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The hippocampus and cortex of the mammalian brain are regions involved in learning and long-term memory. Estrogen and lithium affect similar learning and memory molecular processes. We hypothesized that in ovariectomized mice lithium treatment will enhance genetic factors in the brain that are involved in neuroprotection, learning and memory. Our study used bilaterally ovariectomized (bOVX) C57BL/6J mice treated for one month with 14.2 mM LiCl in their drinking water. Results indicate that LiCl-treated bOVX mice show enhanced cortical increases in mRNA expression of ER-α, NR1, Bcl-2, BDNF, and CaMkII-α; hippocampal mRNA showed no changes. Our results indicate that in bOVX C57BL/6J mice, lithium enhances the expression of specific cortical genes coded for proteins involved in learning, memory and neuroprotection.

Key words: lithium, mRNA, ovariectomy

INTRODUCTION

The literature indicates that estrogen-mediated neuromechanisms enhance synaptic plasticity (Leranth et al. 2002), reduce apoptotic activity in cortical neurons (Honda et al. 2001), and facilitate activation of transcription factor, cyclic adenosine monophosphate (cAMP) responsive element binding protein (CREB) (McEwen 2001). Women typically reach menopause by their late 40s to mid-50s and estrogen replacement therapy (ERT) is commonly prescribed during peri- and post-menopause. Also, pre-menopausal ovariectomized (OVX) women suffering from benign diseases are usually prescribed ERT. Women prescribed ERT are potentially at risk, since there is an increased incidence of lobular carcinoma associated with ERT (Newcomer et al. 2003). Other studies indicate that ERT does not improve cognition during menopause, specifically episodic memory (Henderson 2009). Therefore, an alternative therapy, especially one that provides assistance with estrogen–mediated protein-signaling pathways and positively affects cognition offers far-reaching benefits.

Estrogen influences several neuroanatomically-specific behavioral tasks (Simpkins et al. 1997). Estrogen receptor-α (ER-α) knockout mice and OVX mice perform poorly on hippocampal-dependent behavioral tasks, suggesting that estrogen and ER-α expression influence cognitive-behavioral performance levels (Fugger et al. 2000). Although ER-β is also known to influence behavior, ER-β signaling pathways are more specific to visuospatial performance (e.g., Morris water maze) and anxiety (Bodo and Rissman 2006). Co-localization of ER-α and brain derived neurotrophic factor (BDNF) are observed in pyramidal cells of the hippocampus, and estrogen influences BDNF expression (Solum and Handa 2002). Ligand binding of BDNF to tyrosine receptors initiates a signaling cascade that results in activating CREB (Blanquet et al. 2003), and facilitating CREB-mediated gene expression via *N*-methyl-D-aspartate receptor (NMDAR) signaling pathways (Wheeler and Cooper 2004). Rats with ovarian steroid deprivation (via simulating postpartum depression) show ephemeral gene expression of calcium/calmodulin-dependent protein kinase II (CaMkII), a kinase associated with increasing synaptic plasticity and cognition (Suda et al. 2008).

Lithium, traditionally used to treat bipolar disorder, affects several molecular pathways via glycogen synthase kinase-3beta (GSK-3β) inhibition (Jope

2003) and by inositol monophosphatase inhibition resulting in depleting amounts of free inositol (Harwood 2004). Inhibition of GSK-3β is correlated with reduced apoptotic activity (Hongisto et al. 2003), increased neurotrophic factor expression (Angelucci et al. 2003), and facilitated CREB response element binding (Ozaki and Chuang 1997, Grimes and Jope 2001). Lithium's neuroprotective properties are also ascribed to increased levels of the anti-apoptotic agent B-cell lymphoma/leukemia-2 (Bcl-2) and BDNF – this increase being associated with inhibition of GSK-3β and NMDAR tyrosine phosphorylation in primary rat cultures of cortical cells (Chuang 2005). Studies also show that lithium augments expression of a specific CaMk isoform in rat brain (Rushlow et al. 2009), and that low activity of CaMkII is observed in the cortex of lithium-treated rats (Celano et al. 2003).

