

Chronic Lithium Salt Treatment Reduces CRE/CREB-Directed Gene Transcription and Reverses Its Upregulation by Chronic Psychosocial Stress in Transgenic Reporter Gene Mice

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The molecular mechanism of action of the mood stabilizer lithium is assumed to involve changes in gene expression leading to neuronal adaptation. The transcription factor CREB (cAMP-responsive element binding protein) regulates the expression of many genes and has been implicated in important brain functions and the action of psychogenic agents. We here investigated the effect of lithium on cAMP-responsive element (CRE)/CREB-mediated gene transcription in the brain, using transgenic reporter mice that express the luciferase reporter gene under the control of four copies of the rat somatostatin gene promoter CRE. Chronic (21 days) but not acute (24 h) treatment with lithium (7.5 mmol/kg) significantly decreased CRE/CREB-directed gene expression in hippocampus, cortex, hypothalamus, and striatum to 60–70%, and likewise reduced CREB phosphorylation. As bipolar disorder is also considered as a stress-related disorder, the effect of lithium was determined in mice submitted to a paradigm for chronic psychosocial stress. As shown before, stress for 25 days significantly increased CRE/CREB-directed gene expression in several brain regions by 100–150%. Treatment of stressed mice with lithium decreased stress-induced CRE/CREB-directed gene expression to control levels in nearly all brain regions and likewise reduced CREB phosphorylation. Chronic lithium treatment induced β -catenin accumulation and decreased cAMP levels, indicating an inhibitory effect of lithium on glycogen synthase kinase 3 and the adenylate cyclase/protein kinase A signalling cascade, which are known to modulate CREB activity. We here for the first time show that lithium regulates CRE/CREB-directed gene transcription *in vivo* and suggest CREB as a putative mediator of the neuronal adaptation after chronic lithium treatment.

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INTRODUCTION

Bipolar disorder, also known as manic-depressive illness, is a serious mental illness characterized by alternating episodes of mania and severe depression. It is one of the leading disabilities worldwide (Murray and Lopez, 1997) and can be considered as highly life-threatening due to a high rate of suicide. The etiology of bipolar disorder has been linked to genetic factors (Craddock and Forty, 2006), which lead to a higher individual vulnerability. However, in addition, particularly environmental influences such as

chronic stress are assumed to precipitate the disease (Johnson, 2005). Among the drugs recommended for the treatment of bipolar disorder, lithium salts are of prime importance (Gitlin, 2006). Lithium has been discovered more than 50 years ago as a drug with an acute antimanic action (Cade, 1999). More importantly, lithium is highly effective in preventing the relapse of manic and depressive episodes. In addition to this mood-stabilizing and phase-prophylactic action, lithium is used as an alternative or adjunct in the therapy of severe recurrent depression and is also recommended in bipolar II disorder, which is characterized by mild mood elevations or hypomania and prolonged depressive episodes (Keck and McElroy, 2003). Moreover, it has been proven to reduce the suicide rate more efficiently than other treatments (Tondo *et al*, 1997). In contrast to its acute antimanic action, the mood-stabilizing and antidepressive properties of lithium require chronic administration of up to several months. The

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underlying mechanism of this phase-prophylactic action is still not satisfactorily understood, although recently several molecular targets of lithium have been identified. Signal transduction pathways affected by lithium include the adenylate cyclase/cAMP/protein kinase A (PKA) pathway by inhibition of adenylate cyclase, the inositolphosphate/PKC/calcium system by inhibition of inositol monophosphatase, and glycogen synthase kinase 3 (GSK3)-dependent pathways through inhibition of GSK3, which regulates a number of cytoskeletal and cell cycle proteins and also transcription factors (Quiroz *et al*, 2004). Given that the therapeutic action of lithium in bipolar disorder requires sustained treatment for several months, it is unequivocal that rapid changes in neurotransmitter and signal transduction systems are insufficient to explain the underlying mechanism. Rather long-term complex processes are suggested to be involved, which can be considered as drug-induced neuroplasticity or neuronal adaptation. The molecular basis for these adaptive changes is the expression of genes regulated by transcription factors. An important transcription factor is the cAMP-responsive element binding protein (CREB). Two isoforms of CREB are ubiquitously expressed, alpha-CREB (341aa) and delta-CREB (327), lacking a stretch of 14 aa in the N-terminal part of the molecule (Shaywitz and Greenberg, 1999). CREB belongs to the family of basic region leucine zipper transcription factors and binds as dimer to the cAMP-responsive element (CRE) (Shaywitz and Greenberg, 1999). CREB is phosphorylated at Ser-119 (referring to delta-CREB) or Ser-133 (referring to alpha-CREB) by PKA and several other kinases, which are activated by manifold signalling pathways (Lonze and Ginty, 2002), and then it transactivates transcription by the recruitment of coactivators such as CREB-binding protein (CBP) (Shaywitz and Greenberg, 1999) and TORC (Conkright *et al*, 2003; Kovacs *et al*, 2007). Among the CREB-regulated genes are several genes important for neurotransmission and signalling (Impey *et al*, 2004). Moreover, CREB is involved in adaptive processes such as learning, memory (Silva *et al*, 1998), and opiod-induced addiction (Nestler, 2001). As the signalling pathways affected by lithium can also influence CREB, CREB-directed transcription could be a downstream mediator of lithium action.

A suitable tool for the investigation of the modulation of CREB activity *in vivo* is the use of transgenic reporter mice. We reported previously on the SomCRE-Luc mouse expressing firefly luciferase under the control of four copies of the CRE from the rat somatostatin gene promoter (Boer *et al*, 2007a). The measurement of CRE-directed luciferase expression in different brain regions allows the monitoring of CREB transcriptional activity. CRE-binding proteins other than CREB could, however, be involved.

