Restraint stress influences AP-1 and CREB DNA-binding activity induced by chronic lithium treatment in the rat frontal cortex and hippocampus

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Abstract

The therapeutic efficacy of mood stabilizers may involve the regulation of gene expression mediated by transcription factor activation. In this study, we investigated AP-1 and cAMP response element-binding protein (CREB) DNA-binding activity in the rat frontal cortex and hippocampus of rats fed a control diet, a lithium diet for 7 wk, or 6 wk of lithium, followed by withdrawal for 7 d. Subsequently, animals were exposed to restraint stress or no stress and the DNA-binding activities assessed at 2, 8 and 24 h post-stress. AP-1 activity was increased in both brain regions by lithium, an effect that persisted with lithium discontinuation. Restraint stress induced AP-1 activity in the frontal cortex of the control group. This stress-induced effect on AP-1 activity was attenuated in lithium-treated and lithium-withdrawn animals. AP-1 DNA binding was also induced by stress in the hippocampus of control animals and the activity diminished over time in the lithium and lithium-withdrawn groups. CREB activity also increased in the frontal cortex and hippocampus of the lithium-treated group. Stress increased CREB activity in the frontal cortex of the controls, and was slightly attenuated with lithium treatment. CREB activity in the hippocampus was insensitive to stress. The proteins involved in the AP-1 and CREB transcription complexes were also characterized. Our findings of increased AP-1 and CREB binding after lithium are consistent with lithium’s inhibitory effect on glycogen synthase kinase-3β, which has been shown to negatively regulate AP-1 and CREB transcriptional activity.

Key words: AP-1, CREB, frontal cortex, gel shift, glycogen synthase kinase-3β, hippocampus, lithium, stress.

Introduction

Despite more than 50 yr passing since the initial introduction of lithium as a treatment for mania, the pathophysiology of manic depressive illness remains elusive. In recent years, an increased understanding of the processes involved in several intracellular signalling pathways has provided better insights into the mechanism of action of this prophylactic medication and mood stabilizer. Signal transduction systems known to be targets of lithium include phospholipase C, adenylate cyclase–cAMP and phosphoinositol (PI) pathways, downstream effects on G proteins, protein kinase A (PKA) and protein kinase C (PKC) (Brunello and Tascedda, 2003). Inasmuch as the therapeutic action of lithium in bipolar disorder requires a prolonged period of treatment, it is clear that longer term actions on complex biological processes are involved. These processes are likely to include regulation of neuronal gene expression by important cellular transcription factors. We reported earlier that chronic lithium reduced the basal expression of c-fos mRNA, and attenuated the induced c-fos response to muscarinic stimulation by pilocarpine in rat frontal cortex and hippocampus (Mathé et al., 1995; Miller and Mathé, 1997). Evidence also suggests that lithium may function as a neuroprotectant, regulating neuroplasticity...
and cell survival (Gould and Manji, 2002; Li et al., 2002).

