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Lithium Attenuates Nerve Growth Factor–Induced Activation of AP-1 DNA Binding Activity in PC12 Cells

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This investigation tested if lithium, the primary therapeutic agent for bipolar mood disorder, modulated activation of the AP-1 transcription factor in PC12 cells treated with nerve growth factor (NGF), which induces robust responses in these cells. NGF induced large, time-dependent increases in AP-1 DNA binding activity. Pretreatment with 5 mmol/L lithium for 24 h reduced AP-1 induction by NGF by 42%; shorter treatments and lower concentrations of lithium had smaller inhibitory effects on AP-1. This effect of lithium was not limited to AP-1, as it also inhibited NGF-induced cyclic AMP responsive element (CRE) DNA binding activity. In contrast, activation of AP-1 and CRE by forskolin was unaffected by lithium. AP-1 constituent proteins were differentially susceptible to lithium, as cJun was reduced by 55%, cFos was unaffected by lithium, and an intermediate effect was observed with Jun B. These results reveal that lithium modulates the activation of transcription factors in a neuronal cell model, indicating that selective regulation of gene expression may contribute to the long term in vivo effect of lithium. ***[Neuropsychopharmacology 1:12–17, 1997]*** *© 1997 American College of Neuropsychopharmacology*

KEY WORDS: *AP-1; CRE; Immediate early genes; Jun; Fos; Lithium; Bipolar disorder*

The mechanism of action of lithium, which is the primary treatment of bipolar affective disorder, is not known. Several lines of investigation have shown that lithium modulates the activities of certain receptor-coupled signal transduction processes, such as the phosphoinositide and cyclic AMP second messenger systems (reviewed in Jope and Williams 1994; Manji and Lenox 1994). Selective modulation of gene expression by lithium also has been reported (Wang and Young 1996; reviewed in Jope and Williams 1994); including, for example, those coding for proteins directly involved in signal transduction, such as G-proteins and adenylyl cyclase (Colin et al. 1991; Li et al. 1991). Whether this is due to altered activity of signaling systems or to more direct actions of lithium on transcription processes remains to be determined.

Of potential importance in associating signal transduction activity to gene expression are the immediate early gene protein products, such as members of the Fos and Jun families. The expression of these genes is activated rapidly by specific signaling systems and dimers of the protein products function as transcription factors (e.g., AP-1) to regulate the expression of a variety of genes (Morgan and Curran 1991). In this regard, it is interesting to note that lithium was reported to increase stimulus-induced c-fos mRNA levels in cultured astrocytes (Arenander et al. 1989) and PC12 cells (Kalasapudi et al. 1990; Divish et al. 1991). Whether or not the changes in c-fos mRNA levels in PC12 cells were associated with lithium-induced alterations in protein levels or transcription factor activity was not reported. Later it was shown that in contrast to the implications from the PC12 cell studies, lithium treatment of rats attenuated the in vivo stimulation of AP-1 DNA binding activity in the cerebral cortex (Williams and Jope 1995). This find-

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ing indicated that lithium modulates cellular responses to signaling systems that are coupled to AP-1 mediation of regulated gene expression.

In the present investigation we examined if lithium influenced AP-1 DNA binding activity in rat pheochromocytoma PC12 cells which, compared to in vivo studies with rat brain, provides a model system that facilitates experimental manipulation. Preliminary experiments revealed that exposure of PC12 cells to nerve growth factor (NGF) caused a robust increase in AP-1 DNA binding activity, so this treatment was used as the primary stimulus in these experiments to test if lithium modulated the activation of AP-1.

MATERIALS AND METHODS

Cell Culture

PC12 cells were obtained from Dr. L. A. Green (New York University) and were maintained in RPMI media with 10% horse serum, 5% fetal clone II (Gibco/BRL), 50 U/ml penicillin, and 100 μg/ml streptomycin on collagen-coated tissue culture dishes. Cells were generally passaged twice a week. Before each experiment, cells were seeded in complete media overnight and transferred to serum-free media for 16 h before the initiation of treatments.