We here, for the first time, demonstrate the effect of lithium on CRE/CREB-mediated reporter gene expression *in vivo* using this mouse model. Moreover, as chronic stress has been implicated in the etiology of bipolar disorder and as lithium does not influence the mood of healthy subjects (Calil *et al*, 1990), the effect of lithium on CRE/CREB-directed gene transcription was determined in stressed mice. For this purpose, SomCRE-Luc mice were submitted to an experimental paradigm of chronic psychosocial stress originally designed as a model for depression (Boer *et al*,

2007a; Kudryavtseva *et al*, 1991). We here demonstrate that lithium treatment decreases CRE/CREB-directed transcription in the brain and reverses the stress-induced increase in CREB activity close to control levels.

Our data, thus, provide new evidence for the significance of the transcription factor CREB for the action of lithium.

MATERIALS AND METHODS

Animal Housing Conditions

The generation and characterization of transgenic SomCRE-Luc mice used in this study have been described previously (Boer *et al*, 2007a). The transgene in these mice consists of the luciferase reporter gene under control of four copies of the CRE from the rat somatostatin promoter and a truncated thymidine kinase promoter. Animals were kept on a 12-h light-dark cycle. Food pellets and water were available *ad libitum*. For acute and chronic drug treatment, mice were single-housed in plastic cages (28 cm L × 16 cm W × 13 cm H). All animal studies were conducted according to the National Institutes of Health's Guidelines for Care and Use of Experimental Animals and were approved by the Committee on Animal Care and Use of the local institution and state.

Stress Model

The model of chronic psychosocial stress in mice has been described before (Boer *et al*, 2007a). For this experiment, male transgenic mice (12–15 weeks old) were used. Two mice were housed in a cage separated by a perforated plastic partition. During 25 days, the partition was removed every day (between 1500 and 1800) for 10 min resulting in daily social conflicts. The daily interactions were recorded by two independent observers. After 3–5 days, one mouse developed a dominant aggressive behavior, whereas the other became subordinate. Chasing and biting behavior was defined as dominant, whereas flight, freezing, and vocalization indicated the subordinate posture and thus social stress. Non-stressed mice from the same litter kept separately served as controls. On day 26, 14–16 h after the last stress exposure stressed and control mice were decapitated and brains were dissected.

Lithium Treatment

Acute treatment: 7.5 mmol/kg lithium chloride (Sigma) was applied i.p. at 0900. Mice were decapitated the following day at 0900 and brains were taken out for luciferase assay. Chronic treatment: 7.5 mmol/kg/day lithium chloride was applied i.p. for 21 days. The mice were decapitated on day 22 of the treatment at 0900 and brains were dissected. Chronic lithium treatment in stressed mice: mice were exposed to chronic psychosocial stress. The submissive animal was treated from days 5 to 25 (0900) with lithium (7.5 mmol/kg/day), whereas the daily stress procedure continued. On day 26 at 0900, 16 h after the last stress exposure (24 h after lithium treatment), the mice were decapitated and brains were taken out. Within all treatments controls from the same litter received the solvent only.

Dissection of Brain Regions and Luciferase Assay

Transgenic mice were decapitated 24 h after the last lithium treatment or 14 h after the last stress exposure. The brains were removed and rinsed with ice-cold PBS. Defined regions were dissected in the following order: Olfactory bulb, cerebellum, pons, frontal part of the brain anterior to the optic chiasm containing parts of the prefrontal cortex (PFC) and the striatum, colliculi, hypothalamus, cortex, and hippocampus. Pieces of tissue were frozen in liquid nitrogen and stored at -80°C .

For the luciferase assay, the tissue was homogenized in potassium phosphate buffer (0.1 M K_2HPO_4 , 0.1 M KH_2PO_4 , pH 7.8) supplemented with 1 mM DTT, 4 mM EGTA, 4 mM EDTA, 0.7 mM PMSF, 5 $\mu\text{g}/\text{ml}$ leupeptin, 5 $\mu\text{g}/\text{ml}$ pepstatin, and 5 $\mu\text{g}/\text{ml}$ aprotinin (all from Sigma) and subjected to three cycles of freeze thawing. After centrifugation, 50 μl of supernatants were mixed with 370 μl assay buffer containing 16.5 mM potassium phosphate, 20 mM glycylglycine, 12 mM MgSO_4 , 3.2 mM EDTA, 1 mM DTT, and 2.2 mM ATP (Sigma), and luciferase activity was measured by adding the substrate luciferin (Promega, Mannheim, Germany) in glycylglycine buffer (25 mM glycylglycine, 15 mM MgSO_4 , 4 mM EGTA, 10 mM DTT) with an automatic dispenser (Autolumat 953, Berthold, Bad Wildbach, Germany). Measured relative light units were normalized to protein content determined by Bradford (1976).

Determination of Lithium

Concentrations of lithium were determined in brain homogenates by atomic absorption spectrophotometry using a Spectra A-10 instrument (Varian, Darmstadt, Germany). A 100 μl sample of homogenized brain supernatant was diluted with 800 μl demineralized water and 100 μl of a solution containing 87 mM sodium chloride and 759 mM cesium chloride, respectively. For calibration of the instrument samples containing 0–2.5 mM lithium carbonate were used. At concentrations between 0.4 and 1.2 mM, the CV of imprecision was always below 5.0%. To guarantee accuracy of analysis, the laboratory participates in systematic measures of external quality controls for lithium (Instand e.V., Düsseldorf, Germany).