The observation that the activity of glycogen synthase kinase-3β (GSK-3β) is inhibited by lithium (Stambolic et al., 1996) has opened new avenues that may lead to a better understanding of lithium’s mechanism of action and possible application in several new therapeutic fields. Because GSK-3β phosphorylates numerous cellular substrates including cytoskeletal proteins, cell cycle proteins and a number of key transcription factors, this inhibitory action of lithium is thought to be an important component of its therapeutic action (Gould and Manji, 2005). Two important factors regulated by GSK-3β are Jun and creb response element-binding protein (CREB). Jun and Fos family proteins play an important role in neuronal function in response to a variety of physiological and pathological stimuli by altering the expression of genes containing activator protein-1 (AP-1) sequences that bind Fos:Jun heterodimeric or Jun:Jun homodimeric complexes (Morgan and Curran, 1991). AP-1 appears to play a dual role, inducing cellular apoptosis or cell survival depending upon the cellular context and stimulus (Hess et al., 2004). CREB, which recognizes the consensus sequence for the cAMP response element (CRE), is a key transcription factor whose activity is triggered by signalling cascades involving cAMP/PKA, calcium/calmodulin and extracellular signal-regulated kinase (ERK)/mitogen-activated kinases that result in phosphorylation of CREB (Schaywitz and Greenberg, 1999). Previous reports have shown that chronic lithium induces AP-1 and CREB DNA-binding activity in the frontal cortex and hippocampus (Ozaki and Chuang, 1997). CREB-binding activity in vitro (Grimes and Jope, 2001) and AP-1-binding activity in human and rat cells (Yuan et al., 1998). Earlier, we found that c-fos mRNA expression appears to be stably repressed by chronic lithium in the frontal cortex and hippocampus (Mathé et al., 1995) and that c-fos mRNA induction by acute injection stress was attenuated by chronic lithium in the frontal cortex and hippocampus (Miller and Mathé, 1997). Inasmuch as electroconvulsive therapy is also effective in treating mood disorders, it is of interest that repeated electroconvulsive seizure treatment in rats has also been shown to diminish stress-induced c-fos expression in the brain (Morinobu et al., 1995). Since there is much evidence that chronic lithium treatment impairs PI/ PKC and adenylate cyclase/cAMP/PKA mediated signal transduction (Manji et al., 1995) and decreases CREB phosphorylation (Wang et al., 1999), long-term lithium may alter any of the multiple interdependent factors regulating fos transcription such as AP-1 or CREB, which in its phosphorylated form is an important regulator of Fos (Lucibello et al., 1991). In this report, we examined the interaction of acute restraint stress with chronic lithium treatment and lithium discontinuation on AP-1 and CREB DNA-binding activity.

Methods

Chronic lithium treatment

Male Sprague–Dawley rats were housed five per cage and kept in standard temperature and light conditions with a 12-h light–dark cycle. The rats were allowed 1 wk to acclimatize to their new environment prior to initiation of lithium treatment at age 6 wk. They had free access to water and were fed normal laboratory rat chow (R36 Lactamin, Stockholm, Sweden n = 24) or identical rat chow containing 1.2 g/kg lithium (n = 48) on days 1–5 and on day 6 continued treatment with chow containing 2.4 g/kg lithium for 4 wk, finally receiving chow containing 3.0 g/kg lithium for an additional 2 wk. A subgroup of the lithium-treated animals (n = 24) were withdrawn from the lithium diet at the end of week 6. The remaining lithium-treated rats (n = 24) continued to receive the lithium diet and the control rats (n = 24) continued with the regular rat chow for an additional week. The amount of lithium-supplemented diet consumed by the individual rats was not determined; however, the amount of food consumed by all of the rats within each cage was monitored daily. In addition to free access to water, all lithium-exposed rats had free access to a bottle containing 0.45% NaCl to prevent lithium toxicity (Ellis and Lenox, 1990). Mean serum lithium concentrations were not determined; however, we have previously shown (Mathé et al., 1994) that this dietary treatment results in serum lithium levels within the range of therapeutic maintenance dose levels (0.4–0.8 mm/l) in humans. All of the experiments were conducted in accordance with the Karolinska Institutet’s Guidelines for the Care and Use of Laboratory Animals.

Acute restraint stress

At the end of week 7 of treatments, a subgroup of the control (n = 18), lithium (n = 18) and lithium-withdrawn (n = 18) groups were exposed to a single exposure to restraint stress of 30 min duration in Decapi-cones (Braintree Scientific, Braintree, MA, USA) and then returned to their home cages. The remaining rats [control (n = 6), lithium (n = 6) or lithium-withdrawn (n = 6)] were not subjected to restraint stress, and remained in their home cages during the
stress treatment of the other rats. The rats exposed to restraint stress from the various groups were sacrificed 2 h, 8 h and 24 h following the termination of the restraint stress along with the unstressed rat groups. The study was approved by Stockholm’s Ethical Committee for Animal Experiments. During lithium treatment, the rats groomed normally and no gross behavioural changes or signs of lithium toxicity were observed.

**Brain region preparation**

The rats were sacrificed by decapitation, brains removed and the frontal cortex and hippocampus dissected as described previously (Miller and Mathé, 1997). The tissues were immediately frozen in liquid nitrogen and stored at −80 °C until shipment for analysis of AP-1 and CREB DNA-binding activity. These brain regions have been shown to be relevant sites for the action of lithium because of their regulatory function in mood, emotion and attention.

