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**Enhancement by lithium of cAMP-induced CRE/CREB-directed gene transcription  
conferred by TORC to the CREB basic region leucine zipper domain**

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## SYNOPSIS

The molecular mechanism of action of lithium salts in the treatment of bipolar disorder is not well understood. As their therapeutic action requires chronic treatment, adaptive neuronal processes are suggested to be involved. The molecular basis of this are changes in gene expression regulated by transcription factors like the cAMP-responsive element (CRE) binding protein (CREB). CREB contains a transactivation domain, in which Ser-119 is phosphorylated upon activation, and a basic leucine zipper region (bZip). The bZip serves for CREB dimerization and DNA-binding but also contributes to CREB transactivation by recruiting the coactivator TORC (transducer of regulated CREB). Here, the effect of lithium on CRE/CREB-directed gene transcription was investigated. Electrically excitable cells were transfected with CRE/CREB-driven luciferase reporter genes. LiCl (6 mM or higher) induced an up to 4.7-fold increase in 8BrcAMP-stimulated CRE/CREB-directed transcription. This increase was not due to enhanced Ser-119-phosphorylation or DNA-binding of CREB. Also the known targets inositol-monophosphatase and glycogen-synthase-kinase 3 $\beta$  were not involved as specific GSK3 $\beta$ -inhibitors and inositol replenishment did not mimic and abolish, respectively, the effect of lithium. However, lithium no longer enhanced CREB activity when the CREB-bZip was deleted or the TORC binding site inside the CREB-bZip was specifically mutated (CREB-R300A). Otherwise, TORC overexpression conferred lithium responsiveness to CREB-bZip or the CRE-containing truncated rat somatostatin promoter. This indicates that lithium enhances cAMP-induced CRE/CREB-directed transcription, conferred by TORC to the CREB-bZip. We thus support the hypothesis that lithium salts modulate CRE/CREB-dependent gene transcription and suggest the CREB coactivator TORC as new molecular target of lithium.

**Short title:** Lithium enhances CREB-driven gene transcription

**Keywords:** lithium, cAMP-responsive element binding protein (CREB), basic leucine zipper region (bZip), transducer of regulated CREB (TORC), coactivator, reporter gene assays.

## <sup>2</sup>Abbreviations:

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<sup>2</sup>**Abbreviations:** CRE, cAMP-responsive element; CREB, cAMP-responsive element binding protein; bZip, basic leucine zipper domain; TORC, transducer of regulated CREB; GSK3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; IMP, inositol monophosphatase; EMSA, electromobility shift assay; PKA, protein kinase A

## INTRODUCTION

Lithium salts are widely used in the therapy of bipolar disorder [1]. A hallmark of the therapeutic concept of lithium treatment is the chronic administration. To achieve the mood-stabilizing and phase prophylactic action, treatment with lithium for several months has been shown to be necessary. Though the effectiveness of lithium is indisputable its mechanism of action is still poorly understood. Some molecular targets for lithium have been identified. Among those are enzymes like the adenylyl cyclase, the glycogen synthase kinase 3 $\beta$  and inositol monophosphatase [2]. As monovalent cation lithium is able to inhibit these enzymes and thereby subsequently induces changes in second messenger levels and signalling cascades which are thought to contribute to the therapeutic action of lithium. However, these changes occur immediately and therefore are not sufficient to explain the extremely delayed onset of the therapeutic action. Thus, subsequent to early changes in enzyme activities particularly long lasting adaptive neuronal processes are suggested to be involved. Indeed, lithium-induced plasticity could be shown in hippocampal neurons both in vitro and in vivo after chronic treatment [3]. The molecular basis for these adaptive changes is the expression of genes regulated by transcription factors. Thus, transcription factors could be a target of lithium, initiating changes in gene expression, which can be summarized as neuronal adaptation. One important transcription factor in the brain is CREB (cAMP-responsive element binding protein). CREB is involved in adaptive processes like learning, memory [4] and opioid-induced addiction [5]. Among the CREB-regulated genes there are several involved in neurotransmission and signalling [6].

CREB belongs to the family of basic leucine zipper transcription factors which contain C-terminally a leucine zipper dimerization domain and a basic region, by which the cAMP-responsive element (CRE) is bound [7]. CREB can be activated by various signalling pathways, which converge in the phosphorylation of Ser-119 located in the transactivation domain. Transactivation of transcription is accomplished by the recruitment of coactivators, which interact with the basal transcription machinery. In addition to the long-known coactivator CBP (CREB binding protein) [7] which binds to the phosphorylated transactivation domain, more recently another coactivator has been discovered. TORC (transducer of regulated CREB activity), an 80 kDa protein which exists in three isoforms [8], binds to the leucine zipper domain and contributes to the transcriptional activity of CREB.

There are few studies on the action of lithium on CREB. The majority of them focus on the phosphorylation of CREB [9-11] and its DNA binding [12, 13]. However, the DNA binding of CREB plays a minor role in the regulation of its activity, as also non-phosphorylated CREB is able to bind the CRE [7]. Furthermore it has been shown before [14-16], that phosphorylation is a necessary prerequisite for the transcriptional activation of CREB but not sufficient.

Therefore, CREB activity cannot be determined by measuring its phosphorylation or DNA binding alone but rather by determining the expression of a CRE/CREB-driven gene. Thus, in the present study we focus on the effect of lithium on the transcriptional activity of CREB as revealed by CRE- or Gal4-system-driven luciferase reporter gene constructs. The expression of the reporter gene records the complete process including DNA binding, phosphorylation, and also the recruitment of coactivators.

The present study shows that lithium strongly enhances the cAMP-stimulated CRE-mediated transcription and CREB activity. This effect depends on the basic leucine zipper region. Furthermore, TORC was shown to confer lithium responsiveness to CREB-bZip. Thus, the present study, for the first time, identifies the CREB-bZip in combination with the coactivator TORC as a new molecular target of lithium.

## EXPERIMENTAL

**Plasmids:** Reporter gene constructs used for transfection were 4xSomCRET81Luc [17] containing the luciferase gene under control of four repeats of the CRE from the somatostatin promoter and a truncated thymidine kinase promoter (Fig.1), -65SMSLuc, containing the luciferase gene under control of the truncated rat somatostatin promoter (-65 to +54) [18] and 5xGal4E1B-Luc [19] which contains the luciferase gene under control of 5 repeats of the responsive element for the yeast transcription factor GAL4. Expression vectors for the overexpression of GAL4-CREB fusion proteins were GAL4-CREB<sub>327</sub> [20], encoding full length CREB, GAL4-CREB<sub>261</sub> [21], which encodes a truncated CREB lacking the basic region and leucine zipper domain, and GAL4-CREB-R300A. The GAL4-CREB-R300A mutant was generated by primerless PCR using the following primers (mutation underlined) 5' tgt tta gaa aac gca gtg gca gtg ctt 3' (forward), 5' aag cac tgc cac tgc gtt ttc taa aca 3' (reverse), flanking primers and GAL4-CREB<sub>327</sub> as template. For PCR cloning of GAL4-bZip encoding the basic leucine zipper domain (CREB<sub>262-327</sub>), the primers 5' *cgcgatcctagc*acgaaagagagaggtcc 3' (forward) (BamHI site in italics, coding sequence underlined), 5' *cgc tct aga* **tta tta** atc tga ttt *gtg gca gta aag gtc* 3' (reverse) (XbaI site in italics, stop codons bold, coding region underlined), and GAL4-CREB<sub>327</sub> as template were used followed by cloning into the GAL4 domain-coding vector pSG424. TORC1-coding sequence was kindly provided by Marc Labow, (Novartis Pharma AG, Basel, Switzerland) and subcloned into pcDNA3.

### Cell culture, transfection and stimulation

Hamster insulinoma tumor cells (HIT-T15) [18] were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 5% horse serum, 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were transiently transfected by the DEAE-dextran method [18] with 2 µg of the indicated plasmid per 6-cm dish.

Cytomegalovirus green fluorescent protein expression vectors (1 µg per 6-cm dish) were added as second reporters to control for transfection efficiency. Cells were treated with 1 mM myoinositol 8 h prior to harvest, with 20 mM LiCl or the GSK3β inhibitors SB215763 (30 µM) or SB415286 (50 µM) 7 h prior to harvest, and with 1 or 2 mM 8BrcAMP, 40 mM KCl or 10 µM forskolin 6 h prior to harvest. Cell extracts were prepared 48 h after transfection. The luciferase assay was performed as previously described [18]. Fluorescence of the green fluorescent protein was measured in a Packard FluoroCount with excitation wave length at 485 nm and emission wave length at 530 nm. For the determination of the intracellular lithium concentration, cells were lysed by freeze-thaw cycles and the lithium concentration in supernatants was measured by ion-selective potentiometry using an Easylyte Na/K/Li analyzer (Bedford, USA).

