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Research report

# Dynamics of c-fos and ICER mRNA expression in rat forebrain following lithium chloride injection

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**Abstract**

Lithium is commonly used as a treatment for affective disorders in humans and as a toxin to produce conditioned taste aversions in rats. LiCl administration in rats has been correlated with activation of c-fos and cAMP-mediated gene transcription in many brain regions; however, little is known about the timing or duration of gene activation. We hypothesized that c-fos gene transcription is rapidly stimulated by LiCl, followed later by the expression of the inducible cAMP early repressor (ICER) transcription factor, a negative modulator of cAMP-mediated gene transcription. By in situ hybridization, we analyzed the timecourse of c-fos and ICER mRNA expression in the central nucleus of the amygdala (CeA), the paraventricular nucleus of the hypothalamus (PVN) and the supraoptic nucleus (SON) at seven time points (0, 0.3, 1, 3, 6, 9 and 12 h) after intraperitoneal LiCl injection (0.15 M, 12 ml/kg, 76 mg/kg). Expression of c-fos mRNA peaked between 20 min and 1 h and returned to baseline by 3 h in the CeA, PVN and SON. ICER mRNA was detected in these regions at 20 min, peaked at 1–3 h and returned to nearly baseline 9 h following LiCl injection. The time lag between c-fos mRNA expression and ICER mRNA expression within the same regions is consistent with ICER terminating c-fos gene transcription. However, no refractory period was detected for restimulation of c-fos transcription by a second injection of LiCl during the period of peak ICER mRNA expression, suggesting the involvement of other transcriptional modulators. 

*Theme:* Neurotransmitters, modulators, transporters and receptors

*Topic:* Signal transduction: gene expression

*Keywords:* Immediate-early gene; Conditioned taste aversion; Bipolar disorder; Lithium toxicity; Refractory period

## 1. Introduction

Lithium is one of the most effective therapeutic agents prescribed for the treatment of bipolar (manic–depressive) disorder and depression. The antidepressant effects of lithium require 3–4 weeks of repeated administration and result in many long-term changes, including alterations in multiple neurotransmitter and signal transduction systems and gene expression (for review see [24]). One of the disadvantages in prescribing lithium is its narrow therapeutic index; the toxic dose of lithium is only 2–3 times higher than the therapeutic dose [1]. Symptoms of acute lithium toxicity in humans include nausea, vomiting, diarrhea, tremors, seizures and coma [1]. In rats, a non-emetic species, acute administration of toxic doses of LiCl has been shown to elicit a wide variety of behavioral and physiological effects, including anorexia [31], decreased activity [2], 'lying-on-belly' [33], delayed gastric emptying [27] and hypothermia [51]. LiCl is also widely used as the unconditioned stimulus in the formation of conditioned taste aversions (CTA), a form of learning that results from the pairing of a novel taste with the toxic effects produced by LiCl [30]. Despite its clinical use for over 50 years, however, the central site of action and the cellular and molecular bases of the therapeutic and toxic effects of lithium are poorly understood.

As an initial approach toward elucidating the mechanism of lithium's actions in the brain, expression of the immediate-early gene c-fos can be used as a marker to identify brain regions that are activated by lithium. Several studies

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have shown by either in situ hybridization or immunohistochemistry that doses of lithium chloride (LiCl) sufficient to produce CTA (76 mg/kg or higher) induce c-fos gene expression in rat brain. Increased c-fos mRNA levels have been observed in rat nucleus of the solitary tract (NTS), lateral parabrachial nucleus (latPBN), central nucleus of the amygdala (CeA) and paraventricular nucleus of the hypothalamus (PVN) 40 min following acute injection of LiCl [21]. LiCl also increases c-Fos-like immunoreactivity (c-FLI) in many brain regions, including the area postrema, NTS, latPBN, CeA, PVN, supraoptic nucleus (SON), cortex and hippocampus [5,11,53]. Induction of c-FLI by LiCl in several of these regions has been correlated with some of the behavioral and physiological responses to LiCl. For example, lesions of the area postrema that block acquisition of LiCl-induced CTAs [3,18,20,37,38] also attenuate LiCl-induced c-FLI in the NTS, latPBN, CeA and PVN [46].

In addition to serving as a marker of cellular activation, the c-fos protein (c-Fos) is a transcription factor. As a member of the Fos family, c-Fos heterodimerizes with other leucine zipper proteins, such as c-Jun, to form AP-1 complexes that bind to consensus AP-1 sites in the promoter regions of several genes (for review see [35]). Thus activation of c-fos can initiate a cascade of gene expression that mediates long-term changes in cell function. Such long-term changes likely underlie CTA acquisition or, with repeated chronic administration, lithium's therapeutic effects. To understand these long-term changes, it is important to understand the timecourse of chances in transcription factor gene expression initiated by a single injection of lithium.

Induction of c-fos gene expression by lithium may be mediated by the cAMP signaling pathway and cAMP-activated transcription factors such as cAMP response element-binding protein (CREB). The c-fos gene is inducible by agents that increase intracellular levels of cAMP [4,10,19] and its promoter contains consensus CRE sites to which phosphorylated CREB can bind [8,42]. Functional roles for both c-Fos and CREB have been demonstrated in LiCl-responsive brain regions during acquisition of LiCl-induced CTAs. For example, blocking LiCl-induced expression of c-Fos protein [22] or the constitutive expression of CREB [23] in rat CeA using antisense oligonucleotides impairs formation of LiCl-induced CTAs. Lithium also increases phosphorylation of CREB [32,49] and CRE-binding activity [32].

The inducible cAMP early repressor (ICER) protein is another immediate-early transcription factor that may be responsible for terminating cAMP-stimulated c-fos transcription [9,26]. ICER is the only inducible isoform of CRE-binding proteins encoded by the cAMP response element modulator (CREM) gene [28]. Following induction by elevated cAMP, ICER heterodimerizes with CREB and other CREB-binding proteins, binds to CRIB consensus sequences and potently inhibits CRE-mediated gene transcription [40]. CREM proteins have been shown to bind to CRE elements in the c-fos promoter and inhibit cAMP-stimulated c-fos transcription [9]. Furthermore, induction of c-fos mRNA by forskolin in cultured cells is significantly attenuated, or refractory, during a period of maximal ICER expression induced by prior stimulation with forskolin [26]. LiCl has been shown to induce CREM gene expression in vivo; using in situ hybridization (ISH), Lamprecht and Dudai observed increased CREM mRNA levels in rat CeA 40 min following LiCl injection [21]. The specific induction of ICER expression after LiCl has not been previously described, however.

These prior studies provide compelling evidence, especially in the rat CeA, that LiCl activates both c-fos- and cAMP-mediated gene transcription. However, the timecourse and duration of c-Fos and cAMP-mediated gene expression is not known. Using in situ hybridization, we examined the timecourse of c-fos and ICER mRNA expression in rat CeA and other forebrain regions following intraperitoneal injection of LiCl. We predicted that induction of ICER mRNA would parallel but follow induction of c-fos mRNA and that c-fos mRNA levels would return to basal levels before ICER. To test the hypothesis that c-fos transcription initially stimulated by LiCl is later attenuated by the transcriptional repressor ICER, we investigated the responsiveness of c-fos transcription to a second injection of LiCl during a time at which c-fos mRNA expression had returned to basal levels and ICER mRNA levels were still high. Our hypothesis predicted that if there was a refractory period for c-fos induction during maximal ICER expression, then induction of c-fos by the second injection of LiCl would be significantly attenuated compared to the c-fos induced by a single injection.