**Preparation of brain nuclear extracts**

Nuclear extracts were prepared from frontal cortex and hippocampus samples essentially as described by Kaminska et al. (1996). Briefly, tissues were homogenized in 5 vol. Hepes buffer A [20 mM Hepes (pH 7.9) containing 25% glycerol, 0.84 M NaCl, 1.5 mM MgCl₂, 0.4 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, and protease inhibitors leupeptin (5 µg/ml), aprotinin (5 µg/ml) and pepstatin (2 µg/ml)]. NP-40 was added to 0.4% and vortexed, the homogenates allowed to stand on ice for 1 min, then centrifuged at 2000 g for 10 min in a microfuge. The pellets were resuspended in 1 ml Hepes A and recentrifuged at 3000 g for 10 min. The resulting pellet (crude nuclei) was resuspended in a defined volume, based on tissue weight, of Hepes B [20 mM Hepes (pH 7.9) containing 25% glycerol, 0.84 M NaCl, 1.5 mM MgCl₂, 0.4 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, and protease inhibitors leupeptin (5 µg/ml), aprotinin (5 µg/ml) and pepstatin (2 µg/ml)], incubated on ice with gentle tube inversion every 5 min for 20 min to lyse the nuclei. After centrifugation for 15 min at 15000 g the supernatant containing the nuclear proteins was transferred to a new tube and vortexed gently. A 1 µl sample was taken immediately for protein concentration analysis by the micro Bio-Rad method. The supernatants were aliquoted in volumes equivalent to 30 µg of protein, frozen on dry ice and stored at −80 °C until gel-shift analysis. Protein was determined using the Bio-Rad microassay with bovine serum albumin (BSA) as the standard.

**Labelling of oligonucleotides**

Double-stranded oligonucleotides with consensus sequences for AP-1 (5'-CGCTTGATGAGTCAGCG-C/GAA-3') and CRE (5'-AGAGATTGCCTGACGTCAGAAGCTAG-3') were labelled with [γ-³²P]ATP, 3000 Ci/mmol (PerkinElmer Life Science, Fremont, CA, USA) using the T⁴ polynucleotide kinase reaction according to the manufacturer’s protocol (Promega Corp., Madison, WI, USA). The radiolabelled oligonucleotides were purified by removing the unincorporated label using micro-spin G-25 Sephadex columns (Amersham Biosciences, Piscataway, NJ, USA). Incorporation of ³²P was determined by TCA precipitation with 35–55% recovery with specific activity of 3–5x10⁸ cpm/µg for AP-1 and 1.7–3.2x10⁸ cpm/µg for CREB, respectively.

**Electromobility shift assay: AP-1- and CRE-binding activity**

The gel-shift assay was modified slightly from the protocol described by Hassanain et al. (1993). Briefly, 10 µg of nuclear protein extract were incubated at 25 °C in Hepes binding buffer [4% glycerol, 10 mM Hepes (pH 7.9), 0.5 mM EDTA, 25 mM KCl, 1 µg/ml BSA, 2.5% NP-40] with the addition of 1 µg poly(di-dC) for 15 min at 25 °C in a 9 µl total volume. Radiolabelled AP-1 or CREB (1.3–1.6 nM) was then added in a 1 µl volume and the incubation continued for 20 min at 25 °C. Non-denaturing polyacrylamide gels (4% acrylamide/0.16% bis-acrylamide/2.5% glycerol) in 1× Tris–glycine-EDTA buffer [50 mM Tris, 380 mM glycine, 2 mM EDTA (pH 8)] were pre-electrophoresed at 100 V for 30 min at 4 °C prior to loading the samples. Following the incubation, 1.5 µl of 10× gel-shift loading buffer [250 mM Tris–HCl (pH 7.5), 0.2% Bromophenol Blue, 40% glycerol] was added and the DNA-protein complexes electrophoretically separated at 4 °C at 100 V for 1 h. The gels were vacuum dried and exposed to X-OMAT film using an intensifying screen. Specificity of binding was determined in competition experiments by including excess (88–175 nM) unlabelled double-stranded AP-1 or CREB oligonucleotide in the reaction mixture.