### **Western blot**

For CREB/ phospho-CREB determination, HIT-T15 cells were treated 3 h prior to harvest with 20 mM LiCl and 2 h prior to harvest with 2 mM 8BrcAMP. For measurement of  $\beta$ -catenin accumulation, the cells were treated 6 h prior to harvest with 20 mM LiCl or the GSK3 $\beta$  inhibitors SB215763 (30  $\mu$ M) or SB415286 (50  $\mu$ M). Proteins in the cell lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane by semi dry blotting. CREB, phospho-CREB or  $\beta$ -catenin was immunostained with specific antibodies and respective peroxidase-labeled secondary antibodies. Immunoreactivity was detected using the ECL reaction.

Specific bands were quantified densitometrically and the ratio between intensity of CREB and phospho-CREB from the same lysate was calculated. For  $\beta$ -catenin blots identical loading was assured by GAPDH staining.

### **Electrophoretic mobility shift assay (EMSA)**

For EMSA, HIT-T15 cells were treated with 20 mM LiCl 2 h prior to harvest and with 2 mM 8BrcAMP 1 h prior to harvest. Nuclear extracts were prepared according to Schreiber [22]. The synthetic complementary oligonucleotides SCE [17] with 5'-GATC overhangs were annealed and labelled by a fill-in reaction using [ $\alpha$ - $^{32}$ P]dCTP and Klenow enzyme. Using 11  $\mu$ g protein from the nuclear extracts, the EMSA was performed as described [23]. Briefly, nuclear extracts were incubated in 20  $\mu$ l (total volume) containing 20 mM HEPES pH 7.9, 1 mM EDTA, 0.5 mM dithiothreitol, 5 mM MgCl $_2$ , 70 mM KCl, 10% glycerol, 0.2  $\mu$ g poly(dI-dC) as non-specific competitor and 16 pmol of unlabeled wildtype or mutant SCE as specific competitor, if indicated, for 10 min on ice. Then, 20,000 cpm of the labelled probe were added and incubated 15 min on ice. Proteins were separated on 5% non-denaturing polyacrylamide gel electrophoresis and bound probes detected by autoradiography.

### **Measurement of intracellular calcium [ $\text{Ca}^{2+}$ ] $_i$**

HIT-T15 cells were subcultured on coverslips at 37 $^\circ$  C in medium containing 130 mM NaCl, 4.7 mM KCl, 1.2 mM KH $_2$ PO $_4$ , 1.2 mM MgSO $_4$ , 1.5 mM CaCl $_2$ , 6 mM glucose, 20 mM HEPES and 2% BSA (w/v), aerated with 100% O $_2$  (v/v), pH 7.4. Where indicated, cells were pretreated with 20 mM LiCl for 1 h before loading with 5  $\mu$ M fura-2/AM, in the presence of Pluronic F-127 0.1% (w/v) at 37  $^\circ$ C for 30 min. After loading, the coverslips were washed, mounted in a temperature-controlled superfusion chamber (37 $^\circ$ C), and placed on the platform of a Carl Zeiss Axiovert IM 135 equipped with a 40x Achromat oil immersion objective (Carl Zeiss, Jena, Germany). The chamber was superfused with the same buffer as above with 0.1% BSA (w/v), without 0.1% Pluronic F-127 (w/v) and the respective test agents at a flow rate of 0.75 ml/min. Ca $^{2+}$  measurements were taken on cells of average size and healthy

appearance (round in shape, no membrane blebs). Fura-2 fluorescence from single cells was recorded with a dual excitation spectrofluorometer system (Deltascan 4000, Photon Technology Instruments, Wedel, Germany).  $[Ca^{2+}]_i$  was calculated according to the formula  $[Ca^{2+}]_i = K_d \times B \times (R - R_{min}) / (R_{max} - R)$ , where  $K_d = 225$  nM [24],  $R_{max}$ ,  $R_{min}$  and  $B$  are constants that were determined in the superfusion chamber from solutions containing fura-2 free acid (1  $\mu$ M) and various concentrations of free  $Ca^{2+}$  (data not shown). All records have been corrected for autofluorescence of unloaded cells at each wavelength before the ratio was used.

### Materials

All reagents, unless otherwise indicated, are from Sigma-Aldrich, München, Germany. Stock solutions were prepared in water, except for forskolin, SB-215763 and SB-415286 (DMSO). Controls received the solvent only.  $[\alpha^{32}P]$ dCTP and ECL system was obtained from GE Healthcare (München, Germany). All enzymes were purchased from Fermentas GmbH, St. Leon-Roth, Germany. SB-215763 and SB-415286 were obtained from GlaxoSmithKline, München, Germany. Phospho-CREB antibody was from NewEngland Biolabs, Frankfurt a.M., Germany and  $\beta$ -catenin antibody was from BD biosciences, Heidelberg, Germany.

### Statistics

All analyses (two-way ANOVA with Bonferroni post hoc test, Student's t-test) as indicated in the legends to the figures were performed using the soft ware STATISTICA 7.1, statsoft, Tulsa, USA.



## RESULTS

To investigate the effect of lithium salts on CRE-directed gene transcription, a transient transfection assay was used. The electrically excitable hamster insulinoma tumor cell line HIT was transfected with a DNA construct in which the expression of a reporter gene is controlled by the CRE from the rat somatostatin promoter. This CRE contains the consensus sequence TGACGTCA and is well known as a high affinity CREB binding site that mediates responsiveness to cAMP and  $\text{Ca}^{2+}$  [17]. The construct (Fig. 1) containing four copies of the CRE in front of the truncated viral thymidine kinase promoter and the luciferase gene, has been characterized before in HIT cells [25]. The CRE conferred negligible low basal transcriptional activity (Fig.1). Stimulation with the adenylyl cyclase activator forskolin resulted in a 27.5-fold increase of CRE-mediated transcription, as described before [25]. For lithium treatment, a concentration of 20 mM was chosen. This concentration resulted in an intracellular lithium concentration of  $3.2 \pm 0.2$  mM. Treatment with 20 mM LiCl only slightly increased basal CRE activity (not significant) (Fig.1). In contrast, lithium treatment resulted decreased of forskolin-induced CRE activity by 41% (Fig.1). This decrease is most likely due to the inhibition of the adenylyl cyclase activity by lithium [26] as the measurement of intracellular cAMP levels indicated markedly reduced levels after lithium treatment (data not shown).

### 1. Enhancement by lithium of cAMP-induced transcription

To test, whether, in addition to its effect on cAMP formation, lithium can influence cAMP-induced transcription; HIT cells were stimulated with 8BrcAMP, an analogue for cAMP with a higher stability and increased membrane permeability. Stimulation with 1 mM 8BrcAMP resulted in a 4.7-fold increase in CRE-mediated transcription (Fig. 2, left panel). In contrast to the effect on forskolin stimulation, lithium in combination with 8BrcAMP exerted a robust enhancing effect on CREB activity by 22.3-fold. Relative to the 8BrcAMP-induced stimulation, lithium treatment resulted in a 4.7-fold increase (Fig.2).

As HIT cells are electrically excitable [25], we asked whether lithium could influence calcium-induced CRE-mediated transcription [25]. HIT cells were treated with 40 mM KCl to induce membrane depolarization and subsequent calcium influx. Membrane depolarization led to a 6.5-fold increase of CRE-mediated transcription (Fig. 2, left panel). Lithium treatment increased depolarization-induced CRE activity only 1.39-fold, which was not significant. In contrast, the combined stimulus of 8BrcAMP plus depolarization, which resulted in a 140-fold increase of CRE-mediated gene transcription, was further enhanced by lithium by 1.8fold (Fig. 2, right panel) which was highly significant. Since lithium enhanced transcription not only after stimulation by cAMP but also after stimulation by cAMP plus KCl, lithium may affect transcription not through an increase in cytosolic calcium concentrations. For further

investigation, the intracellular calcium levels of HIT cells after different stimuli were recorded. 20 mM LiCl did not change the basal intracellular  $\text{Ca}^{2+}$  level ( $180 \pm 3$  nM (control) vs.  $176 \pm 5$  nM (lithium)). Stimulation with 2 mM 8BrcAMP increased intracellular  $\text{Ca}^{2+}$  by  $84 \pm 16$  nM. Pretreatment with lithium resulted in a slightly decreased calcium rise ( $38 \pm 11$  nM), which was not significant (Student's t-test). Stimulation with both, 8BrcAMP and KCl, increased  $\text{Ca}^{2+}$  levels by  $226 \pm 25$  nM over basal. Pretreatment with lithium had no effect ( $218 \pm 37$  nM). The results support the notion that the enhancing effect of lithium on CRE-mediated transcription is not caused by elevated intracellular calcium levels.

