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Title:

Stimulation by lithium of the interaction between the transcription factor CREB and its coactivator TORC

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SYNOPSIS

Lithium salts are clinically important drugs used to treat bipolar mood disorder. The mechanisms accounting for the clinical efficacy are not completely understood. Chronic treatment with lithium is required to establish mood stabilization, suggesting the involvement of neuronal plasticity processes. CREB (cAMP response element binding protein) is a transcription factor known to mediate neuronal adaptation. Recently, the CREB-coactivator TORC (transducer of regulated CREB) has been identified as a novel target of lithium and shown to confer an enhancement by lithium of cAMP-induced CREB-directed gene transcription. TORC is sequestered in the cytoplasm and its nuclear translocation controls CREB activity. In the present study, the effect of lithium on TORC function was investigated. Lithium did affect neither the nuclear translocation of TORC nor TORC1 transcriptional activity, but increased the promoter occupancy by TORC1 as revealed by chromatin immunoprecipitation assay. In a mammalian two-hybrid assay as well as in a cell-free GST pull-down assay, lithium enhanced the CREB-TORC1 interaction. Magnesium ions strongly inhibited the interaction between GST-CREB and TORC1 and this effect was reversed by lithium. Thus, our data suggest that, once TORC has entered the nucleus, lithium as a cation directly stimulates the binding of TORC to CREB, leading to an increase in cAMP-induced CREB target gene transcription. This novel mechanism of lithium action is likely to contribute to the clinical mood stabilizing effect of lithium salts.

Keywords: cAMP-response element binding protein (CREB), transducer of regulated CREB (TORC), Lithium, protein-protein interaction, mammalian two-hybrid assay, GST pull-down assay

1 The abbreviations used are:

CREB, cAMP response element binding protein; TORC, transducer of regulated CREB; GSK3, glycogen synthase kinase 3; IMPase, inositol monophosphatase; AC, adenylate cyclase; CRE, cAMP response element; SIK2, salt inducible kinase 2; HIT cells, hamster insulinoma tumor cells; DAPI, 4',6-Diamino-2-phenylindol; ChIP, chromatin immunoprecipitation; qRT-PCR, quantitative real-time PCR; GST, glutathione S-transferase; IPTG, isopropyl β -D-1-thio-galactopyranoside; DTT, dithiothreitol

INTRODUCTION

Lithium salts are clinically important drugs used to treat bipolar mood disorder and other mental diseases. Although some biochemical effects of lithium are known, such as inhibition of glycogen synthase kinase 3 (GSK3)¹, inositol monophosphatase (IMPase), and adenylate cyclase (AC), the molecular mechanism that underlies the clinical effects of lithium has remained unclear [1, 2]. Noteworthy, the mood stabilizing effect of lithium develops slowly, over weeks and months of lithium treatment [3], suggesting that the clinical effects of lithium are secondary to neuronal adaptation and based on a lithium-induced new pattern of gene expression [3, 4]. The transcription factor CREB has been implicated in adaptive responses of the brain to various stimuli [5] and has been suggested to mediate also lithium-induced changes in gene expression [6, 7]. Lithium can influence CREB-directed gene transcription at multiple levels. On the one hand, lithium may affect upstream signaling pathways that converge on CREB including cAMP-, calcium-, and GSK3-mediated signaling pathways [2]. On the other hand, a newly discovered CREB coactivator called transducer of regulated CREB (TORC) has recently been identified as a novel lithium target [8]. Lithium was found to enhance cAMP-induced gene transcription mediated by CREB and its DNA binding site, the cAMP response element (CRE). This stimulation by lithium was not conveyed by protein kinase A, IMPase or GSK3, but was conferred to CREB by TORC [8].