Supershift analyses were performed with 1 µl (1 µg/µl) of TransCruz Gel Supershift antisera (Santa Cruz Biotechnology, Santa Cruz, CA, USA): sc-253, c-Fos; sc-48, FosB; sc-45, c-Jun; sc-73, junB and sc-44, junD or 1 µl sc-58 (0.5 µg/µl) CREB, or 2.5 µl phosphorylated CREB (pcREB, 0.3 µg/µl, Upstate Cell Signaling Technologies, Charlottesville, VA, USA). The antisera were incubated with 10 µg nuclear extracts for 1 h at 4 °C before the addition of
radiolabelled AP-1, or for 2 h at 4°C before the addition of radiolabelled CREB. For both binding activities, the incubation was continued for an additional 20 min at 25°C followed by gel separation as above. Supershifts were also conducted in the absence of extracts with the antibodies as a control for antisera effects on retardation of the labelled probe.

Data and statistical analysis

Differences in binding activities for AP-1 and CREB were quantified by determining the optical density for each sample using a CCD camera video system (Scion Corp., Frederick, MD, USA) and NIH Image J software. Possible differences in body weights and weights of the frontal cortex and hippocampus were statistically evaluated using a one-way ANOVA. Post-hoc comparisons were made with the Student’s–Newman–Keuls procedure. The DNA-binding data were expressed as percentage of the control diet within the diet treatments and within the various times post-stress. The data were subjected to two-factor ANOVA, SigmaStat for Windows with factor A=chronic diet treatments (control, lithium, lithium-withdrawn) and factor B=time after restraint stress (no stress [0], 2 h, 8 h and 24 h) as main effects. Post-hoc comparisons were made using the Student’s–Newman–Keuls procedure.

Results

Effect of chronic lithium and lithium discontinuation on brain and body weight

No significant effect of the chronic lithium regimen was observed in the weights of the frontal cortex (one-way ANOVA: F = 0.894, d.f. = 11, 60, p = 0.551) or hippocampus (F = 0.747, d.f. = 11, 60, p = 0.69). However, body weights were affected by the lithium treatment (F = 55.312, d.f. = 2, 8, p < 0.001). Rats in the lithium group weighed ~25% less than the rats receiving the control diet (366.8 ± 41.2 vs. 488.2 ± 39.8, p < 0.001). The body-weight recovery was rather fast, as the lithium-withdrawn group weighed only 11% less (430.3 ± 37.7) compared to the controls (statistically still a significant difference, p < 0.001). These data are consistent with previous studies in rats (Gilmor et al., 2003; Seggie et al., 1982) but are not in agreement with findings in humans where weight gain has been reported to be associated with lithium maintenance therapy (Nemeroff, 2003; Vestergaard et al., 1988).

Chronic lithium and acute restraint stress effects on AP-1-binding activity

A single specific AP-1-binding band was detected by gel shift in both hippocampus and frontal cortex under our experimental conditions. The specific AP-1 band could be displaced by an excess concentration (100 nM) of unlabelled AP-1 oligonucleotide.

Frontal cortex

Two-factor ANOVA revealed the following effects of lithium and stress on AP-1 DNA-binding activity in the frontal cortex [Factor A (diet treatment): F = 1.163, d.f. = 2, 59, p = 0.319; Factor B (stress): F = 0.442, d.f. = 3, 59, p = 0.724; A × B: F = 4.345, d.f. = 6, 59, p < 0.001]. Post-hoc comparisons revealed that in unstressed animals, AP-1 DNA-binding activity was significantly increased in the frontal cortex after chronic lithium, both at the end of treatment and after lithium withdrawal when compared to animals on the control diet without lithium added (Figure 1). A significant interaction between the dietary treatments and stress was found in the frontal cortex. In animals receiving the control diet, a significant increase in AP-1-binding was observed in frontal cortex, 2 h and 8 h post-stress. In the lithium and lithium-withdrawn groups, AP-1 DNA binding in the frontal cortex was significantly lower when compared to 2 h post-stress time of the regular diet controls. In the control diet group, frontal cortex AP-1 binding was increased by 39.7% at 2 h post-stress. The induction of AP-1 binding seen in the absence of stress in the lithium and lithium-withdrawn groups was attenuated 2 h post-stress by 7% and 23%, respectively.