To examine the concentration-response relationship of the effect of lithium on 8BrcAMP-stimulated CRE activation was dose-dependent, increasing concentrations of lithium were employed. A significant enhancing effect of lithium was detectable at 6 mM (2.3-fold increase of 8BrcAMP-induced activity) (Fig. 3). Higher concentrations (20 and 50 mM LiCl) enhanced 8BrcAMP-induced activity about 4-fold (Fig.3) and 11.4-fold, respectively (data not shown). The dose-response curve did not show a sigmoid characteristic as it reached no maximum. Thus, a half maximal effective dose could not be calculated.

#### Role of the known lithium targets GSK3 $\beta$ and inositol-monophosphatase

Lithium salts are known to inhibit glycogen-synthase-kinase 3 $\beta$  (GSK3 $\beta$ ) and inositol-monophosphatase (IMP) [2]. Therefore the question was addressed whether these two enzymes are involved in the effect of lithium on CRE-mediated gene transcription.

Two different GSK3 $\beta$  inhibitors were used. Both, SB216763 and SB415286 did not mimic the effect of lithium (Fig. 4a). SB216763, SB415286 and lithium induced  $\beta$ -catenin accumulation indicating efficient inhibition of GSK3 $\beta$  (data not shown).

Lithium inhibits IMP and thus depletes inositol in the cell [2]. Therefore, cells were treated concurrently with inositol to replenish a presumed depletion in inositol storage. The addition of inositol did not change the effect of lithium on 8BrcAMP-induced CRE-mediated transcription (Fig. 4b). Thus, these well known biochemical targets of lithium most likely were not responsible for the stimulation of CRE-mediated transcription by lithium.

#### Effect of lithium on CREB phosphorylation and CRE binding

An important step in the activation of CREB is its phosphorylation on Ser-119. Therefore, the ratio of phosphorylated to non-phosphorylated CREB was determined by western blot analysis. Treatment with lithium alone did not change the ratio of P-CREB to CREB (Fig. 5). 8BrcAMP induced a slight 1.3-fold increase in CREB Phosphorylation. This Phosphorylation was not detectably changed by lithium (Fig.5). However, as the values only approached

significance ( $p=0,065$ , one-way ANOVA), probably because of the low 8BrcAMP concentration, these are only tendencies which, nevertheless, most likely do not account for the observed effect of lithium on CRE-mediated transcription.

Another possibility for the action of lithium on CRE-mediated gene transcription could be the modulation of the DNA binding of CREB. Therefore, EMSAs with nuclear extracts from lithium treated cells and the somatostatin CRE were performed. Nuclear CREB binding to the CRE was clearly detectable as shown before [17] (Fig. 6a). The specificity of the band was confirmed in competition experiments. Adding an excess of unlabelled CRE markedly decreased the intensity of the band to 8.7 % of control, whereas or a similar excess of a mutant CRE was almost ineffective (reduction to 76.1% of control).

The DNA binding of CREB was not changed by any treatment (Fig. 6b) ( $p=0.9$ , effect of treatment, two-way-ANOVA). Thus, the enhancement of CRE-mediated gene transcription was not related to an effect of lithium on the binding of CREB to its responsive element as revealed by EMSA.

#### Lithium responsiveness depends on the CREB-bZip

To further investigate the mechanism of the observed action of lithium on CREB-mediated transcription the Gal4 system was employed. Expression vectors encoding the DNA-binding domain of the yeast transcription factor GAL4 fused to the transcription factor of interest were cotransfected with a GAL4 binding site-driven luciferase reporter gene. This allows the investigation of the transactivational potential of the transcription factor of interest, independent from its DNA binding and endogenous background. The GAL4 domain itself did not show any responsiveness to the stimuli used (data not shown).

When full length CREB was fused to the GAL4 DNA-binding domain (GAL4-CREB) the treatment with 8BrcAMP and lithium mimicked the effect observed when using the CRE-driven promoter (Fig. 7). 20 mM LiCl alone had no effect on gene transcription. The combination of lithium with the cAMP stimulus resulted in a robust enhancement of gene transcription (2.9-fold over 8BrcAMP alone (Fig. 7). To test whether the lithium effect depends on the bZip domain of CREB, a truncated mutant of CREB (aa1-261) was fused to GAL4 (GAL4-CREB<sub>261</sub>). This construct contains the transactivation domain of CREB (including Ser-119) but lacks the basic leucine zipper domain of CREB. With GAL4-CREB<sub>261</sub>, the lithium effect was completely abolished (Fig. 7). Stimulation with 20 mM LiCl increased transcription only slightly, 1 mM 8BrcAMP 1.6-fold, and the combination of both stimuli 2.43-fold, which did not reach significance (two-way ANOVA) (Fig. 7). Thus, the basic leucine zipper domain seemed to be crucial to confer the lithium effect on CREB.

## 2. The coactivator TORC confers lithium responsiveness

As the coactivator TORC binds to the bZip [8], the involvement of TORC in the lithium effect was tested. A CREB mutant with an exchange of R300 to A in the bZip has been reported to be deficient in TORC binding [27]. Thus, a GAL4-CREB-R300A mutant was employed (Fig. 8a). As reported before, this mutant responded only poorly to the cAMP stimulus [27] and also lithium failed to induce a significant enhancement (Fig. 8a). To confirm the importance of the bZip and TORC for the lithium effect, a construct containing the Gal4-DNA-binding domain fused to the isolated CREB-bZip was employed (GAL4-bZip). Without cotransfection, the bZip showed no activation by cAMP and lithium (Fig. 8a). However, when TORC1 was overexpressed, the bZip was activated by 8BrcAMP and more importantly, also the lithium effect was restored completely (2.4-fold increase, relative to the cAMP stimulus) (Fig. 8a). GAL4 and GAL4-bZip-R300A were not activated by TORC overexpression (data not shown). Finally, we tested whether TORC could confer the lithium effect also on a native promoter. Therefore, a promoter (-65SMSLuc) containing one CRE in its homologous context was employed. The truncated promoter showed only poor response to either stimulus (Fig. 8b). In contrast, after overexpression of TORC1, lithium was able to enhance the cAMP-stimulated promoter activity 1.94-fold (Fig. 8b). Thus, as shown with the Gal4-system and a native promoter, TORC is able to confer the lithium-induced enhancement of cAMP-stimulated CREB activity.

## DISCUSSION

Lithium salts have been established for more than 50 years as the most effective treatment for bipolar disorder [1]. Lithium salts have an acute silencing action, making them beneficial for the treatment not only of manic phases of bipolar disorder but also of psychotic manic states. More important, they are the only treatment that prevents the cycling between manic and depressive states and also reduces the suicide rate [28]. The requirement of chronic treatment of up to several months implicates the involvement of changes in gene expression based on the modulation of transcription factors like CREB.

CREB has been implicated in several important brain functions. In addition to physiological functions like learning and memory [4] there is evidence that CREB also plays a role in the development of mental diseases [29]. Major depression and bipolar disorder both are thought to be related to changes in the cAMP/PKA/CREB system and treatment with antidepressants and mood stabilizers are suggested to correct for deficits in neuronal signalling [30].

In this study, the action of the clinically well-established mood stabilizer lithium on CRE/CREB-driven gene transcription was investigated. The major findings are i) lithium induces a robust increase in cAMP-stimulated CRE-driven gene transcription and enhances CREB activity; ii) this effect of lithium does not appear to be related to an enhanced CREB-Ser-119 phosphorylation, an increased CREB-DNA binding or the inhibition of GSK3 $\beta$  and IMP; iii) the enhancement by lithium of CREB activity seems to be conferred by the coactivator TORC binding to the CREB basic leucine zipper region.

To study the effect of lithium on CRE/CRB-directed gene transcription, a luciferase reporter gene construct was employed where reporter gene expression is regulated by four repeats of the somatostatin CRE. This CRE contains the palindromic core binding site with the consensus sequence 5'-TGACGTCA-3'. In combination with the flanking regions, the somatostatin CRE forms a binding site with a notably high affinity for CREB [7]. The 4xSomCRET81Luc construct has been thoroughly characterized in the pancreatic  $\beta$ -cell line HIT-T15 [17, 25] as well as in other cells including neuronal cell lines [19]. HIT-cells resemble neuronal cells in important aspects. They are electric excitable, express calcium and potassium channels and also have neuron-specific transcription factors.

