader, Spencer, & Dohanich, 1997; Luine, Richards, Wu, & Beck, 1998; Sandstrom & Williams, 2001; Sandstrom & Williams, 2004; Vaucher et al., 2002). 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, Sakkas, Xydakis, & Creatsas, 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

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the side effects of systemic hormone administration. E_2 is known to increase dendritic spine density (Frick et al., 2004; Woolley & McEwen, 1993) and synaptic protein expression (Stone, Rozovsky, Morgan, Anderson, & Finch, 1998) in the CA1 region of the hippocampus and enhance neurogenesis in the hippocampal dentate gyrus (Galea, Spritzer, Barker, & Pawluski, 2006; Tanapat, Hastings, Reeves, & Gould, 1999). Additionally, acute E_2 administration can activate several intracellular kinase cascades, including phosphatidylinositol 3-kinase (PI3K; Cardona-Gomez, Mendez, & Garcia-Segura, 2002; Mannella & Brinton, 2006; Yokomaku et al., 2003) and extracellular signal-regulated kinase (ERK; Fernandez et al., 2008; Fitzpatrick et al., 2002; Kuroki, Fukushima, Kanda, Mizuno, & Watanabe, 2000; Wade & 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, Pattee, Nagalla, Kelly, & Ronnekleiv, 2004) and the hippocampus (Aenlle, Kumar, Cui, Jackson, & Foster, 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 & Frick, 2006b) and activate the ERK cascade in the dorsal hippocampus (Fernandez et al., 2008; Lewis, Kerr, Orr, & Frick, 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 & 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 Use 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 & 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, Weiss, & Pitha, 1989). This hormone preparation is metabolized within 24 hours (Pitha, Harman, & Michel, 1986) 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 & 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 & 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 h 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 h (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 RN-easy 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 and 280 nm on a SmartSpec 3000 Spectrophotometer (Bio-Rad, 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 instructions. 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 the 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 (C_t) 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 C_t value for the reference samples. The concentrations of unknown samples were determined by substituting the C_t 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.

2.4.1. Western blot analysis

Dorsal hippocampi were homogenized and Western blot analysis performed as previously described (Fernandez et al., 2008; Lewis et al., 2008). Briefly, 6 μg of protein from each homogenate (vehicle-treated, $n = 3$; E_2-treated, $n = 3$) were run on 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 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 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) (see Fig. 1), as it is not only associated with increased memory retention in the Morris water maze (Pizarro, Haro, & Barea-Rodriguez, 2003), but is also known to be up-regulated by E_2 (Olazabal, Pfaff, & Mobbs, 1992). Hsp70-1 mRNA levels increased 3-fold by QPCR ($t(4) = 3.731$, $p = 0.02$; Fig. 1A), 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 h after treatment with E_2 ($t(5) = 4.418$, $p = 0.007$; Fig. 1B).

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, Ramsey, & Carter, 2005), and is thought to exert its memory-enhancing affects by promoting adult neurogenesis (Aberg, Aberg, Hedbacker, Oscarsson, & Eriksson, 2000; Perez-Martin, Azcoitia, Trejo, Sierra, & Garcia-Segura, 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, De Keyser, & Wilczak, 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 ($t(4) = 3.90$, $p = 0.018$; Fig. 2A). Additionally, protein levels of IGFBP2 showed a 2-fold decrease 4 h after E_2 treatment ($t(2.145) = 5.402$, $p = 0.028$; Fig. 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 & McEwen, 1993) and enhance neurogenesis (Galea et al., 2006; Tanapat et al., 1999), 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, Engler, & Sheng, 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 ($t(4) = 3.562$, $p = 0.024$; Fig. 3A), and 1.3-fold increase in protein levels 3 h after treatment ($t(5) = 3.603$, $p = 0.015$; Fig. 3B). 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 &

Table 1
Genes whose expression was significantly up-regulated by E_2 treatment.

