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Combined estradiol and lithium increase ER-α mRNA in embryonic C57BL/6J primary hippocampal cultures

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Estrogen replacement therapy (ERT) is commonly prescribed during menopause. Post-menopausal women also tend to suffer from bipolar disorders and as a result are prescribed mood stabilizers – in addition to ERT. There is a paucity of data on how combined hormones and mood stabilizers interact in regulating gene expression that led us to hypothesize that in primary cultures of mixed brain cells predominated by glia, combined 17β-estradiol (E2) and lithium chloride (LiCl) (E2/LiCl) will alter estrogen receptor-alpha (ER-α) mRNA expression. We quantified mRNA expression of ER-α using the cDNA of treated primary cultures of mixed brain cells from a previous study. Our results indicate that hippocampal cultures predominated by glia increase in ER-α mRNA expression when treated for 48 h with combined E2/LiCl. Our findings may encourage further investigation on the molecular mechanisms involved in combined estrogen and lithium treatment.

Key words: ER-α, estradiol, glia, lithium

Estrogen replacement therapy (ERT) is a common prescription for females during post-menopause, but ERT efficacy remains debatable (Webber et al. 2004). Additionally, unipolar and bipolar disorders occur during menopause and as a result, women are prescribed mood stabilizers in combination with ERT (Burt and Rasgon 2004, Soares and Taylor 2007). Few studies focus on the interacting properties of hormones and mood stabilizers. The paucity of studies is regrettable since both hormones and mood stabilizers influence a myriad of cell signaling pathways by either initiating protein signaling cascades or by modulating these cascades via activating and/or inhibiting specific proteins (McEwen 2001, Jope 2003). Estrogen is diminished during menopause (Blake 2006) and this depletion not only affects women physiologically but also has neurological implications such as enhancing neurodegeneration, reducing synaptic connectivity, and cognitive decline (Gandy 2003). Studies show that estrogen reduces astrocytosis, increases cell proliferation and differentiation of neurons potentially mediated via estrogen receptors (see McEwen 2001, Saravia et al. 2007). A plethora of protein signaling cascades are facilitated by estrogen resulting in gene expression for cell survival, as well as for learning and memory mechanisms among glia and neurons (McEwen 2001). Estrogen receptor α and β (ER-α and ER-β) play roles in facilitating estrogen-mediated glia through extracellular-regulated kinase and phosphatidylinositol 3-kinase/Akt protein signaling (Dhandapani and Brann 2007), as well as neuronal protein signaling cascades (McEwen 2001). Studies also show pyramidal cells of rat hippocampus co-express brain derived neurotrophic factor (BDNF) and ER-α (Solum and Handa 2002), and BDNF drastically decreases in people suffering from neurodegenerative disorders (Sohrabji and Lewis 2006). Estrogen, via ER-α, increases BDNF expression and facilitates BDNF-tyrosine kinase receptor signaling in both glia and neurons (Sohrabji and Lewis 2006). Anti-apoptotic factors are also affected by ER-α. Also, astrocytic co-expression of B-cell lymphoma/leukemia-2 and ER-α in the hippocampus of post-mortem brains of Alzheimer's disease (AD) patients indicates a potential mechanism for ER-α neuroprotection (Dhandapani and Brann 2007). Further, ER-α expression increases in the hippocampus of AD patients compared with controls (Lu et al. 2003).

Lithium is commonly used to treat bipolar disorder (an adjuvant for depression), and is identified as a spe-

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cific inhibitor of glycogen synthase kinase-3-beta (GSK-3β) (Klein and Melton 1996). Molecular implications for lithium continue to be explored. Similar to estrogen, lithium facilitates gene expression responsible for anti-apoptosis, and mechanisms of learning and memory (Manji et al. 1999). Among neurons, lithium-facilitated-GSK-3β inhibition is associated with reducing apoptosis (Hongisto et al. 2003), increasing neurotrophic factors (Angelucci et al. 2003), increasing dopamine receptor subunit 2 (Dziedzicka-Wasylewska and Wędzony 1996), and facilitating DNA binding of transcription factors (Grimes and Jope 2001). Lithium also affects astrocytic pH levels that stimulate myo-inositol transport systems, ultimately affecting protein signaling pathways in astrocytes (Song et al. 2008). Inositol signaling is known to be regulated by proline-, glutamic acid-, and leucine-rich protein-1, a co-regulator of ER-α signaling (Nair and Vadlamudi 2007).