Western Blot in Brain Homogenates

Transgenic mice were decapitated 24 h after the last lithium treatment or 14 h after the last stress exposure; brains were dissected on an ice-cold plate and immediately frozen in liquid nitrogen. Frozen tissue was pulverized under liquid nitrogen. Powder was transferred to boiling SDS sample buffer and boiled for 5 min. Tissue lysates containing CREB/P-CREB were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane by electroblotting. CREB, phospho-CREB, β -catenin, and GAPDH were immunostained with specific antibodies (NewEngland Biolabs, Frankfurt a.M., Germany) and respective peroxidase-labeled secondary antibodies. Immunoreactivity was detected using the ECL reaction (Amersham Pharmacia, Germany).

Specific bands were quantified densitometrically and the ratio between the intensity of phospho-CREB and CREB or β -catenin and GAPDH from the same homogenate was calculated.

Western Blot in Brain Regions from the Cytosolic Fraction

For Western blot of different brain regions, tissue samples were homogenized in hypotonic buffer (10 mM Tris, pH 7.4, containing 1 mM EDTA and protease inhibitors), freeze thawed and centrifuged. Using the supernatant, protein determination (Bradford) was performed and samples corresponding to 4, 6, and 10 μg of total protein were used for 10% SDS-PAGE. β -Catenin and GAPDH were immunostained with specific antibodies (NewEngland Biolabs, Frankfurt a.M., Germany) and respective peroxidase-labeled secondary antibodies. Immunoreactivity was detected using the ECL reaction (Amersham Pharmacia, Germany). Specific bands were quantified densitometrically and the ratio between the intensity of β -catenin and GAPDH from the same homogenate was calculated.

cAMP Measurement by Radioimmunoassay

The cAMP concentration in brain homogenates was measured by radioimmunoassay (RIA) using the [^{125}I]cAMP kit from Perkin Elmer using the non-acetylated standard method following the manufacturer's instructions.

Extraction of Inositol Phosphates from Mouse Brain

Brain samples were frozen at -80°C to reduce any metabolic activity and were stored until further analysis. Three milliliter of 8% (w/v) trichloroacetic acid, 12 μl of 0.2 M EDTA, and 10 μl of 0.1 M sodium fluoride were added to each tissue sample (ca. 0.5 g wet weight). The specimen was homogenized in an ice bath using an Ultra-Turrax homogenizer with a 7 mm diameter (Staufen, Germany) (10 s at 9500 r.p.m., four times). The homogenate was incubated for 20 min on ice and the precipitate was removed by centrifugation at 4000 r.p.m. for 5 min at 4°C (Megafuge 1.0R, Heraeus Instruments, Hanau, Germany).

The completely removed supernatant was incubated for 15 min at 37°C in order to destroy the creatine phosphate. Then, the trichloroacetic acid was removed by three extractions with 5 ml of diethyl ether. Care was taken in order to completely retain the water phase, which was finally neutralized with 50 μl of 1 M triethanolamine to a pH between 6 and 7 and lyophilized. The sample was then filtered and ready for the quantitative analysis by metal-dye detection HPLC (MDD-HPLC) (see below).

HPLC Analysis

MDD-HPLC was performed on MiniQ PC 3.2/3 column (Pharmacia Biotech) using a Kontron system (BioTEK) as described previously (Mayr, 1988) with a slight modification. The flow rate of the elutes was 0.5 ml/min, and the MDD reagent containing 2-(4-pyridylazo) resorcinol was pumped at 0.25 ml/min. The HPLC gradient made by mixing Buffer A (0.2 mM HCl, 15 μM YCl_3) with Buffer B (0.5 M HCl, 15 μM YCl_3) was as follows: 0–3 min, Buffer B increased from 3 to 5%; from 3 to 7.4 min, Buffer B increased from 5 to 10%; from 7.4 to 11.4 min, Buffer B increased slowly from 10 to 24%; from 11.4 to 14.5 min, Buffer B increased from 24 to 45%; from 14.5 to 17 min, Buffer B increased

linearly from 45 to 97%, where it remained for further 4 min; 21–21.1 min, Buffer B decreased to 3%, where it remained for further 5 min. With this gradient, very stable retention times were observed (maximal run-to-run variation range \pm 0.02 min).

Statistics

The analysis of data for luciferase activity and CREB phosphorylation in brain regions was performed by two-factorial ANOVA (region *vs* treatment) followed by Student's *t*-test for unpaired samples. Western blot data were analyzed by one-factorial ANOVA, followed by Student's *t*-test for unpaired samples (Figure 4). Data for cAMP determination were analyzed by Student's *t*-test for unpaired samples (Figure 5). All analyses were performed using the software STATISTICA 7.1, statsoft, Tulsa, USA.

RESULTS

Acute and Chronic Treatment of Transgenic Mice with Lithium

To investigate the effect of lithium salts on CRE/CREB-directed gene transcription *in vivo*, transgenic SomCRE-Luc mice were treated with lithium chloride. An acute treatment for 24 h did not result in significant changes in luciferase activity in any brain region dissected (Table 1). For chronic lithium administration, animals were treated for 21 days with 7.5 mmol/kg lithium. This dose resulted from day 4 on in a 2.48-fold increase in water drinking ($248 \pm 23.1\%$ (SEM)) compared to control. Lithium concentration in brain homogenates as determined by atomic absorption spectrophotometry was 3.43 ± 0.53 mM, indicating sufficiently high lithium levels. In contrast to the acute treatment, chronic lithium caused a decrease in luciferase activity (Figure 1). Luciferase activity was significantly reduced in the PFC (72.4%), the hypothalamus (65.4%), the hippocampus (67.9%), and the cortex (64.7%), whereas the other regions showed only a tendency for diminished luciferase activity.