	Common Name	Fold-change	p-value	PubMed Number		Common Name	Fold-change	p-value	PubMed Number
Structure	***Tubb2a****	2.67	0.042	M28739	Chaperone	***Hsp70-1****	2.11	0.0368	M12573
	Actn4*	2.63	0.0368	NM_021895					
	Snap25*	2.84	0.0487	BC018249	Protein deg	Rbx1	2.05	0.0435	AK005127
	Tmsb4x	2.29	0.0368	NM_021278		Rnf5	2.10	0.0398	NM_019403
	ctn4	2.40	0.0491	BC006677		Bean	21.34	0.0368	AF240460
	Myo6	2.13	0.0435	NM_008662		Pep4	6.54	0.0368	NM_008820
	Myh2	9.73	0.0368	BC008538		Serp23	2.18	0.0311	BC018517
	Esm1	12.28	0.0435	BC020038		Psmd1	2.10	0.0368	AK010596
	Arfl10C	2.66	0.0465	BC013719		Psmb2	2.06	0.0498	NM_011970
	Tpk2	2.45	0.0464	BC019149					
	Mtrp6	2.30	0.0368	BC020019	NP/GF/Rec	Rfrp	2.03	0.0465	NM_021892
	Cct6a	2.26	0.0203	NM_009838		Hgfl	3.69	0.0471	NM_008243
	Ccol	2.28	0.0368	NM_177177		Erbb4	2.32	0.0402	AF059177
						Neurod2	2.54	0.0368	NM_010895
Transcription	***Junb***	2.35	0.0465	NM_008416		Pafah1b1	2.40	0.0368	NM_013625
	Pik3cb	2.13	0.0368	AK003230		Kcna1	2.04	0.0471	NM_010595
	Tcf4	2.24	0.0368	NM_013685		Olfr, put	2.01	0.0368	X89678
	Eef1d	2.07	0.0491	NM_023240		Olfr67	2.78	0.0491	NM_013619
						Gpr124	2.05	0.0368	NM_054044
Metabolism	***Odc***	2.42	0.0487	NM_013614		Igsf1	2.35	0.0464	AY227771
	Got2	2.35	0.0368	NM_010325		Scube1	2.34	0.046	NM_022723
	Atp1b3	2.28	0.0463	NM_007502		Eva	5.09	0.0368	BC015076
	Sult4a1	2.16	0.0368	NM_013873		Cd209e	2.39	0.0368	AF373412
	ATP F1 complex	3.38	0.0203	BC013607		Neph	2.36	0.0471	NM_025684
	Dlat	2.03	0.0203	BC003202					
	Nec1	2.01	0.0368	AK018159	Oxidative Stress	Pxf	2.56	0.0368	AK012785
	Atp6b2	2.07	0.0435	BC012497					
	Pmca1b	2.63	0.0398	AK013291	Inflammation	Tcra	2.49	0.0368	X72904
	E2RG	10.42	0.0368	AK019895		Aldh1a7	2.00	0.0465	NM_011921
	Agk	2.05	0.0368	BC019145		Egln1	2.07	0.0386	BC006903
	Hatpaseb1	2.06	0.0464	BC017127		CS1	2.23	0.0368	AK014517
Transport	SV2	2.13	0.0399	AK013742	Differentiation	Morf	2.04	0.0464	NM_017479
	Rab1b	2.54	0.0368	BC016408		Cd81	2.21	0.049	BC011433
	MLC1	2.11	0.0466	AF449425		Gcm2	2.50	0.0368	NM_008104
	Snta1	2.17	0.0465	NM_009228		Ctf1	2.84	0.0368	NM_007795
	Slc14a1	2.08	0.0389	NM_028122		Alcam	2.07	0.0435	U95030
	Rcc1	2.36	0.0465	BC019807		Mmd	2.37	0.0368	NM_026178
	Itgb4bp	2.22	0.0368	BC015274		Nuf2r	8.42	0.0368	NM_023284
	Ergic1	2.33	0.0464	NM_026170		Ash1l	2.11	0.0389	AF247132

The *p*-values listed in the table represent significance reported after the false discovery rate (FDR) analysis. Genes in 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. *Abbreviations:* Transcription, transcription factor; NP/GF/Rec, neuropeptides, growth factors, or receptors; Protein deg, involved in protein degradation.