## 2. Materials and methods

### *2.1. Animals and drug treatments*

Adult male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were housed individually under a 12-h light (lights on 07:00)–12-h dark cycle at 25°C and had free access to Purina rodent chow and water except following intraperitoneal (i.p.) injections when chow was removed. To minimize handling stress on the day of LiCl injection, rats were handled daily for 5–7 days prior to treatment.

#### *2.1.1. c-fos and ICER mRNA expression following LiCl injection*

Twelve rats (340–420 g) were injected 4–6 h after lights on with either LiCl or NaCl (i.p., 0.15 M, 12 ml/kg; $n=6$ each) and then overdosed with sodium pentobarbitol 1 h following the injection. When completely unresponsive, the rats were perfused transcardially, first with 100 ml

of isotonic saline–0.5% sodium azide–1000 U heparin and then with 400 ml phosphate-buffered 4% paraformaldehyde. The brains were removed, blocked, post-fixed for 2 h and then transferred into 30% sucrose 24 h to 1 week prior to sectioning. Adjacent sections were processed for c-fos or ICER ISH, as described below.

#### 2.1.2. Timecourse of c-fos and ICER expression

Rats (42 total, 250–350 g) were injected with LiCl (i.p., 0.15 M, 12 ml/kg) at approximately 07:00 (just after lights on) and then overdosed with sodium pentobarbitol 0.3, 1, 3, 6, 9 and 12 h later ($n=6$ per timepoint). For the 0 h timepoint, uninjected rats were overdosed approximately 1 h after lights on. Rats were perfused and adjacent forebrain tissue sections from each timepoint were processed in parallel for c-fos and ICER cDNA ISH.

For the ICER timecourse, an additional fifteen rats (three rats per timepoints 0, 0.3, 1, 3 and 6 h following injection of LiCl) were injected and perfused. Sections were processed for ISH with ICER oligonucleotide probe, as described below.

For circadian controls, untreated rats were overdosed with sodium pentobarbitol and perfused 6 h after lights on ($n=3$) and 12 h after lights on (i.e. at lights off; $n=3$).

#### 2.1.3. Refractory c-fos expression

Rats (24 total, 400–800 g) received a first injection of either LiCl or NaCl (i.p., 0.15 M, 12 ml/kg) followed 3 h later by a second injection of LiCl or NaCl. At 20 min following the second injection, rats were overdosed with sodium pentobarbitol and perfused. Thus there were four treatment groups ($n=6$ each): LL rats were given two LiCl injections, NN rats were given two NaCl injections, LN rats were given one LiCl injection, followed by a NaCl injection and NL rats were given one NaCl injection followed by a LiCl injection. Forebrain tissue sections were processed for c-fos ISH, ICER ISH and combined c-Fos immunohistochemistry–c-fos ISH with oligonucleotide probes as described below. Four rats at a time (one from each group) were processed in parallel.

An additional 12 rats (400–525 g) were perfused 1 h instead of 20 min following a second injection. Rats received a first injection of either LiCl (LL+1 h; $n=6$) or NaCl (NL+1 h; $n=6$) followed 3 h later with a second injection of LiCl. Forebrain tissue sections were processed for c-fos cDNA ISH as described below.

### 2.2. In situ hybridization (ISH)

Coronal sections (40-μm thick) were cut on a freezing, sliding microtome and transferred into 20-ml glass scintillation vials containing 2× saline sodium citrate (SSC, 0.15 M NaCl–0.015 M sodium citrate) buffer. Every fourth section from the forebrain through the hypothalamus and amygdala (bregma −0.8 mm to −3.6 mm) was processed for c-fos or ICER ISH. Coordinates were based on Paxinos and Watson's atlas [34].

Free-floating sections were prehybridized for 1.5–3 h at 48°C, or 37°C for oligonucleotide probes, in 1 ml per vial of 50% formamide, 2× SSC, 10% dextran sulfate, 0.7% Ficoll, 0.7% polyvinylpyrrolidone, 0.7% bovine serum albumin (BSA), 85 mM dithiothreitol and 1.4 mg/ml sheared, denatured herring sperm DNA. Sections were then hybridized for 16 h at 48°C or 37°C with heat-denatured $^{35}$S-labeled cDNA ($1.0\times10^7$ cpm per 1 ml buffer per vial). Following hybridization with cDNA probe, sections were washed sequentially in 2×SSC, 2×SSC, 1×SSC, 0.5× SSC, 0.25×SSC, 0.125×SSC, 0.125×SSC at 48°C for 20 min each. Sections hybridized with oligonucleotide probes were washed in 2×SSC, 1×SSC, 0.5×SSC, 0.5×SSC at 37°C for 10 min each. After a brief storage in 0.1 M phosphate buffer (PB), were mounted onto gelatin-coated slides and then apposed to autoradiographic film (BioMax-MR, Kodak) for 1–4 days (c-fos) or 6–12 days (ICER). Following film exposure, several sets of slides were dipped in undiluted Kodak NTB-2 photoemulsion and left in the dark at 4°C for 1–4 weeks. After development, slides were counterstained in cresyl violet and coverslipped.

#### 2.2.1. ISH probes

The c-fos cDNA probe was a full-length 2.1-kb restriction fragment [6]. The ICER cDNA probe was a 166-bp restriction fragment comprising the ICER-specific portion of CREM cDNA [47]. The cDNA probes were labeled with $^{35}$S-dATP (NEN DuPont) by random-priming (Roche Molecular Biochemicals). On two separate timecourse runs, c-fos probes were random-primed simultaneously with both $^{35}$S-dATP and $^{35}$S-dCTP. One set of these data had considerable non background and could not be used for quantitation.

For refractory period experiments and three sets of ICER ISH timecourse experiments, a 48-mer oligonucleotide (5′-CAG TTT CAT CTC CAG TTA CAG CCA TGT TGG GCT TTT GCA TAC AGA GTG-3′) complementary to a portion of the ICER-specific exon of the CREM gene was 3′ end-labeled using terminal deoxymucleotidyl transferase (Roche Molecular Biochemicals) and $^{35}$S-dATP (NEN DuPont or Amersham). A similar, but shorter oligonucleotide was used by Luckman and Cox [25]. For refractory period experiments, a 30-mer oligonucleotide (5′-CGC CTC GTA GTC CGC GTT GAA ACC CGA GAA-3′) complementary to bases 141–170 of rat c-fos mRNA was used. In one experiment, c-fos oligo was tail-labeled with $^{33}$P-dATP in an attempt to improve signal for photoemulsion exposures.

### 2.3. Combined c-Fos immunohistochemistry–c-fos ISH

Free-floating, 40-μm thick forebrain tissue sections were washed twice for 15 min in 0.1 M phosphate-buffered saline (PBS) and then incubated for 30 min in 0.2%

Triton–1% BSA–PBS. After two washes in PBS–BSA for 15 min each, sections were incubated overnight with a rabbit anti-c-Fos antiserum (Ab-5, Oncogene Research) at a dilution of 1:20 000. After two 15-min washes in PBS–BSA, sections were then incubated for 1 h with a biotinylated goat anti-rabbit antibody (Vector Laboratories) at a dilution of 1:200. Antibody complexes were amplified using the Elite Vectastain ABC kit (Vector Laboratories), and visualized by a 5-min reaction in 0.05% 3,3-diaminobenzidine tetrahydrochloride. After two 5-min washes in 0.1 M PB, sections were immediately transferred into 20-ml vials containing 2×SSC, and processed as described for c-fos ISH using c-fos oligonucleotide probe. Following film and photoemulsion exposure, slides were dehydrated by a graded ethanol and xylene series of washes and coverslipped using Permount.