Table 1 Luciferase activity in acutely lithium-treated CRE-Luc mice

	Acute treatment (24 h)	
	Control (solvent)	Lithium
Bulbus	100 \pm 8.52	104.96 \pm 24.14
Cerebellum	100 \pm 10.37	70.53 \pm 13.61
Pons	100 \pm 10.35	91.24 \pm 16.38
Colliculi	100 \pm 9.44	99.71 \pm 20.88
PFC	100 \pm 13.55	122.81 \pm 29.12
Hypothalamus	100 \pm 8.45	93.04 \pm 17.89
Hippocampus	100 \pm 9.19	113.69 \pm 22.50
Cortex	100 \pm 12.11	99.87 \pm 19.3

Acute treatment with lithium was performed in CRE-Luc mice that were not stressed. PFC, prefrontal cortex. Values are mean and expressed as percentage of the mean value in controls (solvent-treated littermates) (\pm SEM) from 10 animals per group.

Chronic Psychosocial Stress and Lithium Treatment

As stress has been implicated in the development of bipolar disorders, the effect of lithium on the CRE/CREB-directed transcription under chronic psychosocial stress was determined. The effect of chronic psychosocial stress on CREB using the sensory contact model has been reported previously (Boer *et al*, 2007a). In this model, the subordinate mouse is chronically stressed by the unavoidable sensory contact of a dominant conspecific. The dominant and subordinate behaviors as well as stress-induced weight loss of the subordinate animal were similar as has been reported before (data not shown). Consistent with previous results, chronic psychosocial stress increased luciferase activity in each brain region tested (Figure 2). Values reached significance in bulbus (272.9%), cerebellum (246.4%), colliculi (206.7%), hippocampus (189.3%), and cortex (202.7%).

To investigate the influence of lithium on CRE/CREB-directed gene transcription under chronic psychosocial stress, subordinate SomCRE-Luc mice in the sensory contact model were treated for 21 days with lithium. Under these conditions, the luciferase activity in most brain regions tested did not differ significantly from control levels (Figure 3). Only in the bulbus, there was still an increase in luciferase activity. Statistical comparison between the groups stress and stress plus lithium treatment revealed significant differences in colliculi, PFC, hippocampus, and cortex (Table 2). For the hypothalamus, the values only approached significance. Thus, chronic lithium treatment predominantly abolished the stress-induced increase of CRE/CREB-directed transcription and resulted in levels similar to controls.

Western Blot Analysis of CREB and Phospho-CREB in Stressed and Chronically Lithium-Treated Mice

The phosphorylation of CREB at Ser-133 is a crucial step in the activation of transcription. We thus examined CREB

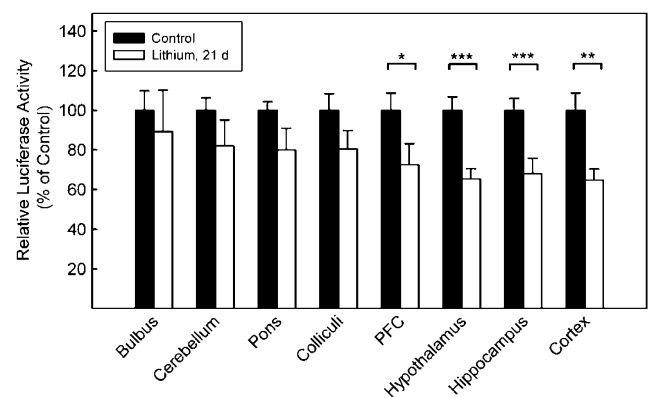


Figure 1 Effect of chronic lithium treatment on CRE/CREB-directed luciferase expression. SomCRE-Luc mice were treated chronically (21 days) with 7.5 mmol/kg/day LiCl. Luciferase activity was measured in homogenates of different brain regions, normalized to protein contents, and expressed as percentage of the mean value in controls (solvent-treated littermates) (means \pm SEM from 13 animals). Chronic treatment decreased luciferase activity in all brain regions reaching statistical significance in PFC, hypothalamus, hippocampus, and cortex. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; as determined by two-way ANOVA followed by Student's *t*-test for unpaired samples. PFC, prefrontal cortex.

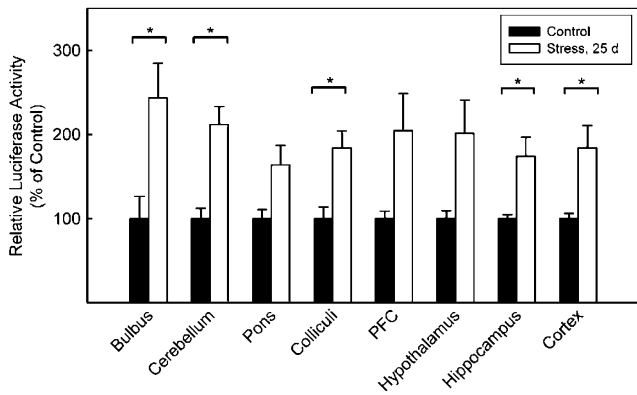


Figure 2 Effect of chronic psychosocial stress on CRE/CREB-directed luciferase expression. Male CRE-Luc mice were either not treated (black bars) or exposed to psychosocial stress for 25 days (white bars). Luciferase activity was measured in homogenates of different brain regions, normalized to protein contents, and expressed as percentage of the mean value in controls (unstressed littermates) (means \pm SEM from 10 animals). Stress increased luciferase activity in all brain regions reaching statistical significance in bulbus olfactorius, cerebellum, colliculi, hippocampus, and cortex. * $p < 0.05$ as determined by two-way ANOVA followed by Student's *t*-test. PFC, prefrontal cortex.