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 ($t(4) = 3.069$, $p = 0.037$; Fig. 3A), and a 1.7-fold increase in protein levels 4 h after E_2 treatment ($t(5) = 2.568$, $p = 0.049$; Fig. 3B). 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 ($t(4) = 4.623$, $p = 0.01$; Fig. 3A), and a 1.9-fold increase in protein levels 4 h after E_2 treatment ($t(5) = 2.887$, $p = 0.034$; Fig. 3B).

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, Gupta, & Knowlton, 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 & Clarke, 1995). Hsp70 is also an integral part of the cytosolic estrogen receptor (ER) protein complex that keeps ER in an inactive state

Table 2
Genes whose expression was significantly down-regulated by E_2 treatment.

	Common Name	Fold Change	p-value	PubMed ID		Common Name	Fold Change	p-value	PubMed ID
Transcription	***Igfbp2*** *	16.67	0.0466	AK011892	Structure	Lox	11.11	0.0368	NM_010728
	Plc	3.57	0.0468	NM_010566		Siat8d	2.17	0.0368	NM_009183
	Inpp5d	3.85	0.0465	NM_011107		Dpt	100.00	0.0368	NM_019759
	Pla2g1b	2.38	0.0389	NM_008342		Mtap4	50.00	0.0435	AK019611
	Rbm8	3.23	0.0368	NM_025875		Pxn10	2.17	0.0203	-
	Smarca3	6.67	0.0368	AF165911		Cypt6	2.22	0.0368	NM_025738
	AF-9	20.00	0.0368	AK008707		Adam18	2.63	0.049	NM_010084
	Brd7	25.00	0.0465	NM_012047		Adam rep	2.17	0.0491	AB112362
	Ikbkb	10.00	0.0368	AF088910					
	Lztfl1	2.13	0.0466	NM_033322	Differentiation	Rho	7.14	0.0368	BC013125
						Wnt4	3.85	0.0368	NM_009523
Transport	Kcnj8	2.13	0.0389	NM_008428		Gli5	3.33	0.0368	BC021517
						Pcdh15	2.86	0.0435	NM_023115
Apoptosis	Birc4	7.14	0.0368	NM_009688		Pax5	8.33	0.0464	NM_008782
						Pax9	2.78	0.0468	NM_011041
Ca2+-assoc	Calb2	2.50	0.0368	BC017646		Ccna1	4.35	0.0203	NM_007628
	Cacng3	2.56	0.0491	NM_019430		Pol theta	2.70	0.0368	AK020790
	Calmbp1	5.00	0.0402	NM_009791		Cdc2l2	2.22	0.0368	NM_007661
	Cacybp	11.11	0.0368	U97327					
	Trpv6	2.17	0.0368	NM_022413	GF/Rec	Pglp	50.00	0.0368	AJ293619
	Pln	3.85	0.0466	AK002622		Cpr2	6.67	0.0401	BC016483
						Gadd45a	20.00	0.0464	NM_007836
Metabolism	Anpep	2.38	0.0368	NM_008486					
	Lrp6	2.17	0.0368	NM_008514	Protein deg	Arih2	2.86	0.0495	NM_011790
	Atic	2.56	0.0465	AK010611		Psg4-1	2.08	0.0464	AK017550
	Fabp4	11.11	0.0465	BC002148					
	Cyp40	50.00	0.0368	AB006034	Immune sys	Cd2	2.38	0.0384	NM_013486
	Nit1	3.03	0.0465	AK004988		Itgae	4.17	0.0435	NM_008399
	Ethi6	20.00	0.0435	AK010551		Tslp	2.22	0.0471	NM_021367
	Pit1	50.00	0.0389	AF196476		Fcnb	3.70	0.0368	AK010913
						BCA3	2.13	0.0465	NM_020616

The *p*-values listed in the table represent significance reported after the false discovery rate (FDR) analysis. Genes in 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. *Abbreviations:* Transcription, transcription factor; Ca^{2+}-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.