Both estrogen and lithium facilitate transcriptional properties of CREB by activating promoting factors of CREB and/or inhibiting negative regulators of CREB (Grimes and Jope 2001, McEwen 2001). We hypothesize that LiCl-treated OVX mice will enhance mRNA expression of factors important in learning, memory and neuroprotection in the brain (namely, ER-α, NMDAR critical subunit NR1, Bcl-2, BDNF and CaMkII-α). To test our hypothesis we treated bilaterally OVX (bOVX LiCl) C57BL/6J mice with 14.2 mM LiCl for 1 month beginning two weeks post-bOVX surgery. Our results show that LiCl treatment enhances genetic factors involved in learning, memory and neuroprotection.

METHODS

Subjects, surgery and treatment

All experiments described in this study were approved by the Florida International University Institutional Animal Care and Use Committee (IACUC protocol #: 08-017). C57BL/6J female mice (n=20) were purchased from Jackson Laboratories. Animals were housed within a facility maintained at 20-22°C, 60% humidity, within polycarbonate transparent cages (26.7×20.6×14 cm) on a 12-hour day-night cycle with free access to water and food.

At 4.5 months of age, mice weighing 21.5 g – 22.5 g were anesthetized with ether and, aseptically, their ovaries were removed bilaterally (e.g. bOVX). Mice

were positioned dorsal side up and for each surgical procedure, parallel to the long axis of the animal's body, an incision ¾ cm in length was made ½ cm from the rostral edge of the bony hip. Each fallopian tube was located bilaterally and both ovaries were removed and the transection site cauterized. Treatment with 14.2 mM LiCl in their drinking water that contained 0.9% saline began at 5 months of age for 1 month; LiCl at 14.2 mM maintains lithium blood content at a sub-therapeutic range (0.1–0.5 mM) (Sadeghipour-Roudsari et al. 1998). The control group (Sham) received drinking water with 0.9% saline. All animals were divided into four groups (LiCl-treated bOVX and Sham; saline-treated bOVX and Sham). Sham animals received the incision, the fallopian tube located and ovaries identified, but not removed, and the incision site was sutured.

Uterine and bone weight

All mice (n=20) were anesthetized with ether and then euthanized via cervical dislocation. Brain, uteri, femora and humeri were removed from each experimental mouse. Each uterus was weighed and inspected to assure complete removal of ovaries during bOVX surgery. Bones were cleaned of soft tissue and dried at 70°C for 24 h, then weighed.

Enzyme linked immunosorbent assay (ELISA)

Prior to cervical dislocation, 500-750 μl of blood was drawn from the tail of each subject (n=20) to measure 17β-Estradiol (E2) level. Murine plasma was isolated via centrifugation at 600×g for 15 min after a 4°C overnight storage with 6% EDTA. We performed the ELISA using an Estradiol EIA kit (96 well kit; Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's protocol. Briefly, murine plasma (100–300 μL) was purified using ethyl ether, organic phase was isolated and the remaining ether was evaporated with a gentle stream of N_2 gas at room temperature. Purified residue was then diluted with EIA buffer (100–300 μL) and assayed in duplicates. After 1 hour incubation with estradiol EIA antiserum and estradiol-acetylcholinesterase tracer, the microplate was developed for 45 min using Ellman's reagent and absorbance units measured using an EL_X Ultra Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT).

Total RNA isolation and reverse transcription (RT)

Total RNA was isolated from brain tissue (n=12) using TRIzol reagent (GIBCO, Carlsbad, CA), according to the manufacturer's protocol. Briefly, the brain was removed from each subject; the cortex and hippocampus from each hemisphere were isolated under a Leica ZOOM 200 dissecting microscope, weighed, and rinsed with Dulbecco's Phosphate Buffer Saline (DPBS). A 1:10 mass: TRIzol reagent was added then homogenized with an IKA homogenizer (at speed 5 for 10 s). Chloroform was added and centrifuged at 12 000×g for 15 min at 4°C. After phase separation, total RNA (aqueous phase) was then precipitated with isopropanol and RNA was pelleted via centrifugation. Total RNA pellet was washed with 75% ethanol and then resuspended in DEPC-treated water. To remove any DNA containments we used RQ1 DNase kit (Promega, Madison, WI). An aliquot of samples was used for concentrations and purity quantification using absorptions at 260 nm and 280 nm.