Nuclear Protein Isolation

Nuclear extracts were isolated from PC12 cells by a procedure adapted from that of Dignam et al. (1983) as previously described (Unlap and Jope 1995). Cells were washed with 5 ml of cold phosphate buffered saline (PBS), harvested, and centrifuged at 500*g* for 10 min at 4°C. The pellet was resuspended in 4 ml of lysis buffer (10 mmol/L Tris-HCl, pH 7.4, 10 mmol/L NaCl, 3 mmol/L $MgCl_2$, and 0.5% NP-40) and lysed by repeatedly drawing through a 5-ml pipet. Nuclei were obtained by centrifugation at 500*g* for 10 min at 4°C, and were lysed in 50 μl of buffer C (20 mmol/L Hepes, pH 7.9, 20% glycerol, 0.3 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT), 0.1 mmol/L β-glycerophosphate, 2 mmol/L vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 μg/ml each of pepstatin A, leupeptin, and aprotinin) on ice for 30 min. Nuclear debris was removed by centrifugation at 16,000*g* for 15 min at 4°C and the protein concentration of the supernatant was determined by the method of Bradford (1976) prior to storage at −70°C.

Electrophoretic Mobility Shift Assay (EMSA) of AP-1 and CRE DNA Binding Activities

The EMSAs were performed as described previously (Unlap and Jope 1995). One picomole of double-stranded oligonucleotide DNA containing the AP-1 (5′-CTAGTGATGAGTCAGCCG-3′) or CRE (5′-AGCTCT CTGACGTCAGC-3′) consensus sequence was labeled at 37°C for 1 h in 20 μl containing 50 mmol/L Tris-HCl, pH 8.0, 5 mmol/L $MgCl_2$, 5 mmol/L DTT, 50 mmol/L KCl, 10 mmol/L each of dATP, dGTP, and dTTP, 50 μg bovine serum albumin, 100 μCi [α^{32}P]dCTP (New England Nuclear), and 40 U Klenow (Gibco/BRL). After 1 h, the samples were diluted to 100 μl with TE (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA) and free probe was removed by centrifugation at 1000 rpm for 45 sec on a Sephadex G-50 column. Binding was carried out in 20 μl containing 20 mmol/L Hepes, pH 7.9, 50 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.5 mmol/L DTT, 4% Ficoll, 1 μg poly dI/dC, 5 μg protein extract, and 5000 cpm of labeled oligonucleotide DNA probe for 30 min at 4°C. The reaction mixture was electrophoresed on a 6% nondenaturing polyacrylamide gel in 0.25 × TBE (22.3 mmol/L Tris, 22.3 mmol/L boric acid, 0.5 mmol/L EDTA) for 1.5 h at 150 V. The gel was vacuum-dried and exposed to XAR-5 film for permanent records. Quantitative values were obtained using a Phosphor Imager (Molecular Dynamics) using exposures within the linear response range, and statistical significance was determined using analysis of variance (ANOVA).

Western Blot

Nuclear protein extracts (20 μg protein) were diluted with Laemmli sample buffer, placed in a boiling water bath for 5 min, electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with antibodies to c-Jun, Jun B, (Oncogene Science) and Fos (provided by Dr. M. Iadorola, NIH). After incubation with the primary antibody, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG and color was developed with 3,3′-diaminobenzidine in the presence of H_2O_2. Immunoblots were subjected to quantitation using a BioRad imaging densitometer and data were analyzed for statistical significance using ANOVA with a post hoc Bonferroni test.

RESULTS

To test if serum in the growth media influenced AP-1 activity, stimulation of AP-1 DNA binding activity by NGF was compared in PC12 cells exposed to serum-free media, 5% serum, or 15% serum (i.e., normal growth media) for 16 h. Figure 1 shows that basal AP-1 DNA binding activity was similar in PC12 cells incubated in serum-free media, 5% serum, or 15% serum, and that the stimulation induced by NGF did not vary significantly among cells grown in each of the three conditions, although it was lowest in 15% serum. There-

fore, serum-free media, which supported the greatest response to NGF, was used in subsequent experiments.

To study the effect of lithium on stimulation of AP-1 and CRE activities in PC12 cells, AP-1 and CRE DNA binding activities were induced by treatment with NGF for varying periods of time in serum-free media (Figure 2). AP-1 DNA binding increased rapidly after the addition of NGF, reached a maximum between 4 and 12 h of treatment, and declined after 12 h but remained elevated at 24 h. CRE DNA binding, which resolved into two bands that were quantitated together, also was increased after NGF treatment, although much less than AP-1, it reached a maximum after 2 h, and remained elevated at 24 h.