We recently published results of a study that focused on combined effects of estradiol (17β-estradiol; E2) and lithium chloride (LiCl) (E2/LiCl) on glial NMDAR subunit NR1 gene expression and glutamate excitotoxicity. We showed that a 48 h E2/LiCl treatment reduced NR1 gene expression and increased glutamate excitotoxicity in mouse hippocampal and cortical primary cultures of mixed brain cells predominated by glia (Valdés and Weeks 2009). In the present study, we use the same cDNA from treated primary mixed brain cell cultures of our previous study to further investigate how combined E2/LiCl influence ER-α gene expression. Our results indicate that after 48 h, E2/LiCl treatment increases ER-α mRNA in C57BL/6J mouse primary mixed hippocampal cell cultures. We recognize that since results are generated from embryonic brain cell cultures, such introduces considerations for processes in development. Still, we believe our results will initiate other investigations to elucidate how hormones and mood stabilizer interact and how they affect brain gene expression and proteins.

Florida International University Institutional Animal Care and Use Committee approved all experiments described in this study (IACUC protocol #: 08-017). For a detailed description of experimental procedures, refer to our previous study (Valdés and Weeks 2009). Briefly, primary mixed hippocampal and cortical cultures were harvested from E18.5 C57BL/6J mice. Disassociated cells were randomly plated (6×10^6 for cortical cultures and 3×10^6 for hippocampal cultures) on poly-D-lysine (100 μg/mL; Sigma-Aldrich, Missouri) coated 12-well plates containing B27/Neurobasal medium (Invitrogen, California) with 0.5mM L-glutamine and 4μg/mL gentamicin. Cells were maintained in a humidified, 5% CO_2 atmosphere incubator with a set temperature of 37°C. Additional 12-well plates, with poly-D-lysine (100 μg/mL) coated coverslips were set aside for immunocytochemical purposes. After 4 days in culture, the media was removed entirely and replaced with fresh media; half of the media was changed every 4 days, thereafter, until E2 and/or LiCl treatment. After twelve days in culture, the media was removed entirely and replaced with media treated with 10 mM LiCl (Sigma-Aldrich, Missouri), 0.04 μM E2 (Sigma-Aldrich, Missouri), or combined E2/LiCl and duration of treatment for each group was 48 h. We chose these specific concentrations for E2 and LiCl, and a 48 h treatment period based on our previous findings (Valdés and Weeks 2009).

Primary mixed cultures were immunolabeled using neurofilament-H (NF-H) (Upstate, Virginia) and glia fibrillary acidic protein (GFAP) (Sigma-Aldrich, Missouri) antibodies. Cells were fixed with 4% paraformaldehyde and permeabilized for 30 min at room temperature with 0.25% DPBST (DPBS with 0.25% [v/v] Triton X-100). Non-specific binding sites were blocked with 10% normal goat serum overnight at 4°C. Primary cultures were then incubated at room temperature with mouse NF-H or GFAP antibodies and visualized using fluorescent-conjugated secondary antibodies. Cells were counterstained with Hoechst 33342 to reveal nuclei, coverslips were mounted on glass slides, and sealed with clear nail polish. Images were captured using a Leica Leitz DM RB fluorescent microscope with a Leica DM 500 camera (Leica, Bannockburn, IL, USA).

Total RNA was isolated from cultures using TRIzol reagent (GIBCO, California), according to manufacturer's protocol. Briefly, cultures were rinsed with ice-cold DPBS, TRIzol (600 μL - 1 mL) was added directly into each well, and then homogenized. Homogenized cells were transferred to a fresh tube, and incubated at room temperature. Chloroform was added and centrifuged at 6,700 x g at 4°C. Total RNA (aqueous phase) was precipitated with isopropanol and glycogen overnight at -20°C. RNA was pelleted via centrifugation at 6,700 x g at 4°C, supernatant decanted and RNA pellet washed with 75% ethanol, then resuspended with DEPC-treated water. DNA contaminants were removed with the RQ1 DNase kit (Promega,

Wisconsin). Total RNA was then reverse transcribed (RT) using SUPERSCRIPT™ II RNase H-free reverse transcriptase (Invitrogen, California), according to the manufacturer's protocol. Briefly, 500 ng of total RNA was reverse transcribed with 0.05 µg/µl of Oligo$(dT)_{12-18}$ at 65°C for 5 min. First strand cDNA was synthesized with 5 mM $MgCl_2$, 10 mM DTT, and 2.5 units Superscript II and incubated at 42°C for 50 min and terminated at 70°C for 15 min. RNase H (Invitrogen, California) was added to cDNA to remove any remaining RNA by incubating at 37°C for 20 min.