First strand cDNA synthesis was performed using SUPERSCRIPT™ III RNase H-free reverse transcriptase (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Briefly, 3 µg of total RNA was reverse transcribed with 0.05 µg/µl of Oligo(dT)$_{20}$ at 65°C for 5 min. First strand cDNA was synthesized with Superscript III/RNase OUT Enzyme mix and incubated at 50°C for 50 min; reaction was terminated at 85°C for 5 min. RNase H (Invitrogen, Carlsbad, CA) was added once first strand was synthesized to remove any remaining RNA. RNase H was incubated at 37°C for 20 min. Samples were stored at -20°C until further processing.

Quantitative real-time polymerase chain reaction (PCR)

First strand cDNA was amplified via real time PCR using SYBR Green PCR master mix (ThermoScientific, Rockford, IL), 200-300 nM of forward and reverse primers using AB 7300 Real Time PCR system. Cycling parameters were set at: 95°C 30 s, 57–63°C 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' (PCR efficiency = 94%); NMDAR subunit NR1: forward primer, 5'-ACTCCCAACGACCACTTCAC-3; reverse primer, 5'-GTAGACGCGCATCATCTCAA-3' (PCR efficiency = 90%); Bcl-2: forward primer, 5'-AGGAGCAGGTGCCTACAAGA-3; reverse primer, 5'-GCATTTTCCCACCACTGTCT-3' (PCR efficiency = 101%); BDNF: forward primer, 5'-ATCCAAATATGGCACAGCAA-3; reverse primer, 5'-TTCTGCCTGAGTTTTGATGC-3' (PCR efficiency = 100%); CaMkII-α forward 5'-GGGTTTGGCTCTTGTATGGA-3', reverse 5'-CTCTCCGTGCTTTTGGTCTC-3' (PCR efficiency = 99%). The endogenous control was HPRT: forward primer, 5'-GGAGCGGTAGCACCTCCT-3'; reverse primer, 5'-AATCCAGCAGGTCAGCAAAG-3' (PCR efficiency = 86%). All samples were compared with a standard curve comprised of pDNA generated using TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA). Readings were normalized by dividing interest gene number of mRNA copies by the housekeeping gene (HPRT) number of copies; the output is this ratio.

Statistical analysis

Data are presented as the mean ± S.E.M and statistical significance was determined by an analysis of variance (ANOVA) or a multiple analysis of variance (MANOVA) followed by a Fisher's least significant difference (LSD) post-hoc procedure. Significant differences were those having a P-value<0.05.

RESULTS

E2 plasma levels

There is an obvious trend in Fig. 1, suggesting a rescue in lithium-treated bOVX mice E2 plasma level. No significant differences, however, were detected in plasma E2 content across treatment groups (Fig. 1; $F_{3,15}$=0.7, P>0.5).

Uterine and bone weight

Statistical analysis using ANOVA indicated that uterine weight was significantly less ($F_{3,16}$=45.1, P<0.001) in bOVX mice (non-treated and treated) compared with Sham mice (non-treated and treated), but post-hoc testing detected no significant differences (P>0.8) between LiCl-treated and non-treated groups

(Fig. 2A). Using MANOVA, we analyzed dry bone weight from experimental mice and noted no significant differences in both humeri (Fig. 2B; $F_{3,28}$=2.1, P>0.1) or femora (Fig. 2C; $F_{3,28}$=2.6 P>0.07) across treatment groups; however, Fisher's LSD post-hoc testing indicated significant decreases in humeri weight (P<0.02) for LiCl-treated bOVX mice compared with LiCl-treated Sham and in femora weight (P<0.02) when LiCl-treated bOVX mice are compared with the other treatment groups (Fig. 2B).

Gene expression is enhanced by LiCl in the cortex of bOVX mice

Statistical analysis using MANOVA indicated significant differences between cortical and hippocampal gene expression: ER-α (Fig. 3A; $F_{1,14}$=6.1, P<0.03), NR1 (Fig. 3B; $F_{1,14}$=13.2, P<0.003), Bcl-2 (Fig. 3C; $F_{1,14}$=36.1, P<0.001), BDNF (Fig. 3D; $F_{1,14}$=20.8, P<0.001), and CaMkII-α (Fig. 3E; $F_{1,14}$=19.2, P<0.001) – ER-α (Fig. 3A), NR1 (Fig. 3B), and CaMkII-α (Fig. 3E) mRNA were expressed higher in the cortex, while Bcl-2 (Fig. 3C) and BDNF (Fig. 3D) showed higher mRNA expression in the hippocampus. Statistical analysis using MANOVA also detected significant differences for ER-α (Fig. 3A; $F_{3,14}$=3.5, P<0.05) and NR1 mRNA (Fig. 3B; $F_{3,14}$=3.6, P<0.05) across treatment groups. Statistical analysis, however, noted no significant differences in mRNA expression across treatment groups for Bcl-2 (Fig. 3C; $F_{3,14}$=1.1, P>0.3), BDNF (Fig. 3D; $F_{3,14}$=1.6, P>0.2), or CaMkII-α (Fig. 3E; $F_{3,14}$=3.3,