To test if lithium modulated NGF-stimulated AP-1 DNA binding, PC12 cells were pretreated with 5 mmol/L LiCl for 2 to 24 h and then NGF was added, and AP-1 activity was measured after 2 h. Lithium (5 mmol/L) pretreatment had small inhibitory effects on NGF-stimulated AP-1 activity when cells were pretreated for 2 to 12 h, but after a 24-h exposure to LiCl the AP-1 response to NGF was inhibited by 42% (Table 1). A 24-h pretreatment with 1 mmol/L LiCl only reduced NGF-induced AP-1 by 9%, but 2 weeks of pretreatment resulted in 24% inhibition. Treatment for 24 h with 5 mmol/L lithium alone, in the absence of NGF, did not alter basal AP-1 DNA binding (103 ± 11% of basal $n = 4$).

To determine if the inhibitory effect of lithium on the response to NGF was specific for the AP-1 transcription factor, CRE DNA binding was also measured. A 24-h pretreatment with 5 mmol/L LiCl reduced by 40% CRE measured 2 h after exposure to NGF (Figure 3). The same treatment with lithium did not significantly reduce forskolin-induced CRE activity.

To determine if the inhibition by lithium of AP-1 activation was specific for the response to NGF, AP-1 was measured after stimulation by forskolin. A 24-h pretreatment with 5 mmol/L LiCl did not significantly alter forskolin-induced AP-1 activity (Figure 4).

Since the AP-1 transcription factor is composed of dimers of members of the Fos and Jun families, we examined if stimulation by NGF of three of the primary constituents, cJun, cFos, and Jun B, were inhibited equivalently by lithium treatment. Immediate early gene protein levels were measured by quantitative immunoblot analysis of PC12 extracts prepared 0.5 to 4 h after NGF treatment, with or without a 24-h pretreatment with 5 mmol/L lithium, and values were compared with untreated control cells (Figure 5). NGF treatment caused time-dependent increases in each of the

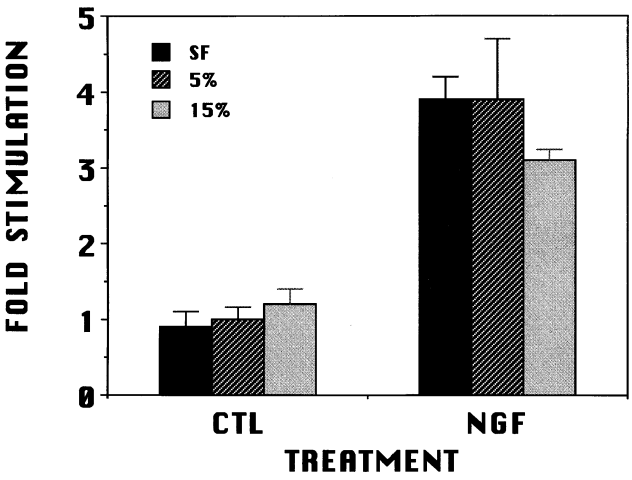

Figure 1. Effects of serum on AP-1 DNA binding. AP-1 DNA binding activity was measured in PC12 cells that had been incubated in media that was serum-free (SF), 5% serum, and 15% serum, and without (CTL) or with a 2-h exposure to NGF (50 ng/ml), as described in the Methods. The figure shows the fold stimulation of AP-1 compared with control cells (maintained in 15% serum and not treated with NGF). Means ± SEM ($n = 3$).