First strand cDNA was amplified via real time PCR using SYBR Green PCR master mix (ABgene, New York), 200-300nM of forward and reverse primers using AB 7300 qPCR apparatus. Cycling parameters were set at: 95°C for 15 s, 57°C or 63°C for 30 s, and extension at 72°C for 30 s, for a total of 40 cycles, followed by a final extension at 72°C for 10 min. The specific primer pairs were: ER-α: forward primer, 5'-AAGGGCAGTCACAATGAACC-3'; reverse primer, 5'-GCCAGGTCATTCTCCACATT-3'; HPRT: forward primer, 5'-GGAGCGGTAGCACCTCCT-3'; reverse primer, 5'-AATCCAGCAGGTCAGCAAAG-3'. All samples were compared with a standard curve comprised of pDNA of HPRT and ER-α generated using TOPO TA Cloning® Kit (Invitrogen, California). Readings were normalized by dividing mRNA starting quantity (SQ) of ER-α by the house-keeping gene HPRT mRNA SQ; output is in picograms (pg) of mRNA. Data are presented as the mean ± S.E.M and statistical significances were determined by an analysis of variance (ANOVA) followed by Bonferroni's post-hoc testing. Significant differences have a P-value<0.05. All results were obtained from two separate experimental procedures with factors and levels randomly determined.

We chose to analyze mRNA expression since both estrogen and lithium indirectly and/or directly affect genomic responses (McEwen 2001, Jope 2003). Quantitative RT-PCR results indicate that ER-α mRNA expression significantly differs depending on cell culture type ($F_{1,8}$=32.59, P<0.001) – hippocampal cultures express higher levels of mRNA compared with cortical cultures (Fig. 1). We also noted significant difference in treatment type ($F_{3,8}$=5.73, P<0.025), and the interaction between treatment and cell culture type ($F_{3,8}$=5.6, P<0.025). Bonferroni's post-hoc testing indicated that combined E2/LiCl significantly increases (P<0.05)