Cyclic AMP is well-known to promote through protein kinase A the phosphorylation of CREB at serine-119 (CREB 327) within the CREB transactivation domain, thereby enhancing its association with the histone acetylase paralogs p300 and CBP [9]. The newly discovered TORC is another essential CREB coactivator [10-14]. Under basal conditions, TORC is sequestered in the cytoplasm through phosphorylation by the salt inducible kinase 2 (SIK2) and other members of the AMPK family of serine/threonine kinases [15-17]. Following exposure to cAMP, TORC is dephosphorylated and translocated to the nucleus, where it associates with the CREB basic region-leucine zipper domain (bZip) [10-14]. In cooperative interaction with CBP, TORC then increases CREB target gene expression [13].

Although TORC has been identified as a novel target of lithium action to regulate gene expression [8], it was unknown how lithium, in the presence of cAMP, enhances the transcriptional activity conferred by TORC to CREB. Therefore, the effect of lithium on TORC function was investigated in the present study.

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EXPERIMENTAL PROCEDURES

Plasmids

Expression vectors for GST-CREB and GST-CREB-R300A were generated by PCR cloning. GAL4-CREB and GAL4-CREB-R300A [8] served as template. After PCR the fragments were subcloned into pGEX2T (GE Healthcare) via *Bam*HI and *Eco*RI restriction sites. The expression vector for human TORC1 under control of a T7 promoter in pcDNA3 has been described before [8]. For ChIP assays, an expression vector for N-terminally FLAG-tagged human TORC1 was generated by PCR cloning using TORC1 as template followed by subcloning into pcDNA3 (Invitrogen). The plasmid 4xSomCRET81Luc has been described before [18]. The reporter gene 5xGal4E1B-Luc [19] contains the luciferase gene under control of 5 repeats of the responsive element for the yeast transcription factor GAL4. For PCR cloning of GAL4-TORC1 and GAL4-TORC1₁₋₄₄ human TORC1 served as template. The resulting DNA was subcloned into the GAL4 DNA-binding domain-coding vector pSG424 [20]. These constructs encode full length TORC1 or the first 44 amino acids, respectively, C-terminally fused to the DNA-binding domain (aa1-147) of GAL4. The expression plasmids for VP16-bZip and VP16-bZip-R300A, encoding the basic leucine zipper domain (CREB₂₆₂₋₃₂₇) fused C-terminally to the transactivation domain of the viral protein VP16, were generated by PCR cloning. GAL4-bZip or GAL4-bZip-R300A, respectively, served as template followed by subcloning into the VP16 domain-coding vector pHKnt-VP16 [21]. Table S1 (supplemental material) gives an overview of all primer sequences. The correct sequence of all constructs was verified by sequencing.

Cell culture and transfection

Hamster insulinoma tumor cells (HIT-T15) [21] were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 5% horse serum, 100 units/ml penicillin and 100 µg/ml streptomycin. For ChIP-assays HIT cells were transiently transfected with 4 µg of expression vector per 6-cm dish using Metafectene (Biontex) according to the manufacturer's instructions. For mammalian two-hybrid assays the DEAE-Dextran method [21] was used to transfect the cells with 2 µg of expression vector and 1 µg cytomegalovirus green fluorescent protein expression vector per 6-cm dish to control for transfection efficiency. Co-transfections were carried out with a constant amount of DNA, which was maintained by adding the empty pBluescript vector (Stratagene). Treatment of cells in the GAL4 assay was with 2 mM 8 Br-cAMP 6 h and with 20 mM LiCl 7 h prior to harvest. For the two hybrid assay, cells were treated with 1 mM 8 Br-cAMP 6 h and with 20 mM LiCl 30 h prior to harvest. Luciferase was determined 48 h after transfection as previously described [21].