### 2.4. Quantitative image analysis

#### 2.4.1. Autoradiographic film densitometry

Pixel density was quantitated from the films using a custom software program (MINDSEYE 1.22B, T. Houpt). Light levels were adjusted to standardize gray levels of tissue background and images were captured in a 10×7.5 mm frame. Densitometry was restricted to hand-drawn outlines of the regions of interest. For each rat, average pixel intensities were obtained from three to four SON sections, two to three PVN sections, four to six CeA sections, two to four PVT sections, four to eight piriform cortex sections, three to eight hippocampus sections, three SCN sections or three to four arcuate sections. After subtracting average tissue background (obtained from eleven to thirteen sections per rat), the individual mean values for each region were then averaged across rats within experimental groups.

#### 2.4.2. Quantitation of c-FLI

Cells expressing darkly-positive, nuclear c-FLI were quantitated using the MINDSEYE software program. Regions were digitally-captured in a 0.72×0.54 mm frame. For the SON, counting was restricted to the area delineated by a hand-drawn outline. Outlining was not necessary for the PVN nor for the CeA because these regions mostly filled the counting frame or had little c-FLI outside the area of interest. Bilateral cell counts were averaged for three sections of the PVN, four sections of the SON and six sections of the CeA for each rat. The individual mean counts for each region were then averaged across rats within experimental groups.

### 2.5. Statistical analysis

Student's *t*-test, analysis of variance (ANOVA) and Newman–Keuls posthoc analyses were performed using commercially available software (GB-STAT PPC SCHOOLPAK, Macintosh version, Dynamic Microsystems). The timecourse data for the PVN failed a test of homogeneity of variance, so the raw data were transformed by square root in order to satisfy the assumptions of the ANOVA test. Significant run effects (determined by randomized block ANOVA) and unequal variances in the refractory period experiment necessitated normalization of c-fos ISH data. Nonparametric tests (Kruskal–Wallis ANOVA and Newman–Keuls posthoc analysis) were performed on both ISH and c-ELI data from this experiment. Results of posthoc tests are shown in the figures.

## 3. Results

### 3.1. Induction of c-fos and ICER mRNA expression 1 h following LiCl injection

As shown in Figs. 1A and 2A, c-fos mRNA expression was significantly induced 1 h following LiCl injection in the central nucleus of the amygdala (CeA), the paraventricular nucleus of the hypothalamus (PVN), the supraoptic nucleus (SON) and the paraventricular thalamic nucleus (PVT, not shown). Regions showing strong hybridization signals following both LiCl and NaCl injections include the cingulate cortex, frontal cortex, parietal cortex, piriform cortex and numerous thalamic nuclei. Moderate hybridization signals were observed in the suprachiasmatic nucleus (SCN) and arcuate nucleus in the hypothalamus of both LiCl- and NaCl-injected rats. Statistical analysis of densitometric data obtained from two sample regions showing hybridization in both LiCl- and NaCl-treated rats (hippocampus and piriform cortex) indicated no significant induction following LiCl injection.

ICER mRNA was significantly induced 1 h following LiCl injection in the CeA, PVN and SON (Figs. 1B and 2B). Moderate ICER hybridization signals were also observed in the SCN, arcuate nucleus, and occasionally in the hippocampus, of rats injected with either LiCl or NaCl.

### 3.2. Timecourse of c-fos and ICER mRNA expression following LiCl injection

Expression of c-fos mRNA was examined by ISH in rat forebrain sections 0, 0.3, 1, 3, 6, 9 and 12 h following LiCl injection at lights on. Representative photomicrographs of photoemulsion-dipped sections at the level of the CeA, PVN and SON are shown in Fig. 3. One-way ANOVA of the densitometric data (Fig. 4) indicated main effects among the different timepoints in the CeA ($F[6,34]=42.6$, $P<0.0001$), PVN ($F[6,34]=10.7$, $P<0.0001$) and SON ($F[6,34]=7.3$, $P<0.0001$). Posthoc analyses indicated that c-fos mRNA levels were significantly elevated 0.3 and 1 h after LiCl in all three regions. There was no significant difference between signals seen at 0.3 h versus 1 h after LiCl. Hybridization signals were no longer above background levels by 3 h after LiCl.

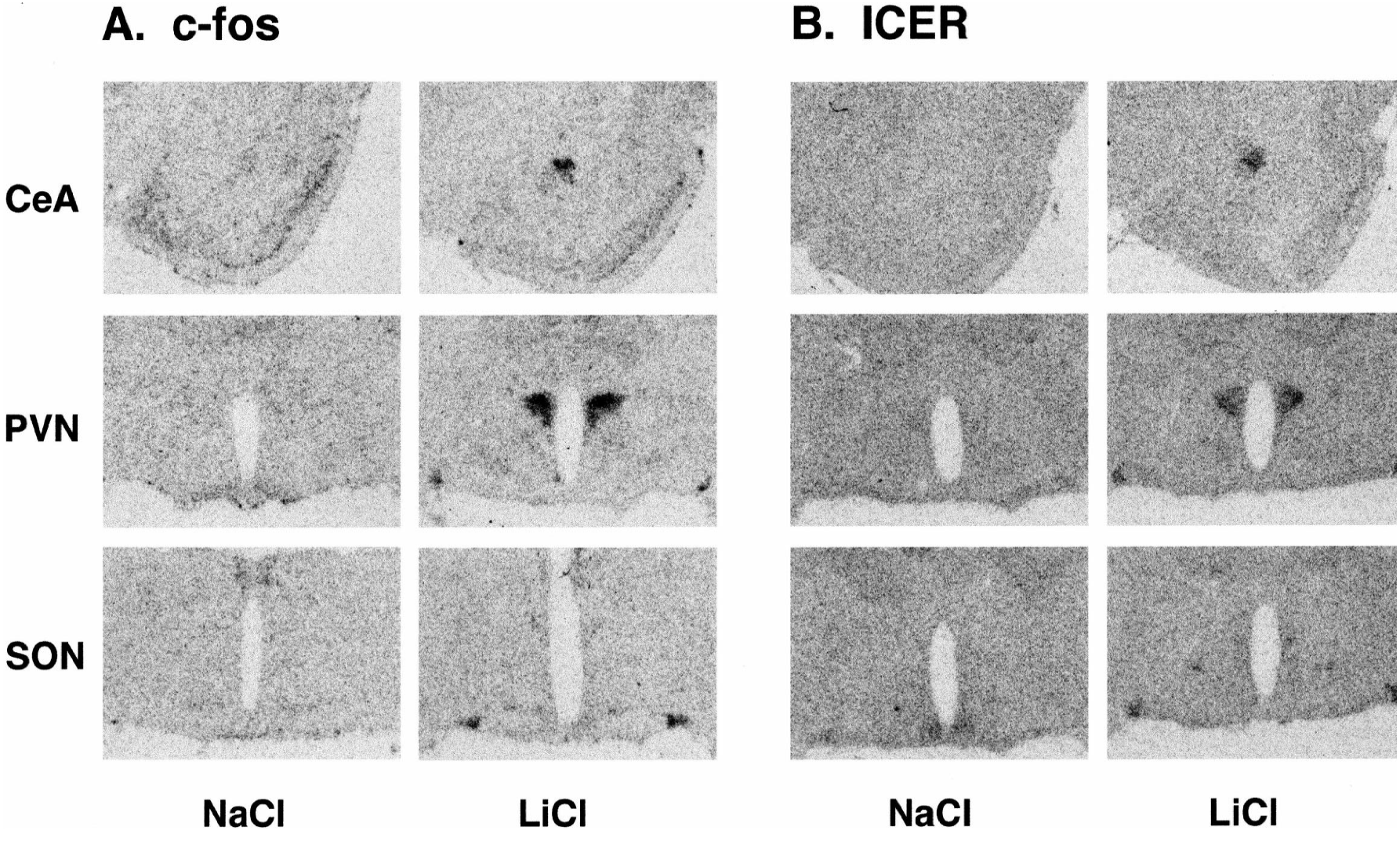


Fig. 1. LiCl-specific induction of c-fos and ICER gene expression in rat forebrain. Representative photomicrographs show c-fos (A) and ICER (B) hybridization in the central nucleus of the amygdala (CeA), paraventricular nucleus of the hypothalamus (PVN) and supraoptic nucleus (SON) 1 h following NaCI or LiCl injection.