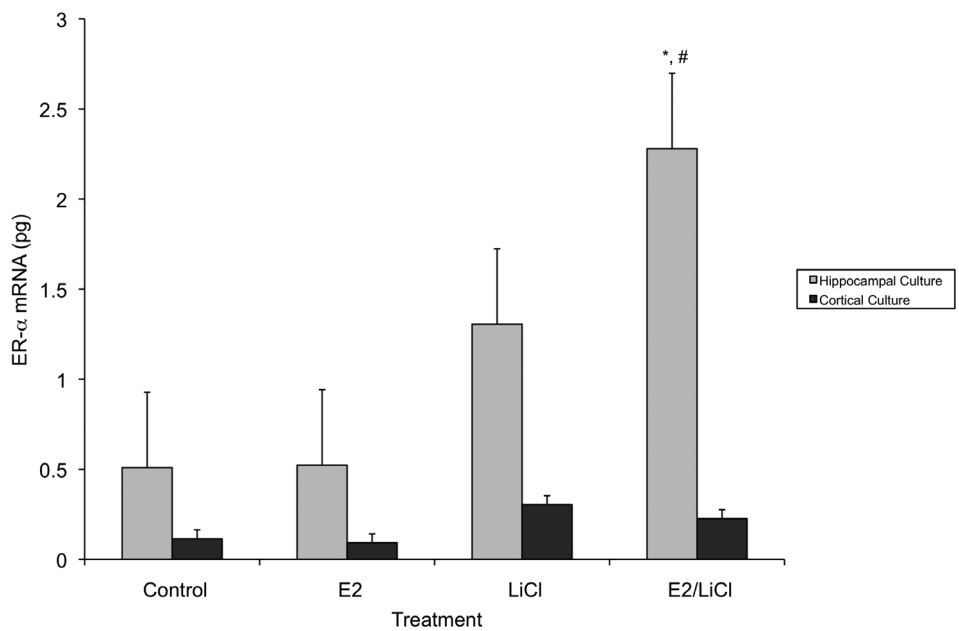

Fig. 1: Real time RT-PCR of ER-α mRNA expression. E18.5 hippocampal (grey bars) and cortical (black bars) primary cell cultures were treated for 48 h with 0.04 µM E2, 10 mM LiCl, combined E2/LiCl, or Control (no treatment). After treatment total RNA was isolated and reverse transcribed. Expression for each gene was normalized using the house-keeping gene HPRT. The figure denotes normalized mRNA expression in picograms (y-axis) and treatment type (x-axis). *, P<0.05 compared with control; #, P<0.05 compared with E2.

ER-α mRNA expression compared with control and E2 treated hippocampal cultures; E2 or LiCl alone does not significantly alter ER-α mRNA expression (Fig. 1). Figure 1 also shows no significant differences in ER-α mRNA expression across treatments for cortical cell cultures ($F_{3,4}$=0.341, P>0.75).

Immunocytochemical images (Fig. 2A) depict high GFAP expression in hippocampal cultures and by design with low NF-H labeling – as noted by the unlabeled surrounding nuclei (Fig. 2B). This coincides with our previous findings that these primary mixed brain cell cultures express high glia-specific mRNA (Valdés and Weeks 2009). Glia predominates in the brain and investigations on how estrogen and lithium affect glial molecular mechanisms are essential for developing translational studies that could benefit post-menopausal women. Glia do express ER-α and expression levels of ER-α vary according to the neuroanatomical region and developmental stage: low levels of ER-α are express in the cortex and hippocampus (Shughrue et al. 1997). Our results (Fig. 1) concur with studies that show murine hippocampus exhibiting higher levels of ER-α than the cortex (Mitra et al. 2003).