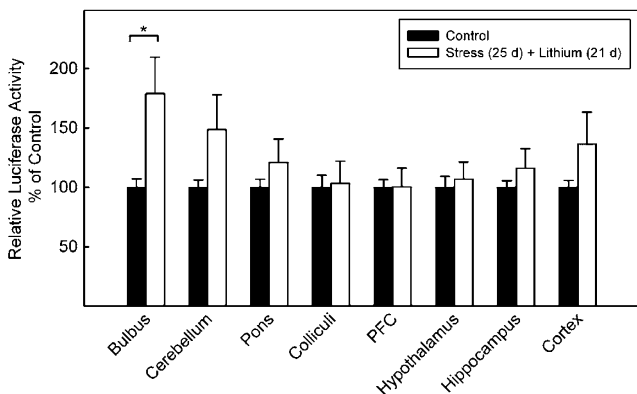


Figure 3 Effect of lithium together with chronic psychosocial stress on CRE/CREB-directed luciferase expression. Male CRE-Luc mice were either solvent treated (black bars) or exposed to chronic social stress for 25 days and treated with lithium for 21 days from days 5–25 of social stress (white bars). Luciferase activity was measured in homogenates of different brain regions, normalized to protein contents, and expressed as percentage of the mean value in controls (solvent-treated littermates) (means \pm SEM from nine animals). Lithium prevented the stress-induced increase in luciferase activity in each brain region except the bulbus olfactorius. * $p < 0.05$ as determined by two-way ANOVA followed by Student's *t*-test. PFC, prefrontal cortex.

phosphorylation in the brains of mice subjected to the various treatments. Chronic lithium treatment did not change the levels of total CREB (Figure 4a and b). In contrast, lithium decreased CREB phosphorylation to 62% (Figure 4b and c). Chronic psychosocial stress induced a two-fold increase in CREB phosphorylation. The stress-induced increase of CREB phosphorylation was completely reversed by concurrent lithium treatment (Figure 4c). Thus, the change in the phosphorylation of CREB mirrored the changes in CREB activity under chronic lithium treatment, stress exposure, or both.

Table 2 Effect of lithium treatment on stress-induced increase in CRE/CREB-directed luciferase expression

Region	Stress (% of control)	Stress+Lithium (% of control)	<i>p</i> -value
Bulbus	243.3 \pm 41.2	178.9 \pm 30.6	0.242
Cerebellum	211.8 \pm 21.4	148.7 \pm 29.3	0.106
Pons	163.5 \pm 23.1	121.0 \pm 19.8	0.192
Colliculi	183.9 \pm 20.0	103.4 \pm 18.6**	0.009
PFC	201.2 \pm 39.7	100.5 \pm 15.7*	0.038
Hypothalamus	204.4 \pm 44.2	106.9 \pm 14.4(*)	0.061
Hippocampus	173.7 \pm 23.1	116.1 \pm 16.6*	0.028
Cortex	184.1 \pm 26.5	136.6 \pm 26.7*	0.042

Abbreviation: PFC, prefrontal cortex.

Male CRE-Luc mice were either only exposed to chronic social stress for 25 days or additionally treated from days 5–25 with lithium (21 days). Luciferase activity was measured in homogenates of different brain regions, normalized to protein contents, and expressed as percentage of mean value of respective controls (non-treated or solvent-treated). Stress: means \pm SEM of 10 animals, stress+lithium: means \pm SEM of nine animals. The *p*-values indicated were determined by two-way ANOVA followed by Student's *t*-test for unpaired samples. * $P < 0.05$; ** $P < 0.01$.

β -Catenin Levels, Inositol Polyphosphates, and cAMP in Chronically Lithium-Treated Mice

Inositol monophosphatase, adenylate cyclase, and GSK3 are known targets of lithium (Quiroz *et al*, 2004). The inhibition of inositol monophosphatase by lithium can induce the depletion of free inositol together with an accumulation of inositol phosphates (Agranoff and Fisher, 2001). Inositol phosphates in brain homogenates of mice treated for 21 days with lithium were determined by metal-dye-detection HPLC. As shown in Figure 5a and Table 3, there was no significant difference in Ins(1,4,5)P₃, Ins(1,3,4,5)P₄, and other inositol phosphate isomer levels between the lithium-treated and the control group. Only a slight increase of Ins(1,4,5)P₃ levels was observed (Figure 5a).

Moreover, the effect of chronic lithium treatment on adenylate cyclase activity was examined by measuring intracellular cAMP levels. By RIA, in brain homogenates cAMP levels were 10–20 pmol/mg. Chronic lithium treatment resulted in a slightly decreased cAMP level of 82%, which was marginally significant ($p = 0.056$) (Figure 5b). Finally, the effect of lithium on GSK3 was determined. Inhibition of GSK3 activity can be measured by determination of β -catenin accumulation. As modulator of the wnt-signalling pathway, GSK3 phosphorylates β -catenin and subsequently induces its degradation. The inhibition of GSK3 should result in accumulation of β -catenin. β -Catenin was clearly detectable by Western blot in brain homogenates; however, only a slight accumulation in mice treated for 21 days with lithium was observed in whole brain homogenates (Figure 6a). It has been reported that GSK3 inhibition by lithium was predominantly detectable in cytosolic fractions (Gould *et al*, 2004). Thus, β -catenin accumulation was determined in cytosolic fractions of those regions showing significant lithium effects on CREB activity.