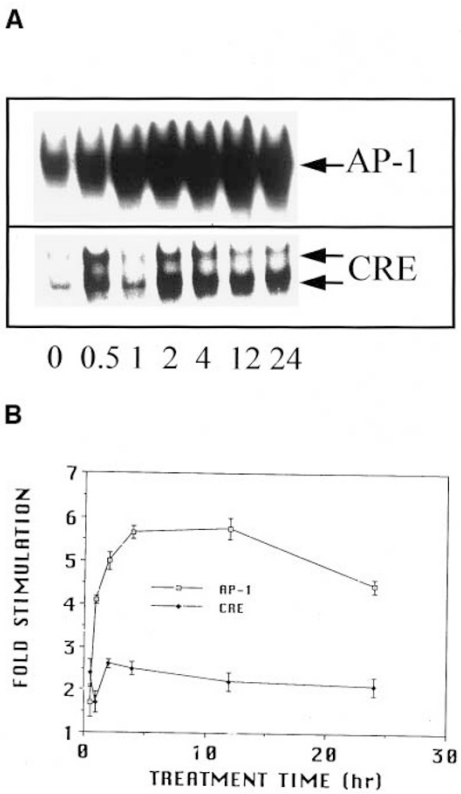

Figure 2. Time course of NGF-stimulated AP-1 and CRE DNA binding activity. PC12 cells were incubated with NGF (50 ng/ml) for 0.5, 1, 2, 4, 12, or 24 h and AP-1 and CRE DNA binding activities were measured as described in the Methods. (**A**) shows the results of a representative EMSA (only the relevant parts of the gels are shown), and (**B**) shows the fold stimulation compared with cells not exposed to NGF. Means ± SEM ($n = 4$).

Table 1. Effects of Duration and Concentration of Lithium Pretreatment on NGF-Induced AP-1 DNA Binding Activity

LiCl (mmol/L)	Pretreatment time (h)	*n*	Percent Stimulation of AP-1		Percent Inhibition by Lithium
			NGF	Lithium + NGF	
5	2	4	505 ± 59	465 ± 73	9 ± 11
5	4	4	545 ± 52	428 ± 50	22 ± 9
5	12	4	518 ± 28	415 ± 40	20 ± 6
5	24	18	492 ± 29	291 ± 24	42 ± 6[a]
1	24	7	446 ± 45	414 ± 25	7 ± 5
1	2 weeks	6	335 ± 10	255 ± 15	24 ± 5[a]

PC12 cells were exposed to lithium as indicated followed by measurement of AP-1 2 h after the addition of 50 ng/ml NGF.

[a] $p < .01$ (ANOVA).

three proteins, with maximum elevations ranging from five- to eight-fold compared with control cells. Pretreatment with lithium severely impaired the NGF-stimulated increase in cJun throughout the time period examined, causing an average 55% (range = 48–65%) inhibition of the elevation of cJun. In marked contrast, lithium had no effect on the NGF-induced increase in cFos at any of the time periods that was examined. Lithium's effects on Jun B levels were more variable than with cJun or cFos, with an inhibitory effect evident at the shorter times of exposure to NGF but not at the longest time period that was examined. These results indicate that inhibition by lithium of NGF-induced AP-1 DNA binding activity was at least in part due to a selective attenuation of the induction of cJun, as well as some inhibition of Jun B, whereas cFos was unaffected.

DISCUSSION

The primary goal of this investigation was to determine if lithium was capable of modulating transcription factor activation, an action that could contribute to long term effects of lithium on cell function by altering signal-induced changes in gene expression. The AP-1 transcription factor was chosen as the primary focus because it is modulated by lithium in rat brain in vivo (Williams and Jope 1995), and PC12 cells were used because they are studied widely as a neuronal model system and cfos expression in these cells was reported to be modulated by lithium (Kalasapudi et al. 1990; Divish et al. 1991). Treatment with NGF was chosen as the stimulus because it is well known to activate robust responses in PC12 cells (Szeberényi and Erhardt 1994), in-

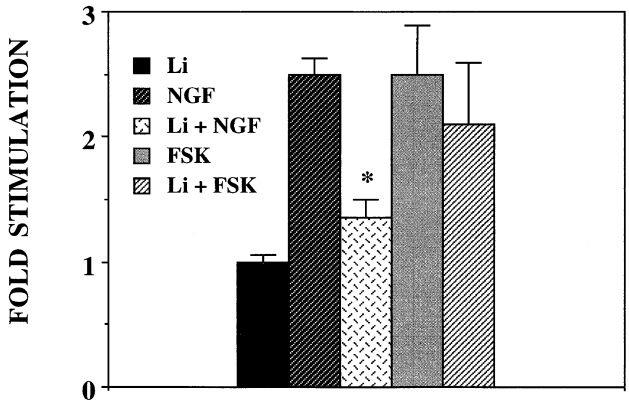

Figure 3. Effect of lithium on CRE activity. PC12 cells were preincubated with 0 or 5 mmol/L LiCl for 24 h and then treated for 2 h with NGF (50 ng/ml) or forskolin (FSK; 10 μmol/L) and CRE DNA binding activity was measured as described in the Methods. Values are given as the fold stimulation compared with untreated control cells. Means ± SEM ($n = 8$). *$p < 0.01$.