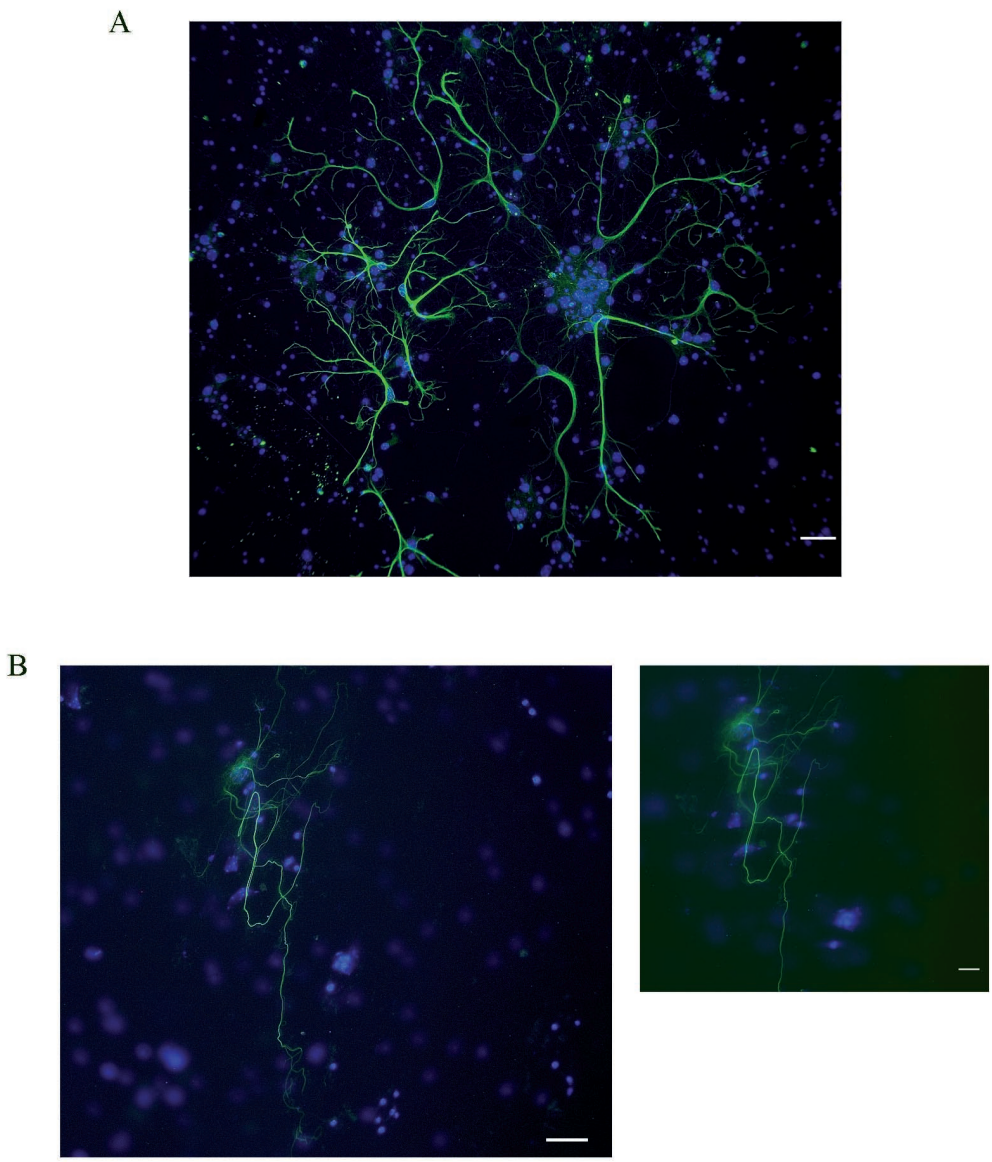

Fig. 2A-2B: Immunocytochemical images of hippocampal cultures labeled with GFAP (A; 200X) and; NF-H (B; 400X) antibodies (green). All nuclei are labeled with Hoechst stain (blue). Scale bar = 2μm.

In our previous study we showed that LiCl reduces NMDA subunit NR1 mRNA expression in E2-treated cultures (Valdés and Weeks 2009). In the present study we show that E2/LiCl-treated cultures show increases in ER-α mRNA expression, however, E2 or LiCl alone does not significantly alter ER-α mRNA expression (Fig. 1). Under conditions of neuronal damage, ER-α expression increases among neonate rat cortical astrocytes, mediated by E2, and this increase aids damaged neurons (Carbonaro et al. 2009). Cortical ER-α mRNA expression was not affected by our treatment parameters, though (Fig. 1). Our cortical cultures did express higher glia-specific mRNA than hippocampal (Valdés and Weeks 2009) and we report in the present study that primary hippocampal cultures show high perikaryon labeling of NF-H (Fig. 2B), which is indicative of neuronal apoptosis (Lee et al. 1999). Additionally, studies show that combined estrogen and lithium increase serotonin in ovariectomized rat striatal and frontal cortex (Morissette and Di Paolo 1996) and serotonin augments neurogenesis (Djavadian 2004). Studies depicting co-localization of ERs among proliferating cells of the dentate gyrus suggest a direct estrogen/ER-interaction modulating neurogenesis (Isgor and Watson 2005). Our study may serve as a platform to further investigate how combined E2/LiCl effects neurogenesis – a topic scarcely explored.

Recent studies using neuronal cell lines show that ER-α gene expression has an E2 dose dependency – reducing ER-α gene expression within 16 h treatment of 0.1 μM E2 (Ng et al. 2009). Other studies indicate that ER-α increases in neonatal rat hippocampus with E2 treatment (Rune et al. 2002). Furthermore, fluxes in ER-α expression levels raises developmental considerations since ER-α expression in rat brain is high during prenatal day 9, declines then increases at the neonatal stage and then declines again with age (Su et al. 2001); this occurs in both the hippocampus and cortex (González et al. 2007). Our cultures are from embryonic mouse brain (E18.5) and this developmental factor may account for differences noted in other publications, but we found no significant differences in ER-α mRNA expression with E2 or LiCl individual treatment (Fig. 1). Our results are generated and interpreted at the transcriptional level but these results indicate that some molecular mechanism responsible for ER-α transcription is being altered due to the combined treatment of estrogen and lithium. We believe, however, knowledge on how estrogen and lithium interact will provide a better understanding on related molecular mechanisms affected by combined ERT and mood stabilizing treatment regiments.

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