β -Catenin levels were significantly increased in PFC (161%) and hypothalamus (145%) of lithium-treated mice;

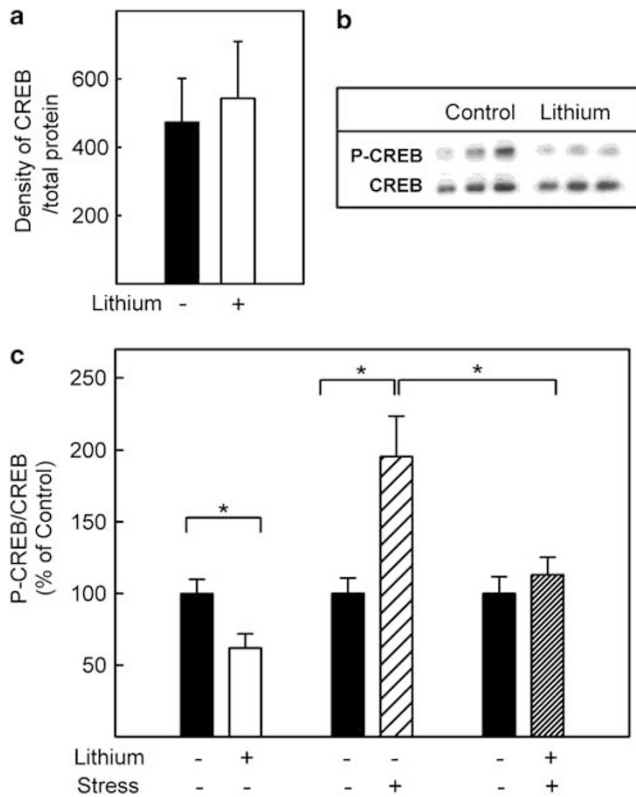


Figure 4 Effect of lithium on CREB Ser 133 phosphorylation. Male CRE-Luc mice were treated for 21 days with lithium or exposed to chronic psychosocial stress with or without concurrent lithium treatment for 21 days. Homogenates of whole brains were subjected to immunoblotting using specific CREB and phospho-CREB (P-Ser 133) antibodies. (a) The intensity of specific CREB bands was determined densitometrically and normalized by protein contents. No effect of lithium on CREB expression level was observed. (b) Typical immunoblot representing specific CREB and phospho-CREB (P-CREB) bands of treated and control animals. (c) The intensity of specific CREB and phospho-CREB bands was determined densitometrically and ratios between CREB and phospho-CREB were calculated. Lithium induced a decrease of CREB phosphorylation. Values are means \pm SEM from seven animals. * $p < 0.05$; one-factorial ANOVA followed by Student's *t*-test for unpaired samples.

in cortex (139%), the increase was marginally significant (Figure 6b).

Thus, in addition to the inhibition of adenylate cyclase, most likely the inhibitory influence of lithium on GSK3 activity correlates with the observed effect on CRE/CREB-directed gene transcription.

DISCUSSION

In the present study, the effect of lithium salts on CRE/CREB-directed gene transcription has been investigated. This was carried out for the first time *in vivo*, using the transgenic reporter mice line SomCRE-Luc (Boer et al, 2007a) that allows the monitoring of the transcriptional activity of CREB in the brain. The major findings are as follows: (i) Chronic but not acute lithium treatment decreased CRE/CREB-directed gene transcription. (ii) Chronic lithium treatment reversed the stress-induced increase of CRE/CREB-directed transcription. (iii) Lithium-induced changes in

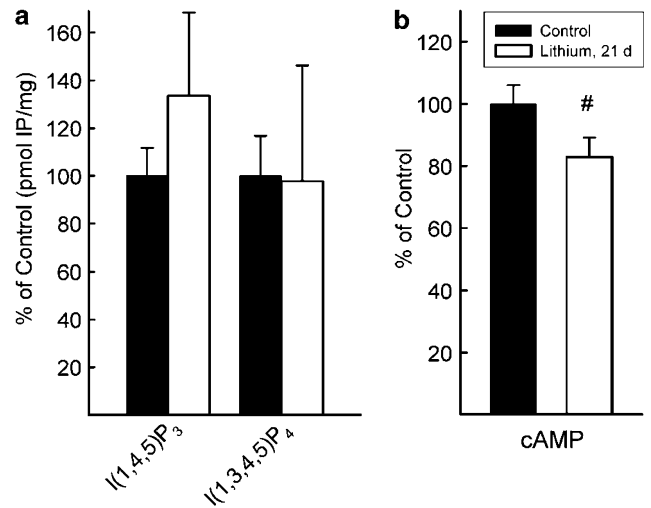


Figure 5 Effect of chronic lithium treatment on inositol phosphate and cAMP levels in brain homogenates. SomCRE-Luc mice were treated with lithium for 21 days. (a) Inositol phosphate levels. Homogenates of whole brains were extracted by trichloroacetic acid/diethylether and submitted to metal-dye-detection HPLC analysis. Values are means \pm SEM from seven animals. (b) cAMP levels of whole brain homogenates were determined by RIA using a cAMP-specific antiserum and the non-acetylated standard method. Values are means \pm SEM from 12 animals. # $p = 0.059$; Student's *t*-test for unpaired samples.

Table 3 Inositol phosphate levels in chronically lithium-treated mice

myo-InsP Isomer ^a	nmol/mg wet tissue weight			
	Control (solvent)		Lithium	
	Mean	SEM	Mean	SEM
Ins(1,4,5)P ₃	1.76	0.21	2.09	0.44
Ins(1,3,4)P ₃	0.07	0.03	0.12	0.07
Ins(1,3,4,5)P ₄	0.31	0.15	0.30	0.15
Ins(1,3,4,6)P ₄	0.61	0.04	0.62	0.02
Ins(1,3,4,5,6)P ₄	0.31	0.15	0.30	0.15
Ins(1,2,3,4,6)P ₅	0.30	0.02	0.29	0.03
D/L-Ins(1,2,3,4,5)P ₅	0.37	0.05	0.34	0.03
D/L-Ins(1,2,4,5,6)P ₅	0.28	0.04	0.26	0.03
Ins(1,3,4,5,6)P ₅	6.16	0.52	6.24	0.26
InsP ₆	15.73	1.15	15.51	0.85

^aIf not denoted, configuration is D.