Figure 1. AP-1 DNA-binding activity in the frontal cortex (% of control diet, mean ± s.e.). Post-hoc comparisons, Student’s–Newman–Keuls: lithium effects: ** p < 0.02 vs. control diet. Stress effects: +++ p < 0.001, + p < 0.05 vs. no stress control diet; ^ p < 0.05, ^^^ p < 0.02 vs. corresponding stress time of control diet.
Two-factor ANOVA revealed the following effects in the hippocampus [Factor A (diet): $F(2, 60) = 0.466$, $p = 0.629$; Factor B (stress): $F(2, 60) = 2.844$, $p = 0.045$; $A \times B$: $F(6, 60) = 2.745$, $p = 0.020$]. Post-hoc comparisons revealed that in unstressed animals, AP-1 DNA-binding activity was significantly increased in the hippocampus at the end of chronic lithium treatment and after lithium withdrawal, compared to control animals not treated with lithium (Figure 2). A significant interaction between the dietary treatments and stress was also observed in the hippocampus. Two hours after restraint stress, a significant increase in AP-1 binding was observed in rats receiving the control diet, but not in rats fed the lithium diet. AP-1 DNA binding was lower at 2 h post-stress only after lithium withdrawal in comparison to controls.

**Chronic lithium and acute restraint stress effects on CREB-binding activity**

Two bands of CREB-binding activity were detected on gels with nuclear extracts derived from the hippocampus as evidenced by displacement of the bands with an excess concentration (100 nM) of CREB oligonucleotide, but two distinct bands were not seen with nuclear extracts derived from the frontal cortex. Data analysis was performed on both bands in the hippocampus and no differences were seen among the groups between bands. The hippocampal data reported represents the upper band.

**Frontal cortex**

Two-factor ANOVA revealed the following effects of lithium and stress on CREB DNA-binding activity in the frontal cortex [Factor A (diet): $F = 19.495$, d.f. = 2, 60, $p < 0.001$; Factor B (stress): $F = 16.747$, d.f. = 3, 60, $p < 0.001$; $A \times B$: $F = 4.489$, d.f. = 6, 60, $p < 0.001$]. An interaction of diet treatment and stress was observed in the frontal cortex. Post-hoc comparisons revealed a significant inducing effect of lithium on CREB DNA-binding activity in the frontal cortex (Figure 3); however, this effect was not seen after lithium withdrawal, the activity returning to that of the controls. Two hours’ post-stress, CREB-binding activity was increased in the frontal cortex in rats receiving the control diet. CREB-binding activity in the lithium group diminished non-significantly 2 h post-stress when compared to the lithium group in the absence of stress, but was no different than the control group at 2 h post-stress. At 8 h post-stress, the CREB activity was significantly higher than the 8 h post-stress control group. At 24 h post-stress the activity was significantly diminished compared to the unstressed lithium-treated group. CREB activity in lithium-withdrawn rats was significantly increased 2 h post-stress in comparison to the activity seen in the same group under unstressed conditions, but was only slightly attenuated compared to the activity seen in the control group at 2 h post-stress.

**Hippocampus**

Two-factor ANOVA revealed the following effects of lithium and stress on CREB DNA-binding activity in the hippocampus [Factor A (diet): $F(2, 60) = 19.495$, d.f. = 2, 60, $p < 0.001$; Factor B (stress): $F(3, 60) = 16.747$, d.f. = 3, 60, $p < 0.001$; $A \times B$: $F(6, 60) = 4.489$, d.f. = 6, 60, $p < 0.001$]. An interaction of diet treatment and stress was observed in the frontal cortex. Post-hoc comparisons revealed a significant inducing effect of lithium on CREB DNA-binding activity in the frontal cortex (Figure 3); however, this effect was not seen after lithium withdrawal, the activity returning to that of the controls. Two hours’ post-stress, CREB-binding activity was increased in the frontal cortex in rats receiving the control diet. CREB-binding activity in the lithium group diminished non-significantly 2 h post-stress when compared to the lithium group in the absence of stress, but was no different than the control group at 2 h post-stress. At 8 h post-stress, the CREB activity was significantly higher than the 8 h post-stress control group. At 24 h post-stress the activity was significantly diminished compared to the unstressed lithium-treated group. CREB activity in lithium-withdrawn rats was significantly increased 2 h post-stress in comparison to the activity seen in the same group under unstressed conditions, but was only slightly attenuated compared to the activity seen in the control group at 2 h post-stress.
the hippocampus [Factor A (diet): $F = 8.199$, d.f. = 2, 60, $p < 0.001$; Factor B (stress): $F = 4.091$, d.f. = 3, 60, $p = 0.010$; A × B: $F = 1.460$, d.f. = 6, 60, $p = 0.207$]. In the absence of stress, post-hoc comparisons revealed a significant increase in CREB DNA-binding activity in the lithium and lithium-withdrawn groups when compared to animals receiving the control diet (Figure 4). In contrast to the frontal cortex, restraint stress did not significantly affect CREB-binding activity in the hippocampus of rats receiving the control diet. CREB activity diminished over time in the lithium and lithium-withdrawn groups post-stress, but the effects were not significant in comparison to the control diet group. However, in the lithium-treated group, at 24 h post-stress, CREB-binding activity was significantly lower than the activity seen in this group in the absence of stress.