P>0.05). No significant differences were noted for the interaction between brain region and treatment group for ER-α ($F_{3,14}$=0.2, P>0.9), NR1 ($F_{3,14}$=2.9, P>0.07), Bcl-2 ($F_{3,14}$=1.0, P>0.4), and BDNF ($F_{3,14}$=0.04, P>0.9), except for CaMkII-α ($F_{3,14}$=3.7, P<0.05).

Since a significant difference was noted for brain region we performed a MANOVA for the hippocampus and cortex, separately. No significant differences in the hippocampus were noted for each gene: ER-α (Fig. 3A; $F_{3,7}$=0.9, P>0.4), NR1 (Fig. 3B; $F_{3,7}$=1.5, P>0.4), Bcl-2 (Fig. 3C; $F_{3,7}$=1.0, P>0.4), BDNF (Fig. 3D; $F_{3,7}$=0.0, P>0.7), and CaMkII-α (Fig. 3E; $F_{3,7}$=0.7, P>0.5). Cortical mRNA, however, showed significant values for all genes except for NR1 (Fig. 3B; $F_{3,7}$=3.8, P>0. 1), ER-α (Fig. 3A; $F_{3,7}$=4.9, P<0.05), Bcl-2 (Fig. 3C; $F_{3,7}$=13.2, P<0.003), BDNF (Fig. 3D; $F_{3,7}$=6.1, P<0.025), and CaMkII-α (Fig. 3E; $F_{3,7}$=4.9, P<0.05). Fisher's LSD post-hoc testing on cortical mRNA indicated that all genes showed significant increases in expression (P<0.05) for LiCl-treated bOVX when compared with all other treatment groups (Fig. 3A-3E).

DISCUSSION

We noted no differences in E2 plasma levels across treatment groups (Fig. 1; experimental mice showed 35–40 pg/ml E2 plasma level – indicative of E2 levels during the proestrous cycle); however, removal of the ovaries resulted in uterine atrophy since we clearly show that uterine weight of bOVX mice was significantly lower compared with Sham mice (Fig. 2A). The non-significant output of E2 plasma levels (Fig. 1) may be due to treatment duration and biological differences between our replicates. Investigations have noted that there is an individual-dependent variation with mood stabilizing drugs (Lerer and Macciardi 2002); however, extending lithium treatment for bOVX mice may cause a significant increase in E2 plasma levels since there is a noticeable trend in our current study (Fig. 1). Lithium is reported to increase cell proliferation and hyperplasia in murine uteri (Gunin et al. 2004), and lithium treatment increases human (Zamani et al. 2009) and murine bone mass (Clement-Lacroix et al. 2005). In our study, we found no alterations in E2 plasma levels across treatment groups (Fig. 1), but reduced uterine weight suggested successful removal of ovaries (LiCl treatment caused no alterations in uterine weight – see Fig. 2A). We also noted that bone
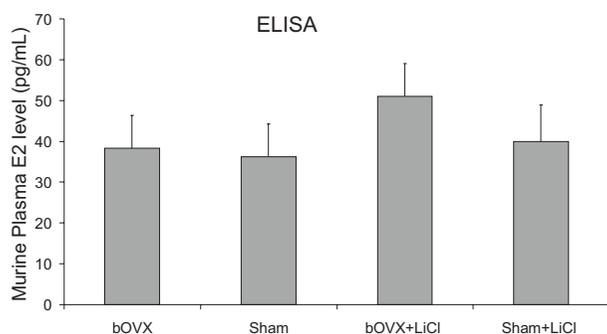

Fig. 1

17β-Estradiol (E2) content in murine plasma as measured via enzyme-linked immunosorbent assay (ELISA). Units for E2 content are in pg/mL (y-axis). Data are presented as the mean ± S.E.M

density actually decreased in LiCl-treated bOVX mice (Fig. 2B and 2C). An explanation eludes us regarding decreased bone mass in LiCl-treated bOVX mice and not non-treated bOVX, since the literature reports that after 3.5 months post-bOVX surgery, murine bone mass decreases (Masuda et al. 1997). Progesterone also diminishes following bOVX surgery (Galeeva and Tuohimaa 2001). Progesterone also affects uterine (Murray and Stone 1989) and bone tissue (Horner 2009). Measuring progesterone plasma levels may provide further insight, and histology on bone and uteri would provide a more detailed analysis on how our treatment parameters affect the architecture of these tissue types.