Lithium induced a robust increase in 8BrcAMP-induced CRE/CREB-directed gene transcription. The dose-response curve showed a significant effect with 6 mM lithium. The measurement of the cytosolic lithium concentration revealed that the intracellular level of lithium was about 6-fold lower than in the cell culture medium. As plasma levels of 1 mM lithium have been proven to be effective in patients, we conclude that lithium at therapeutic concentrations is able to increase CRE/CREB transcriptional activity.

A simple explanation for the effect of lithium could be an induction of calcium influx, since the combination of calcium and cAMP stimuli has been shown to be super additive in the

activation of CRE/CREB-directed gene transcription [17]. However, the combined stimulus, 8BrcAMP plus KCl, was also enhanced by lithium. Moreover, the determination of intracellular calcium concentrations revealed, if anything, rather decreased calcium levels by lithium after cAMP-stimulation. This could be due to an enhanced efflux of calcium caused by a replacement of sodium by lithium in a sodium/calcium exchanger which has been reported previously [31].

Two well-known targets of lithium are IMP and GSK3 $\beta$  [2]. Both do not appear to be involved in the observed effect of lithium on CRE-driven gene transcription. IMP inhibition and subsequent inositol depletion have been suggested to be the common mechanism of action of three mood stabilizing agents [32] as lithium, valproate and carbamazepine stabilized neuronal growth cones in an inositol-reversible manner. However, the action of lithium on CRE/CREB-directed transcription was not affected by the addition of inositol, which should prevent the assumed inositol depletion by lithium. GSK3 $\beta$  has been shown to be inhibited by lithium at concentrations of about 1-2 mM [33] and is discussed to mediate the therapeutic effects of lithium [34]. As CREB is phosphorylated by GSK3 $\beta$  on Ser-115 [35], lithium may influence CREB activity via GSK3 $\beta$ . It has been shown that the phosphorylation of CREB by GSK3 $\beta$  reduces the DNA binding of CREB and thus attenuated CREB activity [12]. However, in the present study, two specific GSK-inhibitors did not mimic the effect of lithium on CRE/CREB-directed gene transcription. Moreover, the EMSA revealed no effect of lithium on CREB DNA-binding. Noteworthy, lithium and the two GSK3 $\beta$  inhibitors induced an accumulation of  $\beta$ -catenin indicating that GSK3 $\beta$  was active and the inhibitors were effective. Thus, inhibition of GSK3 $\beta$  most likely does not contribute to the effect of lithium on cAMP-induced CRE/CREB-directed gene transcription.

As the two best known lithium targets seemed not to be involved we focused on the effect of lithium on CREB activity. Western blot analyses revealed that the robust enhancement of cAMP-induced CRE/CREB-directed gene transcription was not accompanied by an increased phosphorylation of CREB. Unchanged CREB phosphorylation after lithium treatment of an osteoblastic cell line [36] or in cerebellar granule cells of acutely treated rats [11] has been shown before. Moreover, in addition to the lacking impact on CREB phosphorylation it was shown that the lithium effect was not dependent on the Ser-119 containing transactivation domain but required the existence of the CREB-bZip. We here confirm a discrepancy between CRE/CREB-mediated transcription and phosphorylation, which has been reported before in HIT cells after cyclosporine A treatment [14] or in cortical [15] and hippocampal neurons [16]. Until recently, CREB transcription activity was generally explained by the classic transactivation pathway of CREB e.g. phosphorylation of Ser-119 and recruitment of CBP, though the involvement of additional factors has also been assumed [37].

Nowadays, a new coactivator has been discovered; the transducer of regulated CREB (TORC) [8]. TORC confers CREB transactivation by binding to the CREB-bZip independent from phosphorylation at Ser-119. TORC exists in three isoforms and has been shown to be localized under basal conditions in the cytosol. Upon stimulation with cAMP it translocates to the nucleus and binds to the leucine zipper of CREB where it contributes to transcriptional activity. As the effect of lithium on CREB activity and the action of TORC converge at the CREB-bZip, the involvement of TORC into the lithium action was shown. Three approaches were used. First, arginine 300 which has been shown to be crucial for the CREB-TORC interaction was mutated to alanine. Here, lithium failed to induce an enhancement of CREB activity. Second, the isolated CREB-bZip showed activation neither by cAMP nor by lithium; however, overexpression of recombinant TORC completely restored the lithium effect. As the isolated CREB-bZip was not sufficient to mediate the lithium effect, one could assume that the recruitment of endogenous TORC was less stable or occurred with lower affinity. The conformation of the CREB-bZip or its position related to the DNA is presumably suboptimal in the GAL4-bZip construct used here, suggesting that the CREB-TORC interaction is stabilized by other CREB regions than the bZip. Overexpression results in higher concentration of TORC in the nucleus which could overcome these deficits. Third, the somatostatin CRE in its homologous context was employed. The promoter contains the CRE which was used to create the oligomerized promoter in 4xSomCRET81Luc. Overexpression of TORC conferred the responsiveness of the native promoter to lithium, thus indicating that also in the homologous context TORC was mediating the lithium effect.

What could be the consequences of these findings? Generally, lithium could be able to modulate the expression of CRE/CREB-regulated genes [6]. The brain derived neurotrophic factor (BDNF) gene and the antiapoptotic Bcl-2 gene are regulated by CREB which has been implicated in neuroprotective and neuroplasticity processes. It was shown that lithium treatment increased expression of BDNF and Bcl-2 in rat hippocampus and cortex [10], which has been considered to contribute to the neuroprotective action of lithium. Furthermore, in the hippocampus it has been shown, that TORC1 was essential for CREB-induced long-term potentiation, a form of synaptic plasticity involved in long-term-memory formation [16]. The hippocampus plays a pivotal role in the control of mood and emotion. In addition, hippocampal volume reduction was associated with bipolar disorder [38]. Thus, one could assume that the mood-stabilizing action of lithium involves CRE/CREB-TORC-mediated gene expression particularly in this brain region.

We here for the first time provide evidence that lithium plays a direct role in the CRE/CREB-directed gene transcription. We showed that independent from interfering with signalling cascades lithium affects directly CREB activity and suggest the coactivator TORC as a new target for its molecular mechanism of action. This will not only deepen the understanding of

the therapeutic action of lithium salts. It also provides new insight into the regulation of gene expression whereas the interplay between transcription factors and their coactivators can be modulated by ions like lithium.



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## LEGENDS TO THE FIGURES

### Fig. 1

Effect of lithium on forskolin-stimulated CRE/CREB-directed gene transcription. HIT-T15 cells were transfected with the indicated reporter gene construct 4xSomCRET81Luc. 7 h prior to harvest cells were treated with 20 mM LiCl and 6 h prior to harvest with 10  $\mu$ M forskolin. 48 h after transfection luciferase activity was determined. Values are expressed as % of the controls that received solvent only. Values are means of 4 independent experiments  $\pm$  SEM each done in duplicate. FSK: forskolin. TK: thymidine kinase promoter. Luciferase: coding sequence of the firefly luciferase gene. \*\*\*:  $p < 0.001$ , two-way ANOVA, Bonferroni post-hoc test.

### Fig. 2

Effect of lithium on cAMP- and membrane depolarization-induced CRE/CREB-directed gene transcription. HIT-T15 cells were transfected with the reporter gene construct 4xSomCRET81Luc. 7 h prior to harvest cells were treated with 20 mM LiCl and 6 h prior to harvest with 2 mM 8BrcAMP or 45 mM KCl. 48 h after transfection luciferase activity was determined. Values are expressed as % of control (no 8BrcAMP, no KCl). Values are means of 3 independent experiments  $\pm$  SEM each done in duplicate. \*\*\*:  $p < 0.001$ , two-way ANOVA, Bonferroni post-hoc test.

### Fig. 3

Concentration-response curve for the effect on cAMP-induced CRE/CREB-directed gene transcription. HIT-T15 cells were transfected with the reporter gene construct 4xSomCRET81Luc. 7 h prior to harvest cells were treated with increasing concentrations of LiCl (20  $\mu$ M-20 mM) and 6 h prior to harvest with 1 mM 8BrcAMP. 48 h after transfection cells luciferase activity was determined. Values of 8BrcAMP-treated cells that received no LiCl were set to 100%. Values are means of 3 independent experiments  $\pm$  SEM each done in duplicate. \*:  $p < 0.05$ , Student's t-test; \*\*:  $p < 0.01$ , Student's t-test.