Representative autoradiograms of AP-1 DNA binding and CREB DNA binding in the gel-shift assay are shown in Figure 5a–d.

**Transcription factors involved in AP-1 and CREB-binding complexes**

Identification of the DNA complexes involved in the activation of AP-1 and CREB binding was tested in nuclear extracts of the frontal cortex and hippocampus from unstressed and stressed rats fed a control diet, a lithium diet, or 6 wk of lithium diet followed by 1-wk withdrawal. Antisera to c-Fos, FosB, c-Jun and JunD produced a supershift of the specific band of AP-1 binding in the hippocampus and frontal cortex in all treatment groups (Figure 6a,b). Antisera to c-Jun had a more pronounced band-shifting effect in the hippocampus than in the frontal cortex. JunB antisera did not produce a supershift of AP-1 binding, regardless of dietary treatment or stress. Stress did not alter the components of the AP-1 complex in either brain region (some data not shown). CREB antisera produced a marked supershift of the specific AP-1 band in unstressed [Figure 6c(i)] and stressed groups [Figure 6c(ii)], regardless of brain region or treatment. pCREB antisera produced a slight probable shift of the AP-1 complex [Figure 6c(i)] and a decrease in binding intensity [Figure 6c(ii)] rather than a mobility shift (all data not shown).

Supershift analyses of the components of CREB DNA-binding activity showed that CREB antisera supershifted CREB bands in the frontal cortex, regardless of diet treatment or stress (Figure 7a). Using a lower amount of protein (5 μg) in the frontal cortex, a shift by pCREB antisera is also seen (Figure 7a). CREB and pCREB antisera also supershifted CREB bands in the hippocampus (Figure 7b), regardless of dietary treatment or stress (all data not shown). Fos antisera
did not produce a supershift of the CREB bands in either stressed or unstressed groups (some data not shown) regardless of dietary treatment in the frontal cortex or hippocampus. Specificity of binding shown with unlabelled competitor (see Figure 7a, b) and the lack of an effect of pCREB or CREB antisera to retard labelled CREB in the absence of extract, indicate that the shifted bands represent CREB–DNA complexes. Analyses with Jun antisera were not conducted.

**Discussion**

Lithium administered chronically to rats in the diet at a therapeutically relevant dose was found to induce an increase in DNA binding to AP-1 and CRE consensus sequences in the frontal cortex and hippocampus. These data are in agreement with previous observations of an increase in AP-1 and CREB binding in these brain regions after 4 wk of dietary lithium treatment (Ozaki and Chuang, 1997); and an increase in AP-1 binding in the frontal cortex and hippocampus after 14 d of lithium in the diet (Tamura et al., 2002). However, a study by Wang et al. (1999) in SH-SY5Y neuroblastoma cells reported that chronic lithium had no effect on CREB DNA binding. The latter data are in contrast to a more recent study suggesting that CREB-binding activity in SH-SY5Y cells is facilitated by lithium (Grimes and Jope, 2001). After lithium