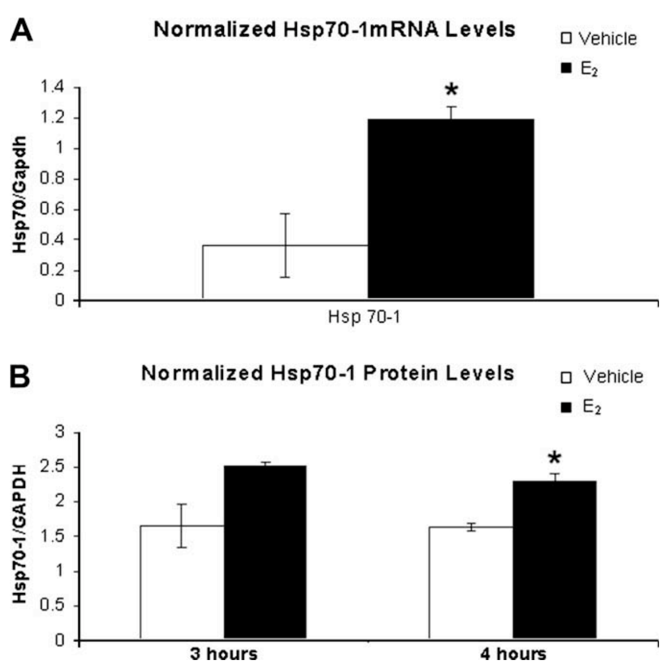

Fig. 1. Heat-shock protein 70-1 (Hsp70-1) gene and protein expression levels following E_2 treatment. (A) Hsp70-1 mRNA expression levels significantly increased 1 h after E_2 treatment. (B) Hsp70-1 protein levels were also significantly higher than vehicle 4 h after E_2 treatment. $*p < 0.05$ relative to vehicle.

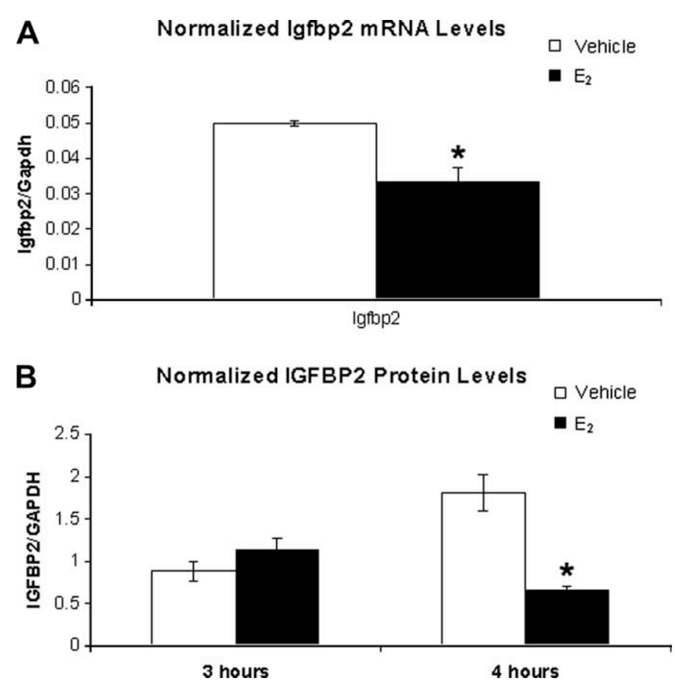

Fig. 2. IGFBP2 gene and protein expression levels following E_2 treatment. (A) Igfbp2 mRNA expression levels significantly decreased 1 hour after E_2 treatment. (B) IGFBP2 protein levels were also significantly lower than vehicle 4 h after E_2 treatment. $*p < 0.05$ relative to vehicle.