### Fig. 4

Effect of GSK3 $\beta$  inhibitors (a) and inositol (b) on cAMP-induced CRE/CREB-directed gene transcription. HIT-T15 cells were transfected with the reporter gene construct 4xSomCRET81Luc. a) 7 h prior to harvest cells were treated with 20 mM LiCl or the GSK3 $\beta$  inhibitors SB215763 (30  $\mu$ M) or SB415286 (50  $\mu$ M) and 6 h prior to harvest with 1 mM 8BrcAMP. b) 8 h prior to harvest 1 mM myoinositol was added, 7 h prior to harvest cells were treated with 20 mM LiCl and 6 h prior to harvest with 1 mM 8BrcAMP. 48 h after

transfection luciferase activity was determined. Values are expressed as % of the controls that received solvent only. Values are means of 3 independent experiments  $\pm$  SEM each done in duplicate. \*\*\*:  $p < 0.001$ , two-way ANOVA, Bonferroni post-hoc test.

**Fig. 5**

Effect of lithium on Ser119 phosphorylation of CREB. HIT-T15 cells were treated 3 h prior to harvest with 20 mM LiCl and 2 h prior to harvest with 2 mM 8BrcAMP. Cell lysates were subjected to western blot analysis and CREB and phospho-CREB immunostained with specific antibodies. a) Immunoblot from a typical experiment. b) Densitometric quantification of the bands. Specific bands of P-CREB and CREB from the same lysate were quantified and the ratio between p-CRE and CREB was calculated. Values are means  $\pm$  SEM of 3 independent experiments.

**Fig. 6**

Effect of lithium on the binding of CREB to the somatostatin CRE as revealed by the EMSA. HIT-T15 cells were treated with 20 mM LiCl 2 h prior to harvest and with 2 mM 8BrcAMP 1 h prior to harvest. Nuclear extracts were incubated with labelled oligodeoxynucleotides containing the somatostatin CRE (SCE). Unlabelled wildtype or mutant oligonucleotides as competitors were added at a 450-fold molar excess. Protein complexes were separated on a non-denaturing SDS gel and probes visualized by autoradiography. a) autoradiography of a typical experiment. The arrow indicates the mutation sensitive complexes b) Densitometric quantification of specific bands. Values are means  $\pm$  SEM from 6 independent experiments.

**Fig. 7**

Effect of lithium on cAMP-induced transcriptional activity of CREB wildtype and CREB mutant lacking the basic leucine zipper region, using the heterologous GAL4-system. HIT-T15 cells were transfected with the Gal4-driven reporter gene construct G5E1B-Luc and GAL4-fusion proteins containing full length CREB or CREB1-261. 7 h prior to harvest cells were treated with 20 mM LiCl and 6 h prior to harvest with 2 mM 8BrcAMP. 48 h after transfection luciferase activity was determined. Values are expressed as % of the respective untreated control. Values are means of 3 independent experiments  $\pm$  SEM each done in duplicate. \*\*\*:  $p < 0.001$ , two-way ANOVA, Bonferroni post-hoc test.

**Fig. 8**

Effect of lithium on TORC-mediated transcription

HIT-T15 cells were transfected with either the Gal4-driven reporter gene construct G5E1B-Luc and GAL4-fusion proteins containing full length CREB mutant R300A or CREBbZip (a) or the truncated rat somatostatin promoter-driven reporter gene -65SMSLuc (b). Expression vector for TORC1 was cotransfected where indicated. 7 h prior to harvest cells were treated with 20 mM LiCl and 6 h prior to harvest with 2 mM 8BrcAMP. 48 h after transfection, luciferase activity was determined. Values are expressed as % of the respective untreated control. Values are means of 3 independent experiments  $\pm$  SEM each done in duplicate.

\*\*\*:  $p < 0.001$ , two-way ANOVA, Bonferroni post-hoc test.



Fig. 1

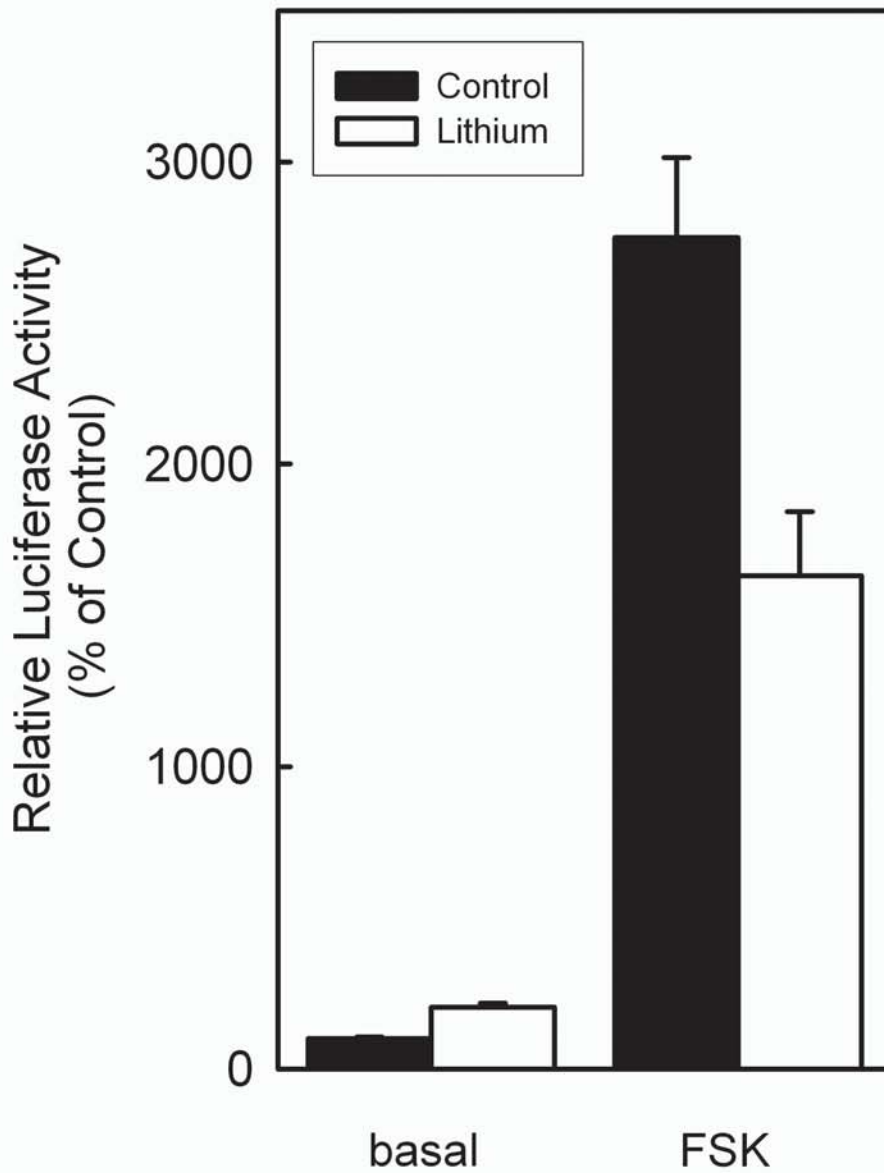
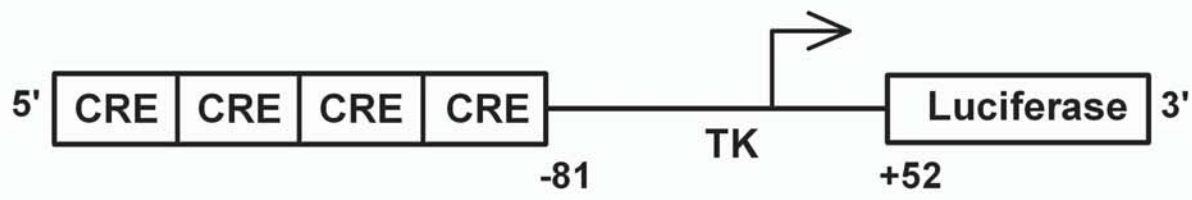