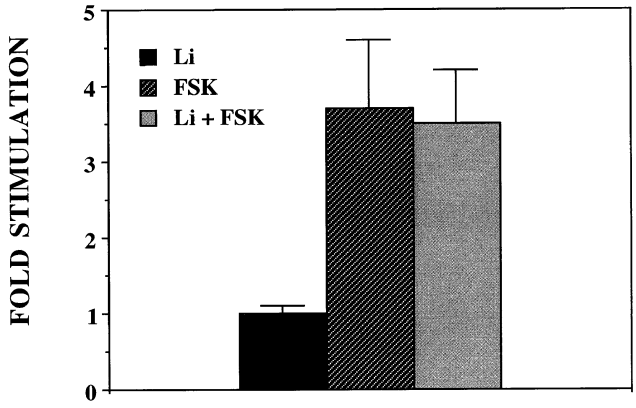

Figure 4. Effect of lithium on forskolin-induced AP-1 activity. PC12 cells were preincubated with 0 or 5 mmol/L LiCl for 24 h, then treated for 2 h with forskolin (FSK; 10 μmol/L), and AP-1 DNA binding activity was measured as described in the Methods. Values are given as the fold stimulation compared with untreated control cells. Means ± SEM ($n = 4$).

cluding increased expression of immediate early genes (Greenberg et al. 1985), and there is increasing evidence of interactions between psychotherapeutic agents and neurotrophin-mediated processes (Nibuya et al. 1995; reviewed in Heninger et al. 1996).

The first major finding of this study was that lithium pretreatment significantly attenuated activation of AP-1 induced by exposure of PC12 cells to NGF. With a 24-h pretreatment, a therapeutically relevant concentration of lithium (1 mmol/L) caused only a small attenuation of NGF-induced AP-1, whereas 5 mmol/L lithium caused a very significant inhibition of this response. Therefore, to obtain a large, reproducible inhibitory effect of lithium, a concentration of 5 mmol/L lithium was used in the majority of the experiments. Although it appears that this higher concentration of lithium was accentuating the response obtained with a therapeutic lithium concentration (1 mmol/L), the results must be interpreted cautiously since it is possible that the effects of 5 mmol/L lithium represent toxic, rather than therapeutic, responses to lithium (Burstein et al. 1985). It is not known if toxic effects of lithium are due to greater magnitude of effects at the same sites modulated by therapeutic levels of lithium, or if they result from different sites of action. Thus, at this point a definitive attribution cannot be made. However, the lack of inhibitory effects of 5 mmol/L lithium on forskolin-induced AP-1 or CRE activation suggest that general cellular toxicity caused by lithium does not account for its inhibition of activation by NGF. The present finding that lithium attenuated NGF-induced AP-1 activation in PC12 cells is in accordance with the finding that chronic lithium treatment of rats at a therapeutic level impaired the in vivo activation of AP-1 in the cerebral cortex (Williams and Jope 1995), an effect also observed with long-term (2 weeks) treatment of PC12 cells with a therapeutic concentration of lithium.

The second major finding of this investigation was that lithium differentially affected the levels of the AP-1 constituent proteins. It was quite surprising to find that the level of cFos was impervious to lithium treatment, whereas the NGF-induced increase in cJun was severely inhibited by lithium. Jun B levels demonstrated a sensitivity to lithium intermediate to that of cJun and cFos. These findings indicate that there is a precise selectivity in lithium's modulation of responses to NGF. This selectivity was also reported in lithium's modulation of NGF-induced increases in G-protein α-subunits in PC12 cells, only some of which were inhibited by lithium treatment (Li and Jope 1995). In that study it was observed that 5 mmol/L lithium did not alter the activation of mitogen-activated protein (MAP) kinase or phospholipase C-γ induced by NGF treatment of PC12 cells. Therefore, the mechanism of action of lithium to inhibit AP-1 activation, and more specifically the induction of cJun, must involve other sites in the signal cascade. Such modulation by lithium also resulted in inhibition of NGF-induced CRE DNA binding activity. Neither of