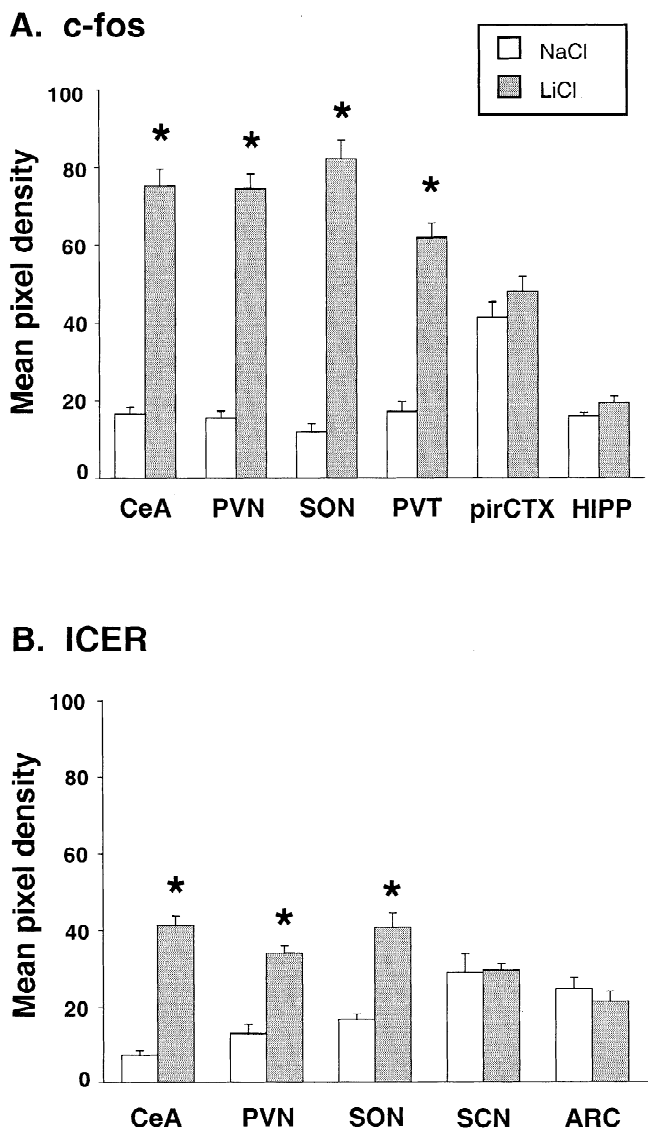


Fig. 2. Quantitation of c-fos (A) and ICER (B) hybridization signals in several rat forebrain regions 1 h following NaCl or LiCl injection. Data represent means±S.E.M. for $n=6$. *, $P<0.05$ compared to NaCl-injected group.

While performing timecourse experiments, we also observed circadian effects on c-fos expression. For example, there was an increase in c-fos hybridization signal in the cortex and thalamus, including the PVT, at both 0 h (lights on) and 12 h (lights off) relative to the 6 h timepoint (see Fig. 5). However, this pattern was observed in both LiCl-treated and untreated rats perfused at 0 h and 12 h (data not shown), indicating a circadian effect in these regions. There was also an intense hybridization signal in the SCN of rats perfused at 0.3 and 1 h timepoints after LiCl injection and at 0 h uninjected (data not shown). While the majority of brain regions showing these circadian effects did not show specific c-fos induction by LiCl injection, circadian induction of c-fos expression in the PVT obscured the timecourse of LiCl-induced c-fos in this region. These data demonstrate that there can be a significant circadian effect on gene expression that may confound timecourse experiments.

ICER mRNA expression was examined in adjacent rat forebrain sections. The most prominent site of LiCl-induced ICER expression in the rat forebrain was the CeA. As seen in Fig. 5, ICER mRNA in the CeA was barely detected 20 min following LiCl, peaked between 1 and 3 h, and was no longer detectable by 9 h. This pattern of expression was observed in four sets of data using random-primed ICER cDNA and three sets of data using tail-labeled ICER oligonucleotide (0, 0.3, 1, 3 and 6 h only). Due to low signal/high background, however, these data could not be quantitated. A comparison of the timecourse of ICER mRNA expression with the timecourse of c-fos mRNA expression shows that ICER expression was delayed with respect to c-fos mRNA expression.

The timecourse of ICER expression in the SON ($n=4$) and PVN ($n=7$) of the hypothalamus following LiCl injection was very similar to that observed in the CeA: ICER mRNA was detectable at 20 min, peaked between 1 and 3 h, and was no longer above background levels 9 h after LiCl (data not shown). On occasion ICER signal above background was detected 12 h after LiCl. A control experiment using untreated rats perfused 6 and 12 h after lights on indicated that there was a circadian induction of ICER mRNA expression in the SON and PVN 12 h after lights on (i.e. at lights off). In some experiments with greater contrast between hybridization signal and background, a small induction of ICER mRNA in the CeA could also be detected at lights off.

### *3.3. Responsiveness of c-fos transcription to a second LiCl injection*

To determine whether the c-fos transcriptional response to LiCl Was refractory during peak ICER mRNA expression, we examined c-fos mRNA expression in the CeA, SON and PVN following a second injection of LiCl given 3 h after the initial LiCl injection. Rats were given an injection of LiCl or NaCl, followed 3 h later by a second injection of LiCl (LL or NL groups) or NaCl (LN or NN groups). Twenty minutes following the second injection, c-fos mRNA and c-Fos protein levels were assessed. Two additional NL and LL groups were examined 1 h after the second LiCl injection (NL+1 h and LL+1 h groups). An example of the data obtained is shown in Fig. 6. Quantitative results are shown in Fig. 7 (c-fos mRNA) and Fig. 8 (c-Fos protein).

Kruskal–Wallis ANOVA indicated a significant overall difference in c-fos mRNA levels among the four groups in the CeA ($\chi^2[3]=13.3$, $P<0.005$), PVN ($\chi^2[3]=9.4$, $P<0.05$) and SON ($\chi^2[3]=14.6$, $P<0.01$). In all three regions, c-fos mRNA was induced following the second injection of LiCl (compare LL to LN), although this induction was statistically significant only in the CeA. There was no significant attenuation in the c-fos response