Fig. 2

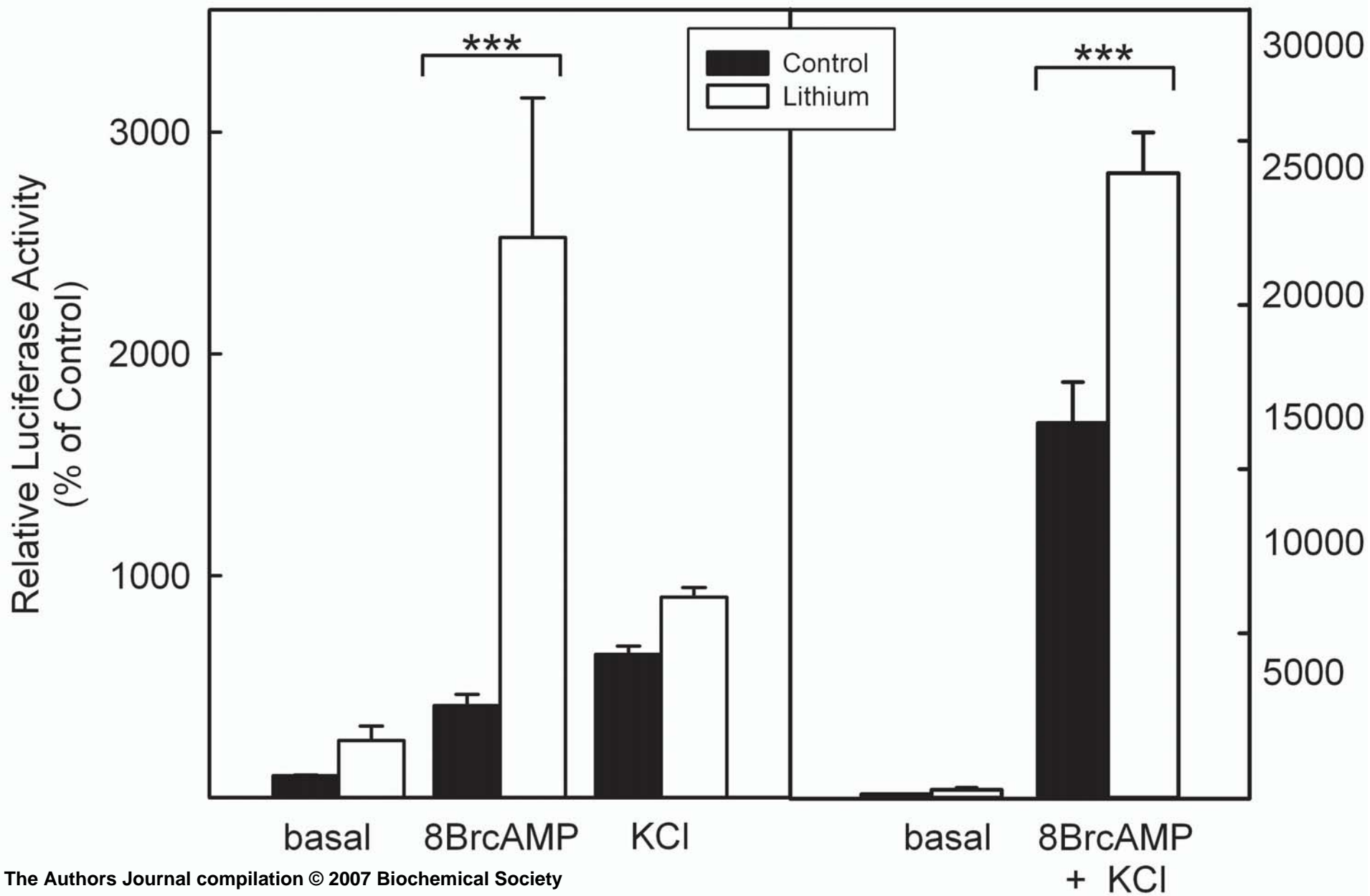


Fig. 3

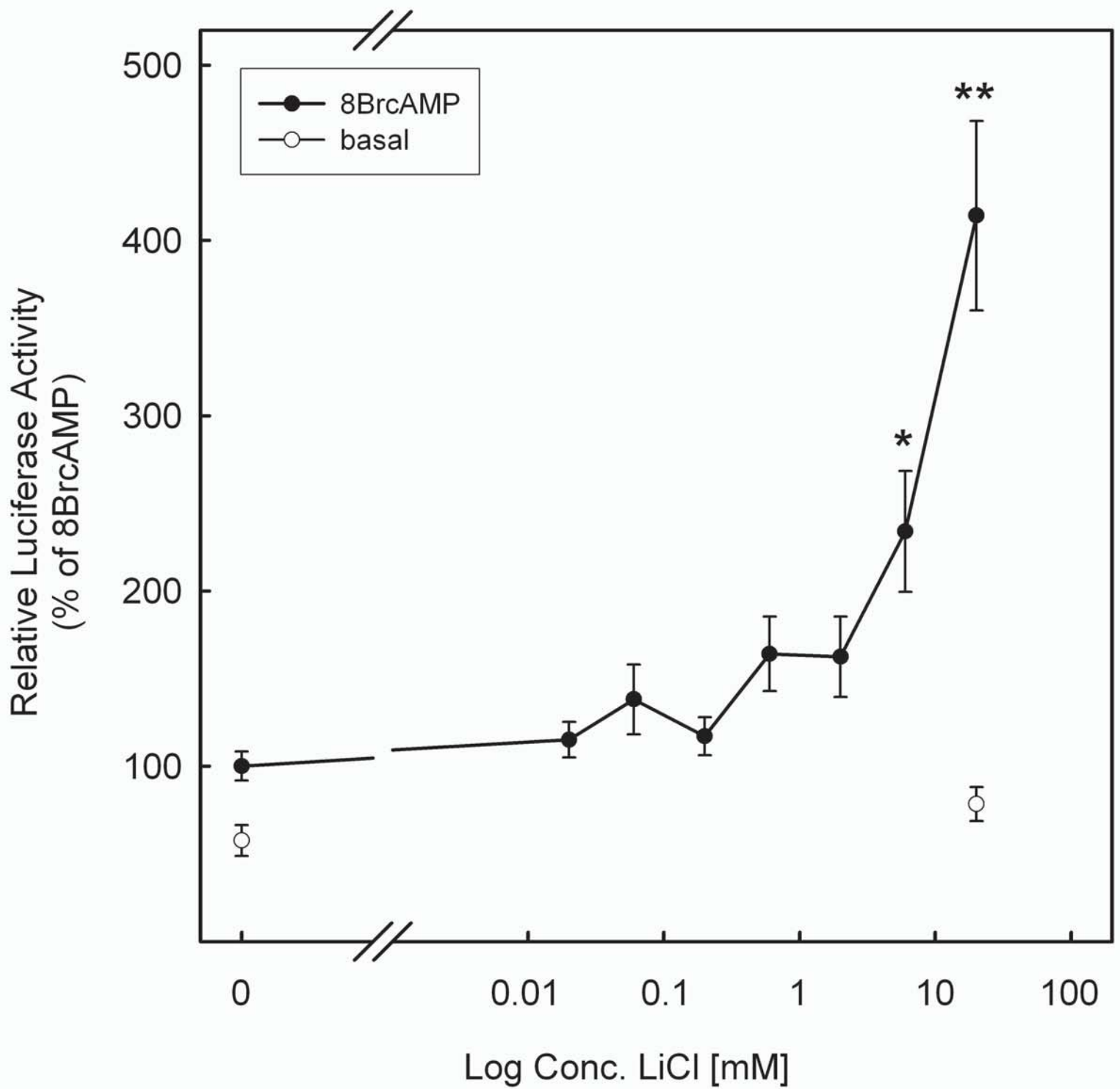