Immunocytochemistry

HIT cells were grown on coverslips in 6-well plates. Treatment with 1 mM or 2 mM 8Br-cAMP and with 20 mM LiCl was 30 min and 90 min prior to fixation, respectively. Fixation and antibody incubation were performed as described [22]. panTORC antibody (Calbiochem) was used as primary antibody against TORC proteins. The secondary antibody was anti-rabbit IgG AlexaFluor[®] 488 (Invitrogen). The coverslips were mounted on slides using Vectashield mounting medium with 4',6-Diamino-2-phenylindol (DAPI) (Linaris). The slides were analyzed using a Zeiss Axiovert 200 microscope (Software: OpenLab[™] 3.1 for Macintosh OS9.2 by Improvion Ltd.).

RNA extraction and reverse transcription

Total RNA was extracted from HIT cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from total RNA by reverse transcription using oligo(dT)₁₅ primer and M-MLV Reverse Transcriptase (Promega)

according to the instructions of the manufacturer. Analysis of the mRNA level of TORC proteins in HIT cells was performed by PCR using primers for human TORC1, TORC2, TORC3, and hamster β -actin as internal standard (sequences see table S2 – supplemental material), followed by agarose gel electrophoresis and densitometric analysis of the bands.

Chromatin Immunoprecipitation (ChIP) Assay and quantitative real time PCR (qRT-PCR)

Chromatin immunoprecipitation was performed as described [23] with some modifications. Transfected HIT cells were treated with 2 mM 8Br-cAMP and with 20 mM LiCl 30 min and 90 min, respectively, prior to cross-linking with 1% (v/v) formaldehyde. Cells were harvested, resuspended in cell lysis buffer (10 mM Tris/HCl pH 8.0, 10 mM NaCl, 0.2% (v/v) Nonidet-P40, protease inhibitors), and homogenized using a 1-ml Dounce homogenizer. After centrifugation, the pellet was resuspended in nuclei lysis buffer (50 mM Tris/HCl pH 8.1, 10 mM EDTA, 1% (w/v) SDS, protease inhibitors). The DNA was sheared by sonication and the cell debris was pelleted by centrifugation. The supernatant was collected and 10% were taken as input control. Immunoclearing, immunoprecipitation, and purification of DNA were performed as described [24]. qRT-PCR was performed using the fluorescent reporter probe method. The 4 repeats of the CRE of the 4xSomCRET81Luc were chosen as DNA sample to measure efficiency of the ChIP assay. The following primers were used: 5'-GCAATAGCATCACAAATTCACAAA-3' (forward primer) and 5'-CCGCCCCGACTGCAT-3' (reverse primer) combined with the fluorescent reporter probe 5'-CGAATTCGCCGATCTCGAGCTC-3' (modifications: 5'-Fluorescein; 3'-TAMRA). For the qRT-PCR, the reaction mix from Eurogentec, containing the AmpliTaq[®] DNA polymerase (Applied Biosystems), was used and prepared according to the manufacturer's instruction. Each sample was measured in triplicate on a 384 well plate in each 18 μ l volume using the ABI Prism 7900HT Sequence Detection System and was analysed with the SDS 2.1 Software (Applied Biosystems).

Expression and purification of GST-fusion proteins from bacteria

Competent cells from *E. coli* strain DH5 α were transformed by heat shock with expression vectors coding for GST-CREB and GST-CREB-R300A. Bacteria were grown in 1L LB medium at 37°C. The expression of the GST-fusion proteins was induced by 1 mM isopropyl β -D-1-thio-galactopyranoside (IPTG) for 4 h. The bacteria were harvested in sonication buffer (20 mM HEPES pH 7.5, 1 M NaCl, 1 mM dithiothreitol [DTT], 1 mM phenylmethansulfonylfluoride [PMSF]) and stored at -80°C. The suspension was thawed on ice and the bacteria disrupted by sonication. The cell debris was pelleted by centrifugation and the GST-fusion proteins were extracted from the supernatant by affinity chromatography using glutathione-agarose beads. The isolated proteins immobilized on agarose were stored on ice.