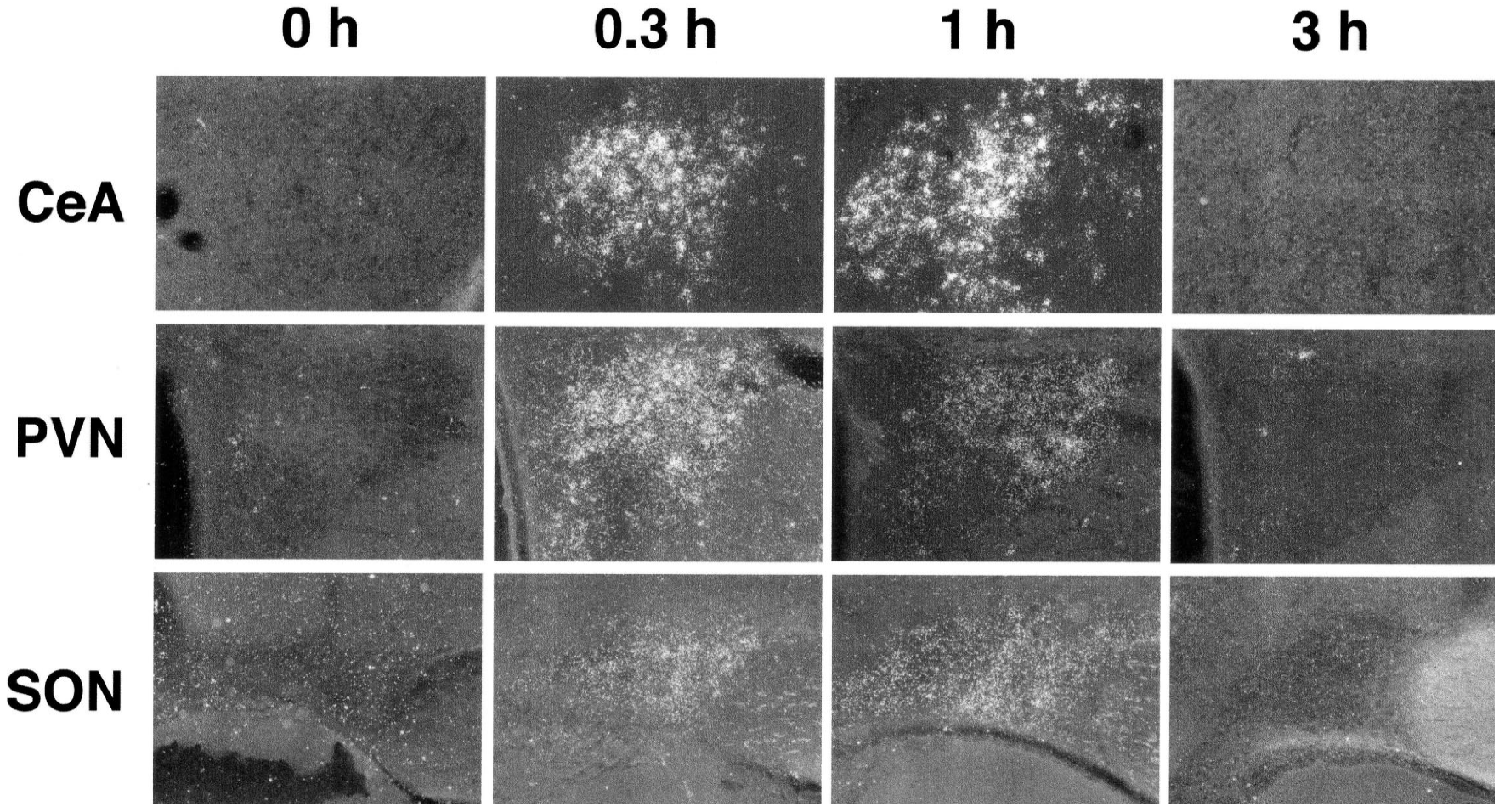


Fig. 3. Timecourse of c-fos mRNA expression following LiCl injection in rat CeA, PVN and SON. Shown are representative dark-field photomicrographs of photoemulsion-dipped sections 0, 0.3, 1 and 3 h following LiCl injection.

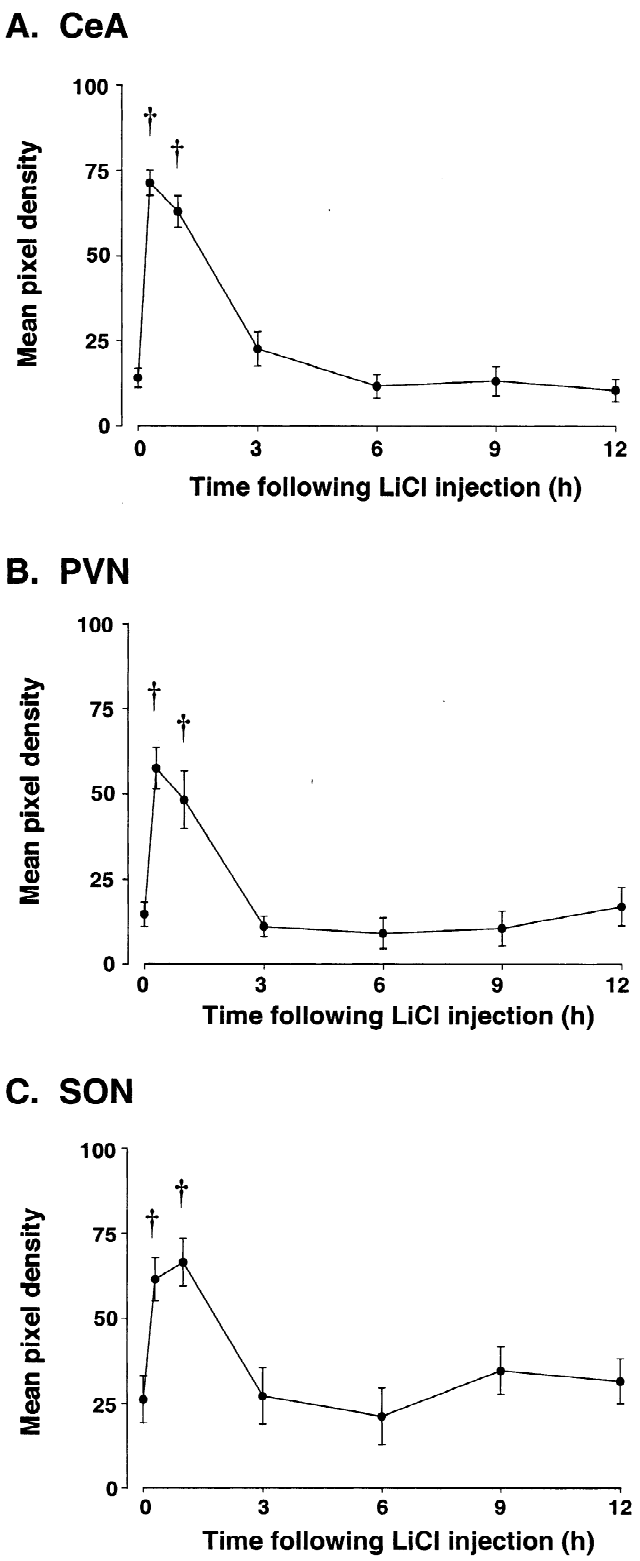


Fig. 4. Quantitation of c-fos timecourse data in CeA (A), PVN (B) and SON (C). Data represent means±S.E.M. for $n=5$. †, $P<0.01$ compared to 0 h datapoint.

to the second injection of LiCl (compare NL and LL) in any region, although there was a trend toward significant attenuation in the SON.

The refractory effects induced by a preceding LiCl injection might be seen not as a decrease in amplitude of expression but as a decreased duration of c-fos expression after a second injection. Therefore we expanded our analysis to look at c-fos mRNA levels 1 h following the second injection of LiCl. To improve quantitation, ISH was performed using a c-fos cDNA probe. Strong c-fos hybridization signals were evident in the CeA, PVN and SON of both NL+1 h and LL+1 h groups of rats. Student's *t*-test analysis of the densitometric data (shown in Fig. 7) indicated that there was no significant difference between NL+1 h and LL+1 h groups in the SON, PVN or CeA. Therefore, there was no attenuation of the response to a second injection of LiCl 3 h after the first injection of LiCl.

To confirm that the first LiCl injection did induce c-fos gene expression, c-Fos protein expression was analyzed by immunohistochemistry (Fig. 8). Kruskal–Wallis ANOVA indicated a significant difference in c-FLI positive cell counts among the groups in the CeA ($\chi^2[3]=16.7$, $P<0.001$) and PVN ($\chi^2[3]=10.4$, $P<0.05$). There was an increase in c-FLI positive cell numbers in rats that had received LiCl 200 min (LN or LL groups) prior to perfusion.