SomCRE-Luc mice or wild-type mice were treated chronically (21 days) with 7.5 mmol/kg/day LiCl. Inositol phosphate levels were determined from whole brain homogenates. Tissue was extracted by trichloroacetic acid/diethylether and submitted to metal-dye-detection HPLC analysis. Values are means \pm SEM from seven animals.

CREB transcriptional activity were parallel to changes in CREB phosphorylation.

Lithium salts exert two therapeutic effects: an acute antimanic action and a delayed mood-stabilizing and phase prophylactic action, which requires chronic treatment of up to several months (Gitlin, 2006). The latter one is assumed

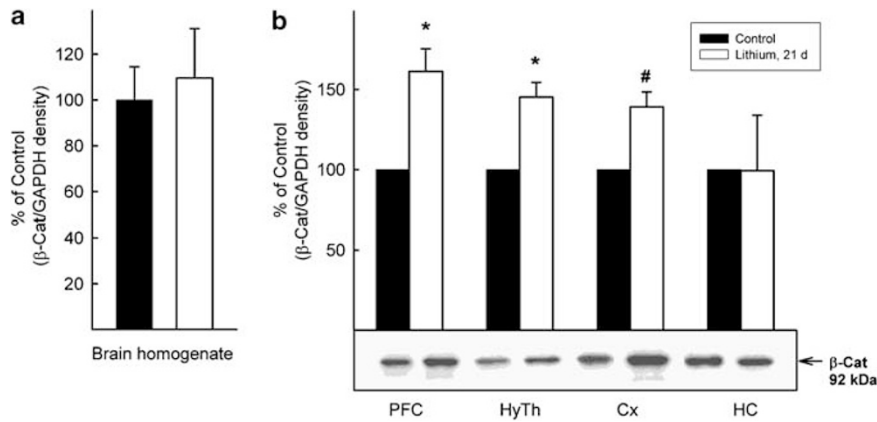


Figure 6 Effect of chronic lithium treatment on β -catenin accumulation in brain homogenates and cytosolic extracts from distinct regions. (a) Immunoblots with β -catenin and GAPDH antibodies from brain homogenates. (b) Immunoblots with β -catenin and GAPDH antibodies from cytosolic extracts of cortex (Cx), PFC, hypothalamus (HyTh), and hippocampus (HC). A representative immunoblot showing specific β -catenin bands is shown. The intensity of the bands was determined densitometrically and ratios between β -catenin and GAPDH were calculated. Solvent-treated littermates served as controls. Values are means \pm SEM from 12 animals (a) or 4 animals (b). * $p < 0.05$; # $p = 0.051$: two-factorial ANOVA followed by Student's *t*-test for unpaired samples.

to affect transcriptional processes, which induce changes in gene expression. Our results indicate that chronic but not acute lithium treatment inhibits CRE/CREB-directed gene expression in the brain. Previous studies investigated the effect of lithium on CREB phosphorylation. Our results are in line with the studies of Chen *et al* (1999), showing that chronic but not acute lithium administration reduced phospho-CREB levels in rat cortex and hippocampus. Also by Tardito *et al* (2006), a decreased CREB phosphorylation in rat hippocampus after 5 weeks of lithium treatment has been observed. However, it was also found that only acute treatment resulted in a decrease in CREB phosphorylation (Rantamaki *et al*, 2006) or that chronic lithium treatment caused an increase in CREB phosphorylation (Einat *et al*, 2003; Kim *et al*, 2004). Son *et al* (2003) found an increase in CREB phosphorylation in rat cerebellar granule cells only after acute treatment for 2 days. As we did not find any changes in gene expression after acute treatment, we did not determine CREB phosphorylation after 24 h. The reason for these diverging results is not clear. Noteworthy, the phosphorylation of CREB at Ser-133 is required but not sufficient for CREB transcriptional activity (Ravnskjaer *et al*, 2007). In contrast to the assessment of CREB phosphorylation, determining the effect of lithium on CRE-directed gene expression, as carried out in the present study for the first time *in vivo*, is a more comprehensive approach that measures CRE and CREB transcriptional activity. The present study thus suggests CREB as a putative mediator of the neuronal adaptation under chronic lithium treatment. The inhibition by lithium of CRE/CREB-directed gene transcription was detectable throughout the brain, but was most pronounced in the hippocampus, the hypothalamus, and cortical regions. This indicates that CREB activity in those regions involved in feeling, motivation, and emotional processes is most likely affected by lithium. Our results are in line with studies on the effect of lithium on the pattern of gene expression. By microarrays, more than 50 genes have been identified, which all were downregulated at least by factor 2, including genes believed to be regulated by CREB (Bosetti *et al*, 2002). No gene being upregulated has been

identified. This study underlines the inhibitory effect of chronic lithium treatment on CREB transcriptional activity.

Chronic psychosocial stress is an accepted animal model for depression, as the somatic effects on mice or rats resemble those in humans. The effects of psychosocial stress on CRE/CREB-directed gene transcription and CREB phosphorylation have been reported recently (Boer *et al*, 2007a). As bipolar disorder is considered as a stress-induced mental illness, we aimed to treat stress-exposed mice with lithium. Before, it was shown that the antidepressant imipramine was able to reverse the stress-induced CREB activity (Boer *et al*, 2007a). Interestingly, lithium likewise attenuates the stress effect and reverses CRE/CREB-directed gene transcription and CREB phosphorylation to control levels in most brain regions. Thus, regarding CREB activity, lithium can be considered as to have the same action as the tricyclic antidepressant imipramine. Indeed, lithium serves as an adjunct in treatment-resistant depression and also has proven to be effective in bipolar disorder type II where depressive episodes are predominant (Keck and McElroy, 2003). The animal model of chronic psychosocial stress cannot, of course, display the complexity of mental illness. Nevertheless, our findings resemble the results of postmortem studies. CREB phosphorylation has been investigated in suicide victims. In the amygdala, CREB phosphorylation was increased, whereas under lithium treatment, the phosphorylation of CREB was significantly lower in the same region (Young *et al*, 2004).