Previous research from our laboratory showed that male C57BL/6J mice treated with 14.2 mM LiCl display enhanced performance in a Morris Water Maze and an object recognition task over a 5 month treatment period (C-H Volmar, personal communication). Atomic absorption spectroscopy analyses of these male mice showed that treatment with 14.2 mM LiCl in their drinking water maintained a sub-therapeutic level of about 0.2 mM lithium in blood samples (CH Volmar, personal communication) – we found higher dosages lethal to bOVX mice. Blood samples at therapeutic levels range between 0.6-1.5 mM (Sadeghipour-Roudsari et al. 1998). Our present findings indicate that LiCl-treated bOVX mice showed enhanced cortical mRNA expression for ER-α (Fig. 3A), NR1 (Fig. 3B), Bcl-2 (Fig. 3C), BDNF (Fig. 3D) and CaMkII-α (Fig. 3E) compared with all other treatment groups.

Our results show increased mRNA expression is brain region-specific (Fig. 3A–3E; occurring in the cortex but not the hippocampus). These findings are consistent with the literature that lithium modulates several genes (Manji et al. 2001) and that this modulation is brain-region specific (Jakobsen and Wiborg 1998). Increased gene expression only occurring in LiCl-treated bOVX mice may be due to the bimodal action of lithium. Although we noted no differences in plasma levels of E2 (Fig. 1), removal of ovaries does cause a withdrawal of circulating hormones, but small amounts of hormones known as neurosteroids are still produced in the brain (Sierra 2004). The bimodal action of lithium may be a plausible explanation of our results. Jope (1999) proposes a bimodal model for lithium, such that lithium regulates positive and negative cell signaling mecha-
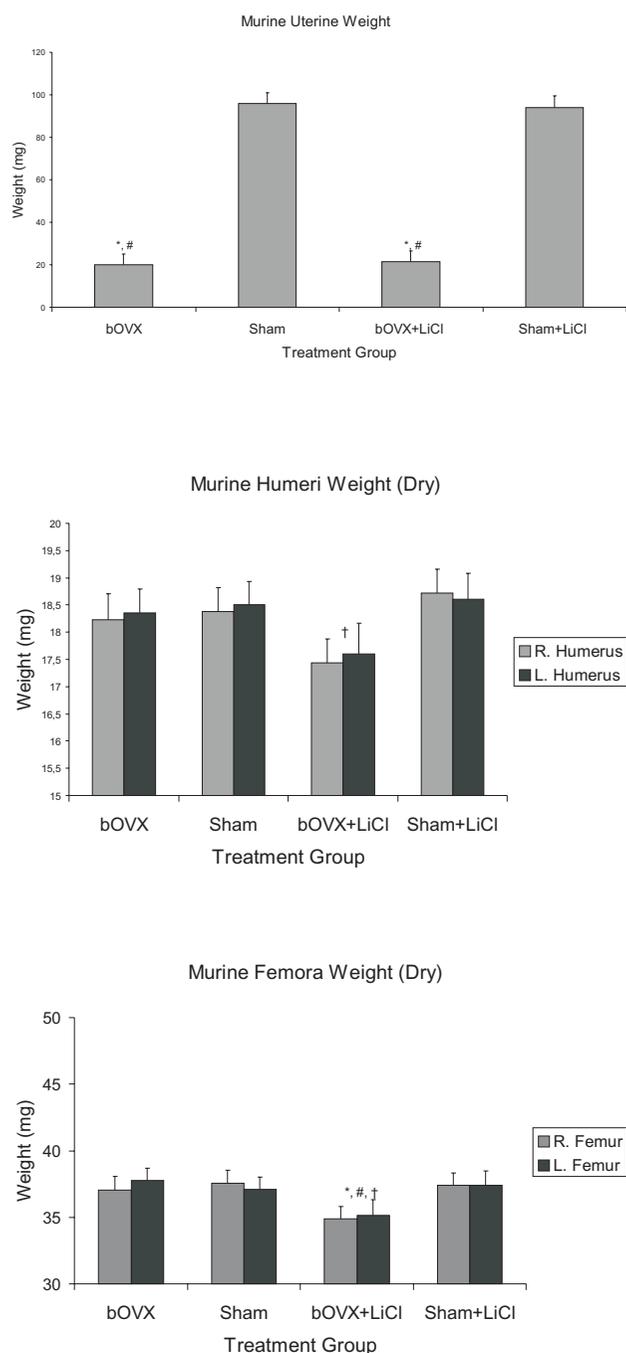