To confirm that ICER was induced by the first LiCl injection, ICER mRNA was examined in adjacent sections ($n=2$ rats per group). Expression of ICER mRNA in the CeA (shown in Fig. 6), as well as in the SON and PVN (not shown), was observed 200 min after the first LiCl injection (LN or LL groups).

## 4. Discussion

In summary, we have shown that (1) expression of the immediate-early genes c-fos and ICER was induced by LiCl injection, but not by NaCl injection, in the CeA, PVN and SON regions of rat-forebrain; (2) c-fos mRNA levels in these brain regions peaked 20 min to 1 h following LiCl injection and returned to background levels by 3 h; (3) ICER mRNA levels in these brain regions peaked 1–3 h following LiCl injection and returned to baseline by 9 h; and (4) c-fos transcription in the SON, PVN and CeA was induced by a second injection of LiCl 3 h after the first injection. We conclude that (1) c-fos and ICER mRNAs are rapidly and transiently induced by LiCl in the SON, PVN and CeA, with the peak of ICER expression delayed relative to the peak of c-fos expression; and (2) c-fos transcriptional response to LiCl in these brain regions is not refractory during peak ICER mRNA expression resulting from an earlier exposure to LiCl. While the timecourse of c-fos and ICER mRNA induction by LiCl is consistent with the hypothesis that ICER terminates LiCl-stimulated c-fos transcription, the lack of a refractory period suggests the involvement of other negative modulators.

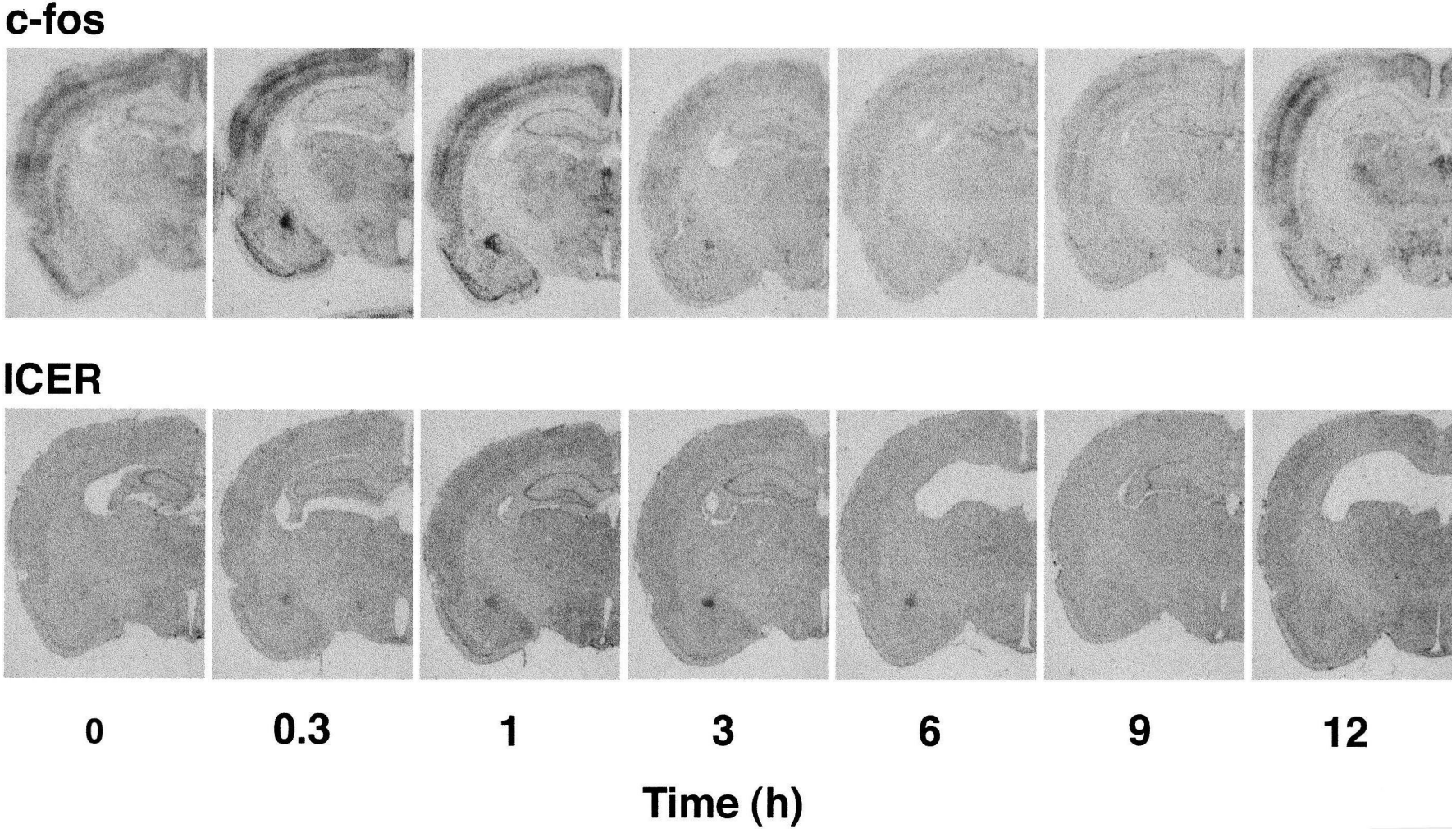


Fig. 5. Temporal comparison of c-fos and ICER mRNA expression in the CeA following LiCl injection. Note the circadian induction of c-fos in the cortex and thalamus, including the paraventricular nucleus of the thalamus, at the 0, 0.3, 1 and 12 h timepoints.