\[\text{Figure 6. Supershift analyses of AP-1 binding in (a) hippocampus and (b) frontal cortex illustrating supershifted band of the binding complex with c-Fos, FosB, c-Jun and JunD antisera but not JunB antisera, and (c) [panels (i), (ii)] illustrating supershift of the AP-1 complex with CREB antisera and weak supershift with pCREB antisera in the frontal cortex and hippocampus. The following abbreviations are used: FC, frontal cortex; HC, hippocampus; SS, supershifted band; C, control; Li, lithium; Wd, lithium withdrawal; C2, Li2 or Wd2, 2 h post-stress.}\]
withdrawal, CREB-binding activity in the frontal cortex decreased to that of the control group, but remained elevated during withdrawal in the hippocampus. Our data are the first to report a persistent induction of AP-1 binding during discontinuation of lithium in the frontal cortex and hippocampus and on CREB binding after withdrawal in the hippocampus. In a study in which rats were administered lithium for 3–14 d, achieving therapeutic levels, the half-life of lithium in the rat (6 h) was found to be shorter than that in humans (28 h) (Wood et al., 1986). This finding indicates that lithium levels would be markedly decreased following 1 wk of withdrawal. Thus, the persistence of the lithium-induced increase in AP-1 and CREB binding during lithium discontinuation is unlikely to be due to the presence of lithium and suggests persistent changes in the expression of neuroprotective proteins encoded by genes with AP-1 or CRE sites in their promoter.

Stress is believed to be an important factor in the vulnerability to a variety of mental illnesses including depression and bipolar disorder. In addition, the frontal cortex and hippocampus are prominent brain regions believed to be involved in the processing of not only emotional but also stressful information. Acute stress induces c-fos mRNA expression in rat frontal cortex (Bing et al., 1991; Mathé et al., 1995) and hippocampus (Morinobu et al., 1995). Fos protein (Melia et al., 1994) and c-jun mRNA expression are also induced by acute restraint stress (Lino de Oliveira et al., 1997). This neuronal activation is an integral part of the stress response to a variety of stimuli and involves activation of the hypothalamic–pituitary–adrenal (HPA) axis. In this report, we found that acute restraint stress induces AP-1 in the frontal cortex and hippocampus of animals fed a control or regular diet. Our findings are consistent with a previous finding that 90 min of restraint stress up-regulated the

Figure 7. Supershift analyses of CREB binding in (a) frontal cortex and (b) hippocampus illustrating supershifted band of the binding complex with CREB or pCREB antisera, but not c-Fos antisera. As shown in the two far right lanes of (a) and (b), CREB bands were displaced by excess of unlabelled CREB (100 nM), or in the absence of nuclear extract, CREB antisera or pCREB antisera did not alter retardation of the labelled probe. The following abbreviations are used: SS, supershifted band; C, control; Li, lithium; C2 or Li2, 2 h post-stress.
induction of AP-1 activity in the frontal cortex, but at odds with a lack of effect of stress on AP-1 activity in the hippocampus (Tamura et al., 2002). We found that the stress-induced increase in AP-1-binding activity was attenuated in both brain regions of chronic lithium-treated animals consistent with our earlier report showing that lithium attenuates stress-induced increases in c-fos expression in these same brain regions. However, Tamura et al. (2002) reported that pre-treatment of animals with lithium in the diet for 14 d did not reduce AP-1-binding activity induced by acute restraint stress. The differences in these data may reflect a much shorter treatment with lithium in the study by Tamura et al. (2002).

Few studies have examined the interaction of acute stress and lithium treatment on CREB activity. While an induction of CREB binding is seen in the frontal cortex under no stress conditions, with lithium treatment, a marked attenuation in CREB activity was observed only at 24 h post-stress. After lithium discontinuation, stress produced a similar effect to that of the control group, with a significant induction in CREB activity in the frontal cortex. In contrast, CREB-binding activity in the hippocampus appears to be insensitive to acute stress in control, lithium-treated and lithium-withdrawn animals.

Taken together, the observations of lithium-induced increases in the activity of two important transcription factors and the attenuation of the stress-induced increase in these activities by chronic lithium support a prophylactic action of lithium in bipolar disorder.