until it binds its estrogens and translocates to the nucleus (Whitesell & 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 (Takahashi et al., 1994), heart (Hamilton et al., 2004), and brain, where it may show differential

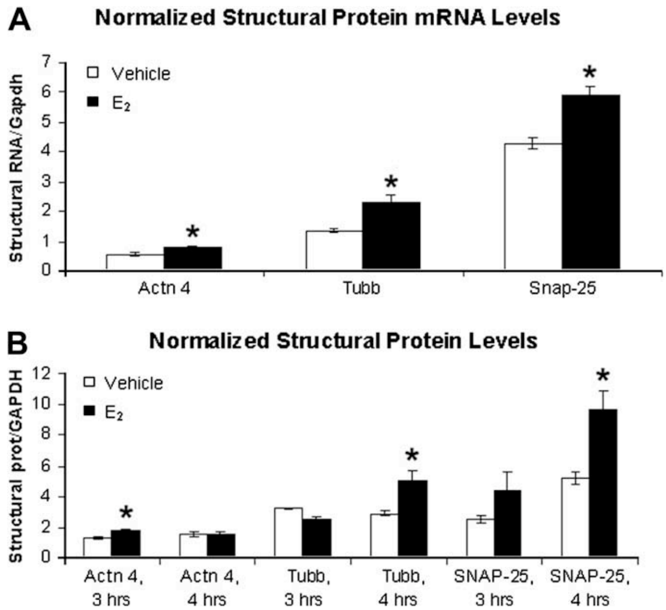

Fig. 3. Structural protein gene and protein expression levels following E_2 treatment. (A) Actn4, Tubb, and Snap-25 mRNA expression levels were all significantly increased 1 hour after E_2 treatment. (B) Actn4 protein levels were increased 3 h after E_2 treatment, whereas, SNAP-25 and Tubb protein levels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

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, Mariucci, Tantucci, Brushchelli, & Giuditta, 1999). Further, when Hsp72 is knocked out in mice, this deletion prevents spatial memory acquisition in the radial arm maze (Ambrosini, Mariucci, Tantucci, Van Hooijdonk, & Ammassari-Teule, 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 (Bezchlibnyk, Wang, Shao, & Young, 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, Brywe, & Isgaard, 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, Sanz, & Mendez, 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, Dictenburg, Plociniak, Afroz, & Bassell, 2005), endocytosis and exocytosis of neurotransmitters (Chin, Nugent, Raynor, Vavalle, & Li, 2000), cell division during neurogenesis (German & Eisch, 2004), neuronal plasticity (Bianchi, Hagan, & Heidbreder, 2005), and dendritic spine morphogenesis (Sekino, Kojima, & Shirao, 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 which 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, Adamson, & Penny, 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, Paschall, Zhou, & Davis, 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, Kipp, & Joe, 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, Acconcia, Rayala, & Kumar, 2006). On the other hand, studies in breast cancer cells show that E_2 actually disrupts microtubule formation (Aizu-Yokota, Ichinoseki, & Sato, 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, Hattiangady, & Shetty, 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 (Galea et al., 2006; Tanapat et al., 1999), 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 kDa (Snap-25). SNAP-25 is a part of the synaptic vesicle docking and fusion process and is essential for neurotransmitter release (Wang & 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, Razani, Ogren, & Meister, 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 h after LTP stimulation (Roberts, Morris, & O'Shaughnessy, 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 & 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 & Frick, 2006b) can influence the mRNA expression levels of genes involved in learning and memory within 60 min of injection, and the protein expression levels within 4h. 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.

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