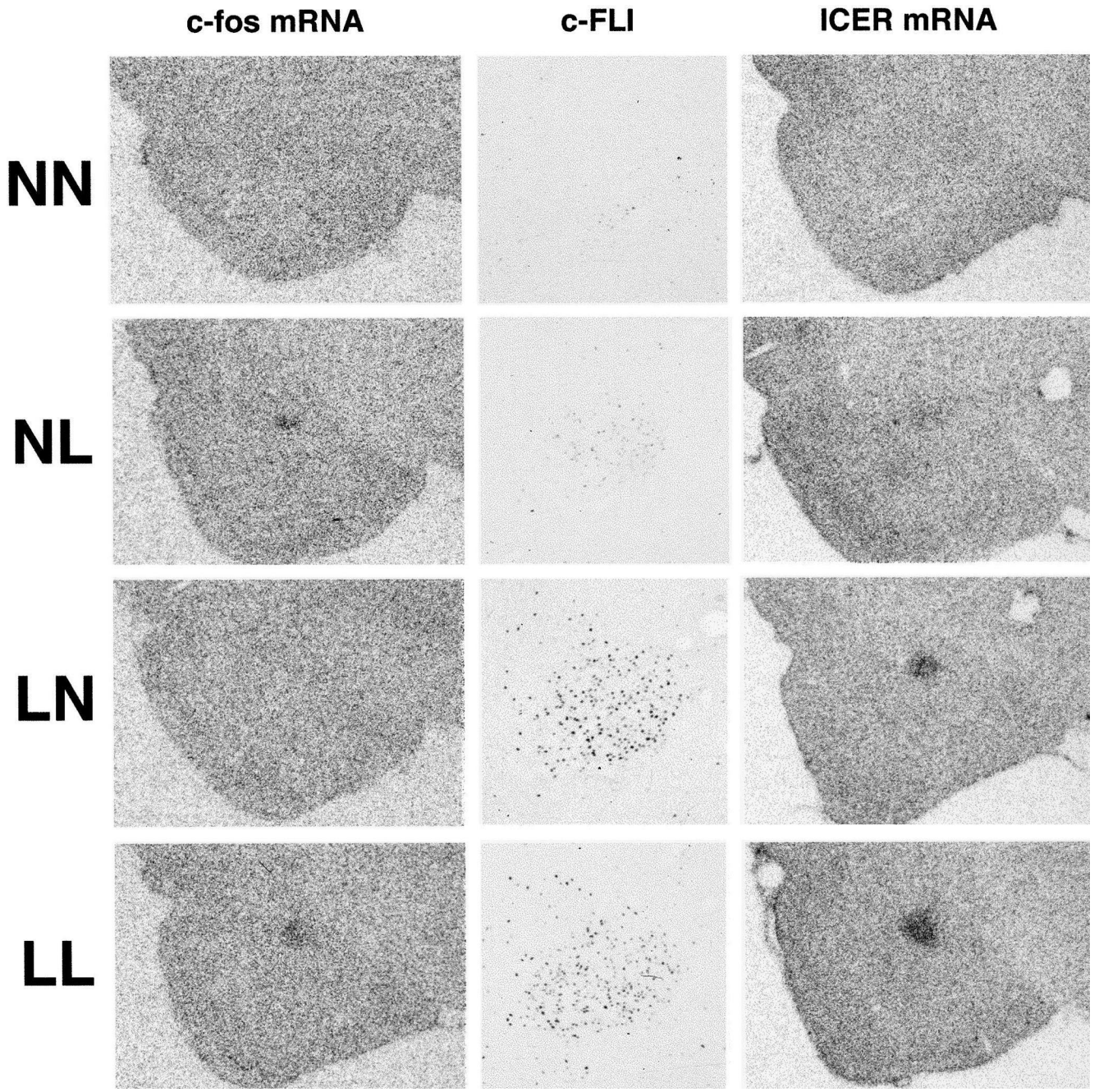


Fig. 6. Induction of c-fos in the CeA in response to a second injection of LiCl. Shown are representative photomicrographs of c-fos mRNA, c-Fos protein (c-FLI) and ICER mRNA for NN, NL, LN and LL treatment groups receiving i.p. injection of NaCI (N-) or LiCl (L-) followed 3 h later by a second injection of NaCl (NN or LN) or LiCl (NL or LL). Rats were perfused 20 min after the second injection.

The information obtained from expression timecourse studies is important in several respects. In general, it indicates how long a stimulus induces active transcription and establishes a sequence of molecular events which may then be correlated to neurochemical and behavioral consequences. When studying transcription factors, knowledge of the promoter elements present in both the transcription factor gene itself and in the genes targeted by the transcription factor complements timecourse information in assisting elucidation of the cascade of molecular events. Understanding the timecourse of early-response gene expression is also important to determine the time required to reset the acute response to stimuli for which repeated administration produces the desired effect, such as that which occurs for lithium in the treatment of bipolar or depressive disorders. Furthermore, analysis of refractory response periods can yield information about the potential interplay of positive and negative transcriptional modulators of gene expression.

The pattern of c-fos mRNA expression induced by LiCl

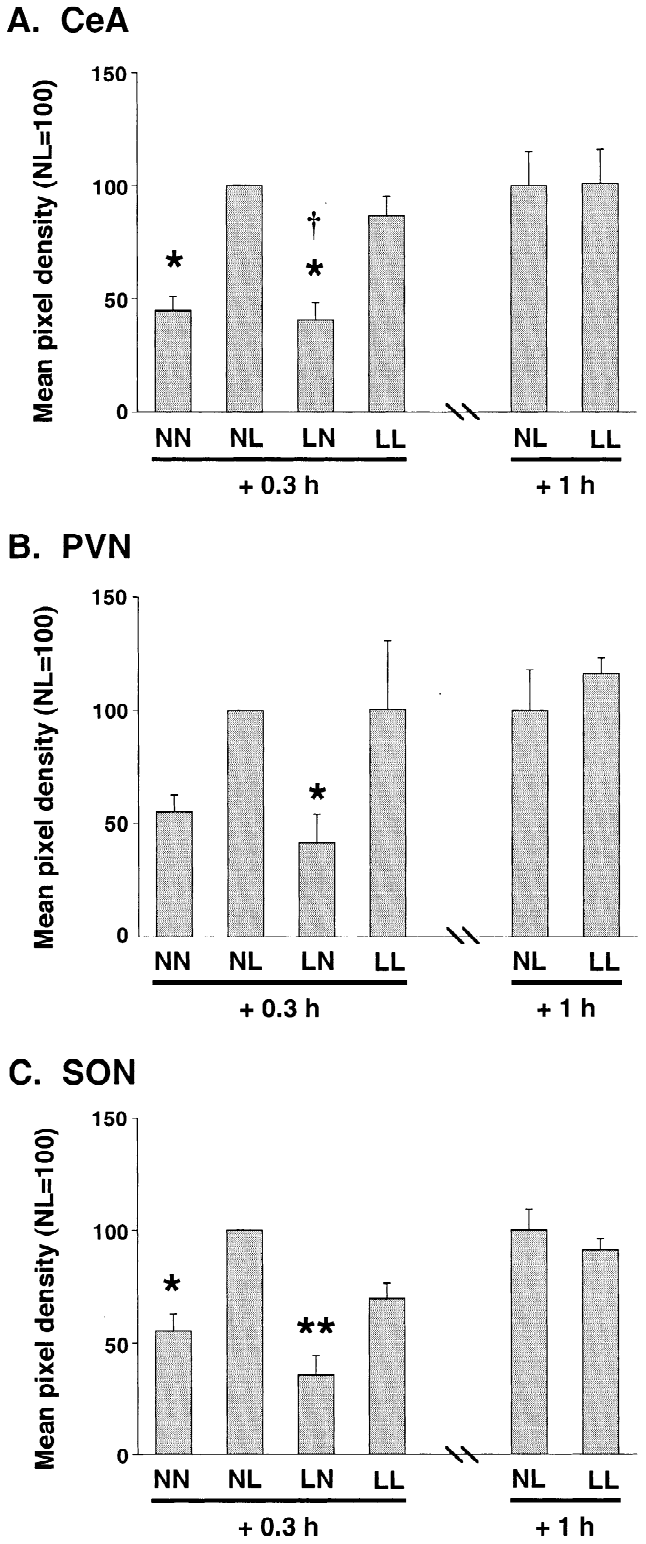


Fig. 7. Quantitation of c-fos mRNA in CeA (A), PVN (B) and SON (C) after a second injection of LiCl. Rats were perfused either 0.3 or 1 h after the second injection as indicated. Data for NN, NL, LN and LL groups represent means±S.E.M. for $n=5$ normalized within experimental runs to NL. Data for NL+1 h and LL+1 h groups represent means±S.E.M. for $n=6$ acquired in a separate experiment and normalized to the NL+1 h group. There was no significant difference between NL and LL groups, nor between NL+1 h and LL+1 h groups, in any region, indicating the lack of refractory response to the second LiCl injection. *, $P<0.05$, **, $P<0.01$ compared to NL group. †, $P<0.05$ compared to LL group.