What could be the underlying mechanism for the inhibitory action of lithium on CREB activity? As the lithium-induced changes in CRE/CREB activity were accompanied by similar lithium-induced changes in CREB phosphorylation, lithium appears to interfere with signalling pathways that lead to the phosphorylation of CREB at Ser-133. The phosphorylation of CREB at Ser-133 is required for CREB activity. It is the prerequisite for the binding of the coactivator CBP, which facilitates transcription by its histone acetyltransferase activity rendering the DNA more accessible for DNA polymerase II (Shaywitz and Greenberg, 1999). CBP has been shown to be crucial for CREB activity

and disruption of one CBP allele leads to the Rubinstein-Taybi syndrome with mental retardation (Shaywitz and Greenberg, 1999).

There are three well-known targets of lithium in signalling pathways: inositol monophosphatase, adenylate cyclase, and GSK3, which all could be involved in the regulation of CREB activity (Johannessen *et al*, 2004).

First, a group of at least four related phosphomonoesterases has been shown to be inhibited by lithium (York *et al*, 1995). Among those, inhibition of the inositol monophosphatase was suggested to be a common mechanism of action of three mood stabilizers (Williams *et al*, 2002). Inhibition of the inositol monophosphatase has been shown to increase inositol monophosphates and to decrease myoinositol concentrations in rat brain (O'Donnell *et al*, 2000), although in humans, this effect has not consistently been observed (Silverstone *et al*, 2005). In our study, chronic lithium treatment did not result in significant changes in the levels of those inositol phosphates that have specific roles in cell signalling (Table 3 and Figure 5a). Second, lithium can influence the cAMP-PKA-CREB signalling pathway and does so in at least two different ways. On the one hand, lithium is well known to inhibit adenylate cyclase (Mork and Geisler, 1987) leading to a decrease in cAMP levels and inhibition of CREB phosphorylation and transcriptional activity. On the other hand, lithium has recently been shown in a cultured cell line to stimulate the coactivator TORC at the CREB basic region leucine zipper domain leading to an increase in cAMP-induced CREB transcriptional activity (Boer *et al*, 2007b). After activation of the cAMP signalling pathway through stimulation of adenylate cyclase, the former effect of lithium predominates, as lithium inhibits CRE/CREB-directed transcription induced by the adenylate cyclase activator forskolin (Boer *et al*, 2007b). This lithium action could explain the decrease in CREB phosphorylation and CREB activity observed in the present study *in vivo*, because PKA is well known to phosphorylate CREB at Ser-133 (Johannessen *et al*, 2004) and because, under the experimental conditions used, there was a slight lithium-induced decrease in brain cAMP concentrations, which approaches significance. Moreover, lithium may also inhibit PKA (Jensen and Mork, 1997; Mori *et al*, 1996).

Third, β -catenin is activated by the wnt-signalling pathway and serves as transcription factor. GSK3 interferes with this pathway by phosphorylating β -catenin and thereby inducing its rapid degradation (Jope and Bijur, 2002). In the present study, lithium treatment elevated β -catenin levels in cortex, PFC, and hypothalamus, indicating that lithium inhibited GSK3 under the conditions used. The influence of GSK3 on CREB has been discussed vividly in the past. A secondary phosphorylation of CREB at Ser-129 by GSK3 has been suggested to be required for full CREB transcriptional activity (Fiol *et al*, 1994; Tyson *et al*, 2002). On the other hand, it was shown that GSK3 phosphorylation of CREB impaired CREB-DNA binding (Grimes and Jope, 2001). As in the regions with lithium-induced β -catenin accumulation, CREB activity was diminished in the present study, the possibility exists that GSK3-induced phosphorylation of Ser-129 may have been necessary for full CREB activation. However, in hippocampus, β -catenin accumulation was not observed.

The lithium-induced decreases in CREB phosphorylation at Ser-133 lead to decrease in CREB transcriptional activity (Shaywitz and Greenberg, 1999) and are thus sufficient to explain the observed lithium-induced changes in CRE-directed transcription. They are therefore the most likely underlying cause. However, additional mechanism may contribute. One of these mechanisms could be an influence on CRE/CREB-directed gene transcription by ICER (inducible cAMP early repressor), which can be induced by lithium (Spencer and Houpt, 2001). ICER antagonizes CREB DNA binding and thereby interferes with CRE/CREB-directed transcription. ICER has been shown to be expressed in rat brain, including the hippocampus (Konopka *et al*, 1998). Thus, adenylate cyclase and GSK3 inhibition as well as ICER induction may contribute to the observed lithium effect, possibly to different degrees dependent on the brain regions.

Lithium has been suggested to exert its therapeutic action by interfering with multiple signalling pathways (Quiroz *et al*, 2004). By showing that lithium inhibits CREB activity in wide spread brain areas and also normalizes stress-induced upregulation of CREB activity possibly through several pathways, the present study underlines the complexity of lithium-induced effects and suggests CREB as an important downstream molecular target of lithium, providing a link to multiple effects on gene expression and lithium-induced neuronal adaptation.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare that except from income received from the primary employers, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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