The supershift studies revealed that the Fos family members, c-Fos, FosB and Jun family members, c-Jun and JunD, are components of AP-1 binding in the frontal cortex and hippocampus. CREB also appears to be a component of the AP-1 complex. The rather weak supershift of the AP-1 complex by c-Jun and pCREB antisera but robust supershift produced by JunD, FosB and CREB antisera suggest that the latter proteins may be significant participants in possible homodimeric or heterodimeric complexes. Other Fos family members were not tested directly, such as Fra-1, Fra-2 and ΔFosB; thus, we cannot exclude the possibility that they may participate in the complex. CREB and pCREB were found to be components of CREB binding in the frontal cortex and hippocampus. In contrast to observing that CREB is a component of the AP-1 complex, c-Fos does not appear to be involved in the CREB-binding complex. The observation that pCREB antisera produced a reduction in overall binding intensity associated with the appearance of a weak supershift of the AP-1 complex, is at odds with the strong supershift of the AP-1 complex with pCREB antisera reported by Ozaki and Chuang (1997). Interestingly, using identical pCREB antisera, we observed a strong mobility shift of the CREB complex as did Ozaki and Chuang, and an increase in overall CREB-binding intensity, in contrast to a decrease in AP-1-binding intensity in the hippocampus and weak mobility shift of the AP-1 complex in the frontal cortex.

Lithium treatment, as shown by us and Ozaki and Chuang (1997) and stress do not appear to influence the characteristics of the AP-1 and CREB protein complexes. In particular, chronic lithium treatment in the absence of stress, and control rats subjected to restraint stress, both lead to induction of AP-1 DNA binding with the expression of AP-1 complex proteins that are identical. The supershift analyses indicate the participation of nuclear proteins in the AP-1 complex are immunoreactive with antisera to c-Fos, FosB, c-Jun, JunD, CREB and albeit weakly, pCREB, under these different and opposite conditions.

It is well established that lithium impairs PI/PKA signalling via G protein receptor coupling and signalling through PKC and calcium/calmodulin-dependent kinases. These signalling pathways directly or indirectly activate several transcription factors including members of the AP-1 family and CREB. Activation of the PKA path is one mechanism leading to CREB-mediated transcription. Activation of receptors coupled to PI hydrolysis can also lead to activation of PKA, PKC and influence CREB and AP-1. Current evidence indicates that these signalling pathways interact with GSK-3β. GSK-3β, recently recognized to be an important effector in the action of lithium, is a component of the Wnt signalling pathway (Corbella and Vieta, 2003). Lithium appears to mimic activation of Wnt signalling by increasing β-catenin levels, a response known to occur with GSK-3β inhibition. GSK-3β is normally constitutively active and its regulation is tightly controlled by phosphorylation which inhibits its activity. When active, GSK-3β regulates the phosphorylation of β-catenin which is degraded. In its dephosphorylated state β-catenin is translocated to the nucleus where it acts as a transcription factor at lymphoid enhancer factor (LEF) sites. GSK-3β has many cellular effects including regulation
of cytoskeletal proteins and is believed to be important in determining cell survival/cell death decisions (Manji and Duman, 2001). GSK-3β also regulates the transcriptional activity of CREB and AP-1. A number of findings provide evidence for an inhibitory effect of GSK-3β, through phosphorylation mechanisms, on CREB and AP-1 (Grimes and Jope, 2001; Nikolakaki et al., 1993). Phosphorylation of Jun by GSK-3β inhibits the binding of Jun to AP-1 sequences, and because Jun is a prominent component of AP-1 this may explain why lithium due to its inhibitory action on GSK-3β activity promotes AP-1 activity, as we have demonstrated. Although not as well studied, GSK-3β appears to functionally reduce CREB activity. Grimes and Jope (2001) have provided evidence from studies in which GSK-3β was overexpressed in SH-SY5Y cells, that GSK-3β inhibits CREB DNA-binding activity, while lithium enhances CREB activity. Thus, the inhibitory action of lithium on GSK-3β activity is also consistent with our findings of increased CREB binding in the frontal cortex and hippocampus after chronic lithium.

In summary, the DNA-binding activity of AP-1 and CREB is significantly increased by chronic lithium. The persistence of these changes after lithium withdrawal suggests a prominent role for these transcription factors in mediating the therapeutic efficacy of this mood stabilizer. Moreover, our observation that stress interacts with lithium to reduce the stress-inducing effect on AP-1, and to some degree CREB activity, supports a purported neuroprotective role of lithium. The targets of AP-1 and CREB activation that may be involved in lithium’s therapeutic action remain elusive. Identification of these targets may provide new insights into the mechanisms responsible for the anti-manic and mood-stabilizing action of lithium and the development of novel therapies.

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Statement of Interest

None.

References