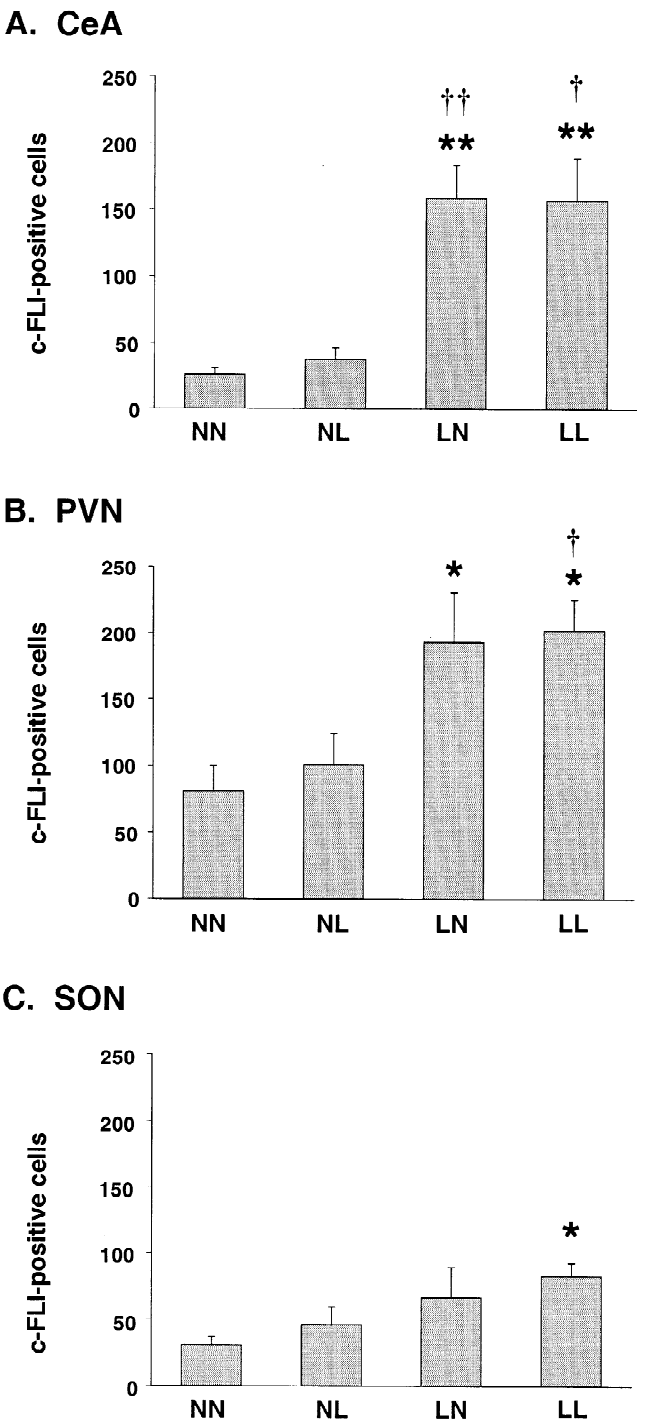


Fig. 8. Quantitation of c-Fos-like immunoreactive (c-FLI)-positive cells in CeA (A), PVN (B) and SON (C) after a second injection of LiCl. Data represent means±S.E.M. for $n=6$. *, $P<0.05$, **, $P<0.01$ compared to NN; †, $P<0.05$, ††, $P<0.01$ compared to NL.

agrees with the pattern of LiCl-induced c-Fos protein expression observed by others [11,36,43,53]. This data also confirms and extends ISH results reported by Lamprecht and Dudai [21], who observed a large increase in c-fos mRNA in the PVN and CeA and a small increase in

CREM mRNA in the CeA 40 min following LiCl injection. There are also reports of LiCl-induced increases in c-FLI in rat cortex and hippocampus [5,53] which we did not see at the mRNA level; however, the reported increases of c-FLI were small (17–26%) and may be below the sensitivity of our ISH assay.

As expected, there was a rapid and transient induction of c-fos mRNA expression by LiCl. Because the c-fos promoter contains a cAMP-response element (CRE) and at least five other response elements, there are several transcription factors regulated by a variety of second messenger systems that may play a role in this induction [13]. Although the co-activation of c-fos and ICER suggests activation of cAMP pathways, evidence exists for the activation of other pathways by LiCl; for example, phosphorylation of MAP kinase is observed in mouse insular cortex and CeA 30 min after LiCl injection [49]. MAP kinase phosphorylates and activates serum response element (SRE)-binding proteins which increase c-fos transcription through the SRE site in the c-fos promoter [35]. The relative contributions of the cAMP, MAP kinase, and other second messenger systems to c-fos gene regulation by LiCl are unknown.

The similar timecourse of c-fos mRNA expression in the CeA, PVN and SON following LiCl injection was somewhat unexpected because we have observed different timecourses of c-Fos protein expression across these brain regions [16]. Others have reported a similar rapid induction of c-fos mRNA by an acute stimulus, although the time at which c-fos mRNA returns to basal levels of expression varies from 1 to 4 h depending on the stimulus and tissue [17,29,45,52]. The exact timecourse of c-fos mRNA expression must depend on the rate of mRNA degradation (controlled by instability determinants in the c-fos mRNA) or on the termination of c-fos transcription by the decay of positive transcriptional modulators or the induction of negative transcriptional modulators [13,35]. Mechanisms described for the down-regulation of c-fos transcription include dephosphorylation of CREB by protein phosphatase PP1 [12], and repression of the c-fos promoter by c-Fos [41] or CREM/ICER proteins [9].

Our results showed that ICER mRNA was specifically and transiently induced in rat CeA, PVN and SON by LiCl injection. ICER is transcribed by a cAMP-dependent mechanism from a second intronic promoter (P2) located within the CREM gene [28]. The P2 promoter contains four CRE motifs [28] and no other known response elements; therefore, induction of ICER by LiCl is highly indicative of activation of cAMP-mediated gene expression by LiCl in these brain regions. The timecourse of ICER expression in rat CeA, PVN and SON after LiCl injection was similar to that observed by others in rat brain or adrenal after other stressful stimuli; ICER mRNA is typically observed within 15–30 min with peak expression occurring 1–3 h following induction in vivo [7,25,52]. Thus, while c-fos expression in the CeA, SON and PVN had returned to basal levels 3 h following LiCl injection, ICER mRNA was highly expressed in these regions. The delay in peak ICER expression until after the peak of c-fos expression is consistent with the hypothesis that ICER terminates LiCl-induced c-fos gene transcription.

As a test of this hypothesis, we determined if c-fos transcription induced by LiCl was refractory to restimulation during maximal ICER expression. Others have seen evidence of refractory responsiveness to restimulation of c-fos mRNA expression during maximal ICER mRNA expression in cell culture [26] or during expression of Fos protein in rodent brain after acute seizure [29,52]. However, our data indicated that c-fos induction by LiCl was not refractory when ICER mRNA and c-Fos protein levels were high. The possibility remains, however, that ICER and c-fos were not expressed within the same cells or that the timing of the second injection was not optimal to detect a refractory period. Alternatively, ICER might not be responsible for terminating LiCl-induced c-fos transcription mediated by cAMP-independent factors such as MAP kinase.

Understanding the activation of immediate-early genes in response to a single LiCl injection is an important first step in understanding the long-term changes in gene expression elicited by lithium, such as those involved in the acquisition of CTAs. Presentation of a novel tastant prior to a single injection of LiCl is sufficient to form a CTA in rats [30].

With multiple pairings, LiCl-induced CTAs have been shown to persist as long as 6 months [15]. However, expression of a CTA more than 6 h after pairing requires protein synthesis [14,22,39,44,50]. Thus, the expression of new genes is required within 6 h to consolidate a CTA. This study provides evidence that LiCl induces gene expression of c-fos and ICER transcription factors within that 6-h window. These transcription factors, as well as others such as FosB and JunB [48], may regulate subsequent waves of gene transcription to effect long-term changes such as CTA consolidation. Studies investigating the pattern of gene expression elicited by LiCl and a novel tastant during this timecourse will be important for elucidating the molecular mechanisms underlying CTA acquisition. Similarly, understanding the pattern of gene expression elicited by a single dose or chronic administration of lithium will be important for elucidating the mechanisms underlying the therapeutic effects of lithium.

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