Fig. 4

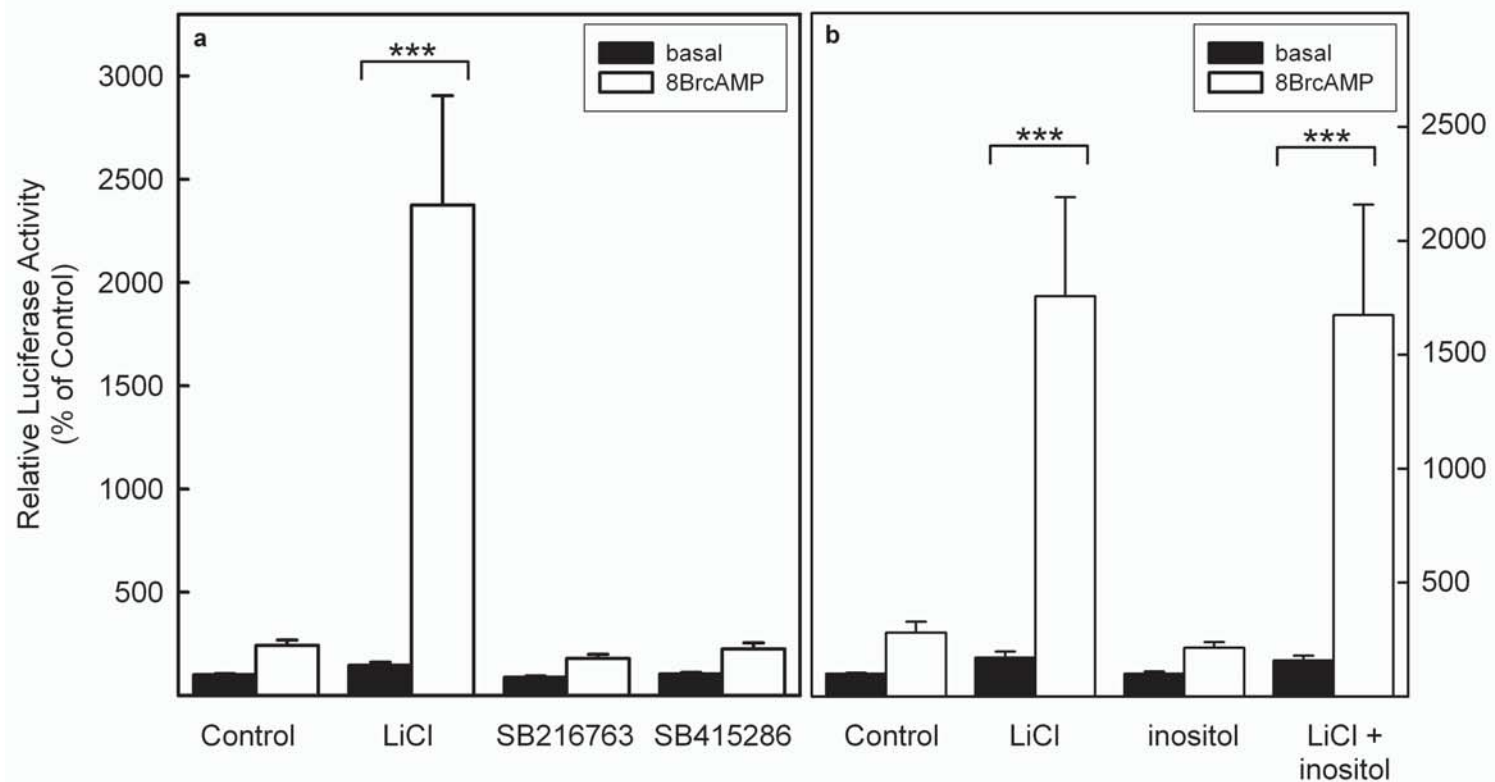
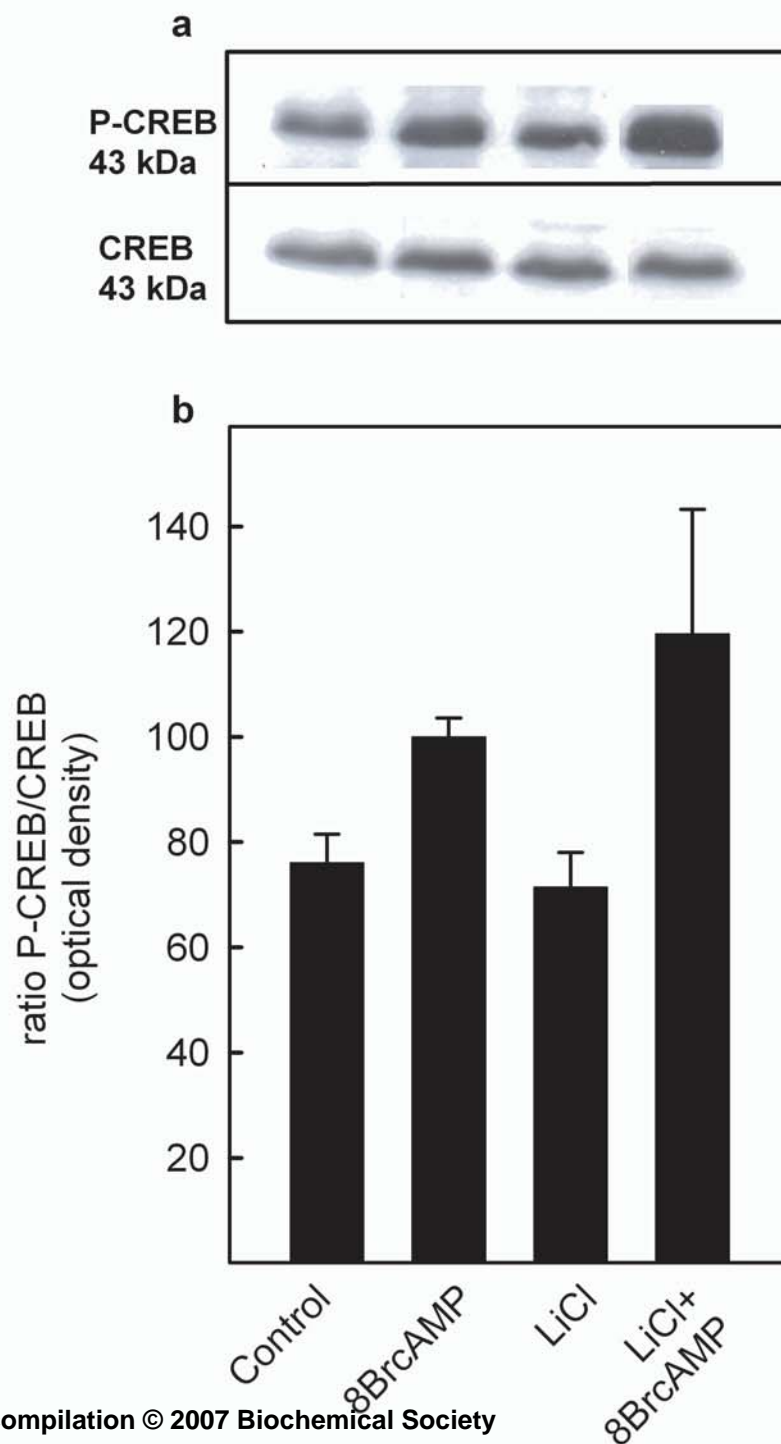