Fig. 2A-2C
Uterine (A) and bone weight (B & C) presented in mg (y-axis). The humeri (B; right = grey bars; left = black bars) and femora (C; right = grey bars; left = black bars) were used for dry bone weight. *, represents significant increase compared to bOVX; #, represents significant increase compared to Sham; †, represents significant increase compared to Sham+LiCl. Data are presented as the mean ± S.E.M Data are presented as the mean ± S.E.M
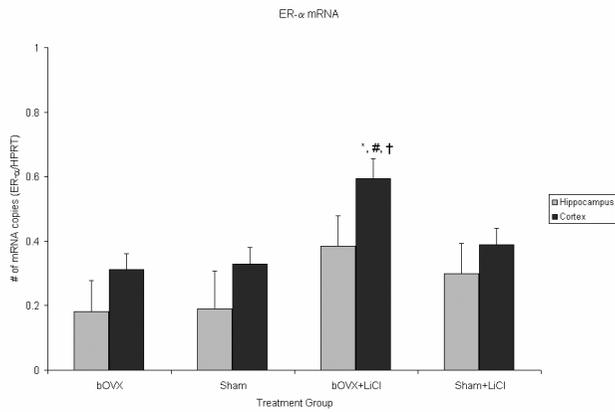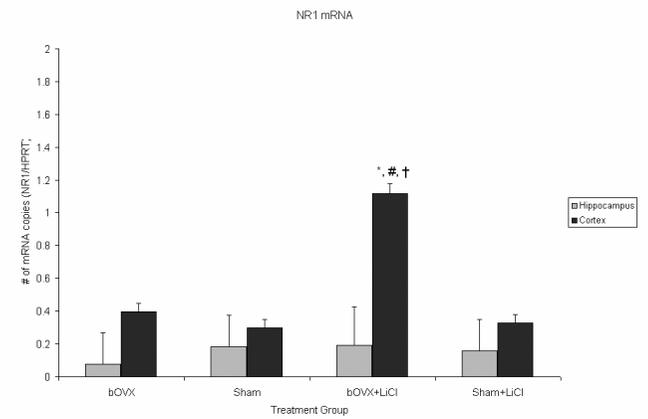
Figure 3A