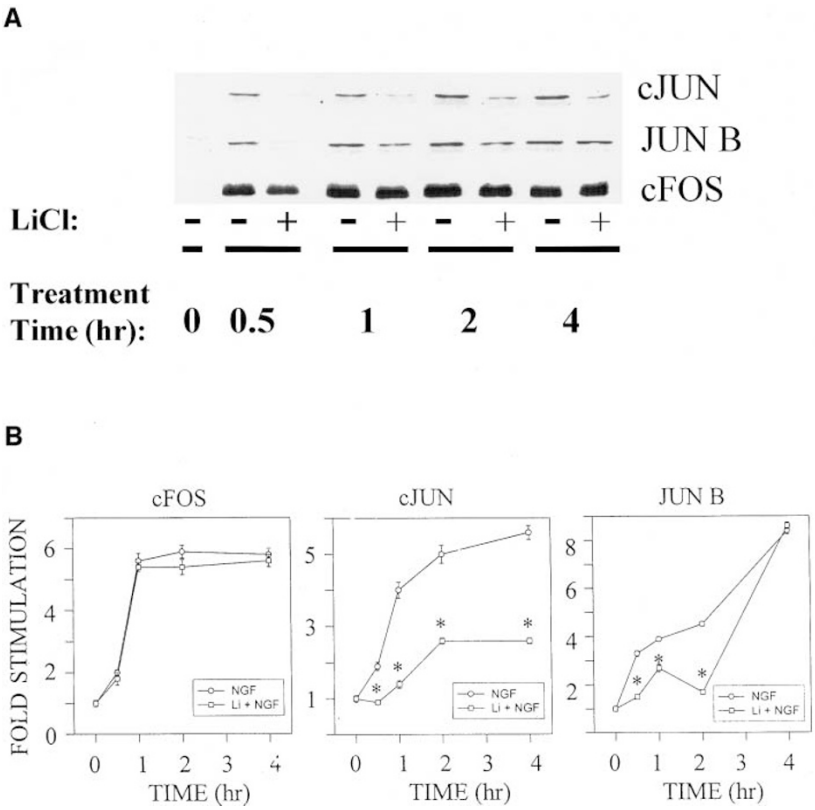

Figure 5. Effects of lithium on NGF-induced increases in cJun, Jun B, and cFos. PC12 cells were grown in the absence or presence of 5 mmol/L LiCl for 24 h followed by treatment with NGF (50 ng/ml) for 0.5, 1, 2, or 4 h. The levels of cFos, cJun, and Jun B were measured by quantitative immunoblots as described in the Methods. (**A**) shows representative immunoblots, (**B**) shows quantitative analysis of the data as the fold stimulation by NGF compared with untreated cells. Values shown at 0 h were obtained from lithium-treated or untreated cells that were not exposed to NGF. Means ± SEM (n = 6–16), *$p < .01$.

the inhibitory effects of lithium on AP-1 or CRE appeared to be a direct action on preformed transcription factors since lithium did not affect forskolin-induced AP-1 or CRE DNA binding activity. Thus, lithium treatment impaired a postreceptor site in the NGF-induced signaling cascade situated before the formation and action of the transcription factors. Since intracellular signaling processes are commonly utilized by multiple receptor-linked systems, it seems likely that lithium's effects are not limited to modulating the response to NGF, but other receptor stimulants have not yet been investigated.

There is increasing evidence that a critical effect of lithium that is potentially related to its therapeutic efficacy in affective disorders is its modulation of gene expression (reviewed in Jope and Williams 1994; Manji and Lenox 1994). Selective effects on gene expression could be attained by altering signal transduction processes, which lead to transcription factor activation, or actions further downstream affecting transcription factor activity. Studies using NGF-induced signaling as a model indicate that lithium is capable of acting at sites downstream of signal activation since only selective components of the signal response (e.g., cJun levels and only some G-protein subtypes) are modulated while others are impervious to lithium (e.g., cFos levels, MAP kinase activation). This selectivity can be predicted to be extended to the expression of genes modulated by AP-1. The AP-1 transcription factor is a heterogeneous mixture of Jun homodimers and Jun/Fos heterodimers, and it is thought that the composition of the AP-1 dimers contributes to the selectivity of effects on gene expression (Morgan and Curran 1991). Thus, the differential effect of lithium on the AP-1 constituent proteins provides a mechanism for lithium to selectively affect the expression of a subset of genes regulated by the AP-1 transcription factor.

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