Fig. 5



**Fig. 6**

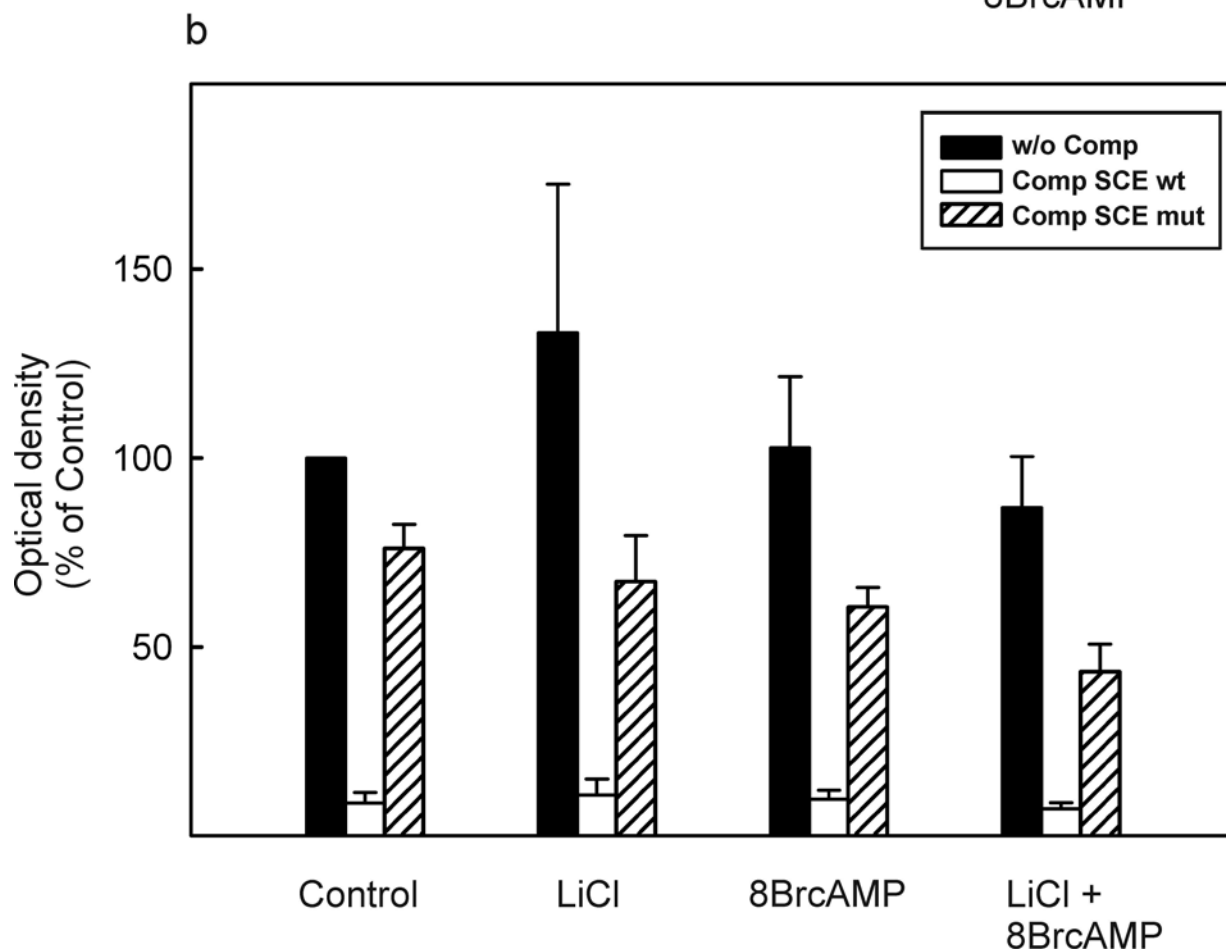
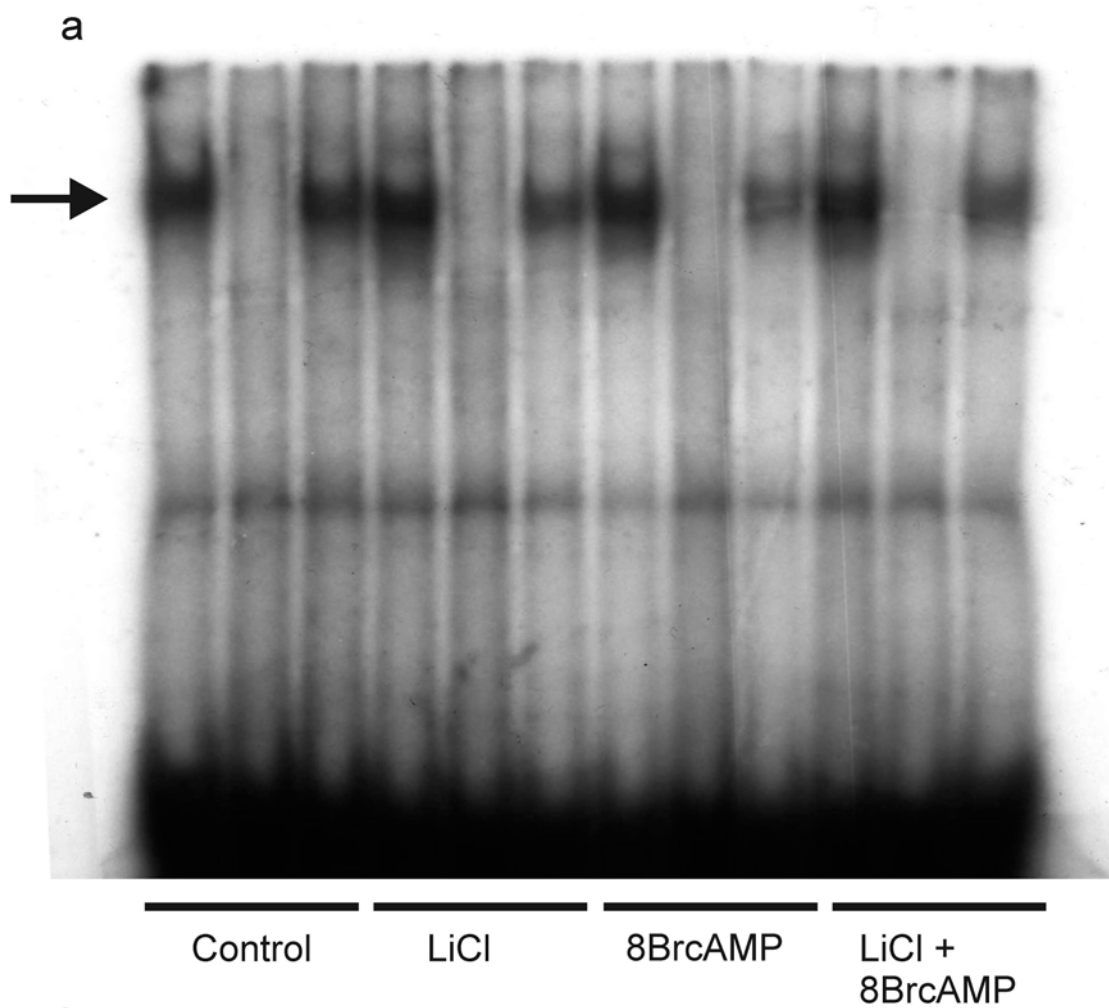


Fig. 7

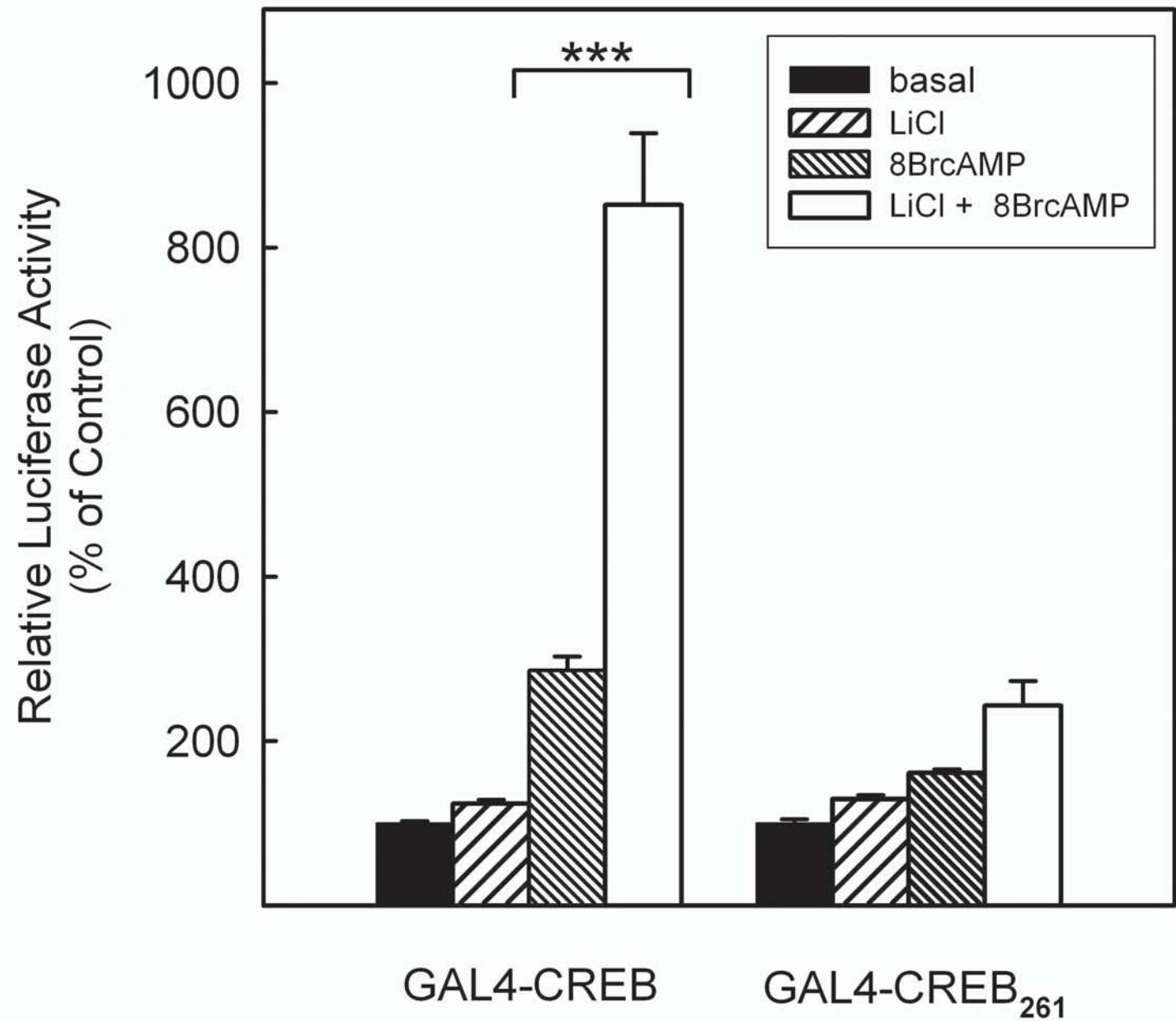


Fig. 8