ER-α mRNA

Figure 3B

NR1 mRNA

Figure 3C

Bcl-2 mRNA

Figure 3D

BDNF mRNA

Figure 3E

CaMkII-α mRNA
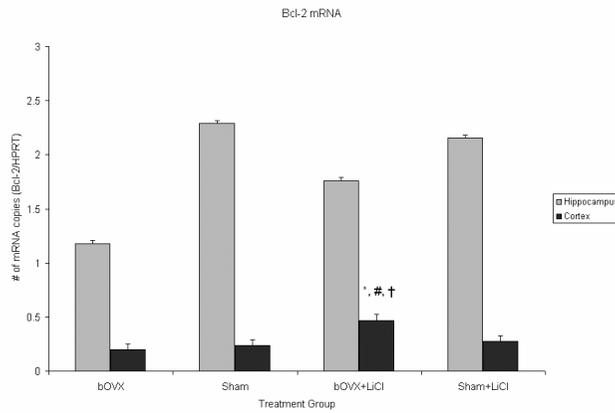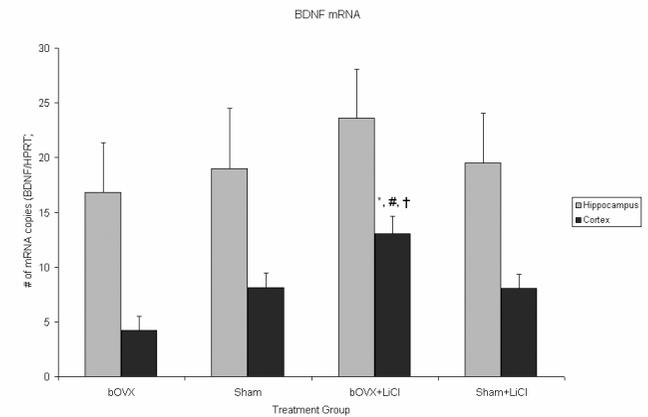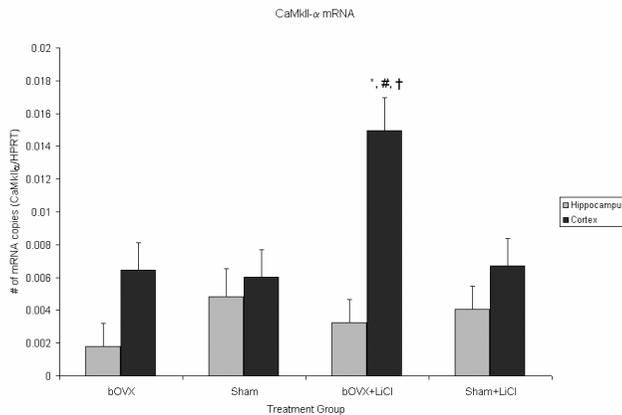
Fig. 3A-3E

Hippocampal (grey bars) and cortical (black bars) mRNA expression for ER-α (A), NR1 (B), Bcl-2 (C), BDNF (D), and CaMkII-α (E) using quantitative real time PCR. Units are represented as the number of mRNA copies (y-axis) for each respective gene (ratio of gene/HPRT). *, represents significant increase compared to bOVX; #, represents significant increase compared to Sham; †, represents significant increase compared to Sham+LiCl. Data are presented as the mean ± S.E.M

nisms by stabilizing extreme and/or minimized levels of integral factors involved in these cell signaling mechanisms. Lithium's bimodal mechanism may facilitate neurosteroidal-mediated brain cell signaling in a hormone-deprived system (e.g., bOVX). Studies do indicate that the molecular effects of lithium vary from acute to chronic treatment (Lenox and Watson 1994).

Although no significant differences were noted, genetic expression did decrease for non-treated bOVX mice in the hippocampus compared with Sham mice; and there is, at least graphically, a notable rescue for hippocampal genes NR1 (Fig. 3B), Bcl-2 (Fig. 3C) and CaMkII-α (Fig. 3E) in LiCl-treated bOVX mice. We currently have preliminary data indicating that protein levels are augmented in the hippocampus of LiCl-treated bOVX mice (data not shown). We do, however, need additional studies using bOVX mice with chronic versus acute lithium treatment and further investigations on how our treatment parameters affect protein levels of ER-α, NR1, Bcl-2, BDNF and CaMkII-α, are necessary to explain the neuroanatomical discrepancies noted in the current study.

CONCLUSION

Studies have shown that ERT reduces the development of neurodegenerative diseases and improves cognition (Garcia-Segura et al. 2001, Wise 2002), however, the latter has been recently challenged (Henderson 2009). It seems that risks associated with ERT outweigh its benefits, since ERT also increases incidences of breast cancer (Newcomer et al. 2003). Selective estrogen receptor modulators (SERMs) provide an alternative to ERT but studies show that SERMs do not improve cognitive functioning (Natale et al. 2004, Palmer et al. 2008). Post-menopausal and OVX pre-menopausal women may also suffer from cognitive decline if estrogen withdrawal is not immediately remedied (Sherwin 2005). Estrogen is essential for normal brain function by facilitating factors involved in learning, memory and neuroprotection. An alternative therapy that mimics the beneficial aspects of estrogen, without the harmful effects will serve as a better treatment for women with depleted ovarian steroids. The current study indicates that in bOVX mice, lithium enhances brain region-specific genetic factors that are involved in learning and mem-

ory (Fig. 3A–3E). Our data provides insight into potential positive clinical implications for lithium and how it may have beneficial promise for post-menopausal women or pre-menopausal OVX women. We believe that this study should serve to drive further investigations into lithium's potential benefits as an estrogen-mediated signaling modulator in an ovarian steroidal-deprived system.

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