[³⁵S]-labeling of proteins

[³⁵S]-labeled human TORC1 was generated by *in vitro* transcription / translation using the TNT T7 coupled reticulocyte lysate system (Promega) according to the manufacturer's instruction including T7 polymerase and [³⁵S]methionine (1000 Ci/mmol) (GE Healthcare). Human TORC1-coding sequence under control of the T7 promoter in pcDNA3 served as template.

GST pull-down assay

The GST pull-down assay using GST-CREB (wt/R300A) and [³⁵S]TORC1 was performed as described [23]. Briefly, the agarose-bound proteins were washed with reaction buffer. Buffers contained 20 mM Tris/HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% (v/v) Nonidet-P40, 1

mM DTT, and 1 mM PMSF. Considering the valency of lithium and magnesium, the salt concentration was balanced with NaCl to 100 mM to prevent effects due to changes in ionic strength of the buffer (for further details Table S3 – supplemental material). For the binding reaction, 3.5 μ l of [³⁵S]TORC1 were added to equal amounts of GST-fusion protein in 250 μ l reaction buffer and incubated overnight at 4°C. The samples were analyzed by SDS-PAGE. The gel was dried and the radioactively labeled proteins were visualized using a phosphor imager (Fuji Film BAS 1800 II) and analyzed densitometrically using the software AIDA 4.15 (raytest Isotopenmessgeräte).

Statistics

Statistical data analysis (two-way ANOVA with Student's *t* test) was performed using the software STATISTICA 7 (Statsoft).

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RESULTS

Nuclear translocation of TORC proteins

Nuclear translocation of TORC has been shown to be a key regulator for CREB transcriptional activity [10, 11, 14, 25, 26]. By immunocytochemistry the effect of lithium and cAMP on the cellular localization of endogenous TORC proteins in HIT cells was investigated. HIT cells, like neurons, are electrically excitable [27] and share with neurons the expression of proteins like the scaffold protein JNK-interacting protein (JIP)-1b / islet-brain (IB)1 [28], or the transcription factors Pax6 [29, 30] and BETA2 / NeuroD [29, 31]. CREB-directed transcription has been well characterized in HIT cells [8, 18, 21, 27, 32, 33] including the biology of TORC [14]. Endogenous TORC was mainly retained in the cytoplasm under resting conditions (Figure 1A, upper panel). Treatment of HIT cells with 8Br-cAMP induced in a concentration-dependent manner the translocation of TORC into the nucleus (Figure 1A, middle panel; Figure 1B). These results are consistent with other reports in which the nuclear translocation of TORC in different cell lines and also primary hippocampal neurons was inducible by treatment with forskolin, a potent activator of adenylate cyclase [10, 14, 26, 34]. Furthermore, the nuclear translocation of TORC was induced in HIT cells by high potassium-induced membrane depolarization and blocked by the calcineurin inhibitor cyclosporine A (data not shown), consistent with a previous report [14]. In HIT cells, LiCl did not affect the translocation of TORC (Figure 1A, lower panel), neither alone nor in combination with 8Br-cAMP (Figure 1B).

The panTORC antibody used in the immunocytochemistry experiments can detect all three isoforms of TORC as it is directed against the first 42 amino acids which are highly conserved among the TORCs [11]. To identify the isoforms expressed in HIT cells, total RNA was extracted and examined regarding the expression level of the different isoforms. This analysis revealed an 8-fold higher expression of TORC1 over TORC2 and the absence of TORC3 (Figure S1, Supplemental Material). Noteworthy, TORC1 is the isoform particularly copious also in brain tissues like hippocampus, cerebral cortex, and cerebellum [11, 26, 34]. Therefore, we focussed on TORC1 in the further investigations.

Transactivation by TORC1

To investigate the effect of lithium on TORC1 transcriptional activity, the GAL4 system was employed. In a luciferase reporter gene assay, HIT cells were transfected with an expression vector coding for TORC1 fused to the DNA-binding domain of the yeast transcription factor GAL4. The promoter of the reporter gene contained 5 repeats of the GAL4-binding site (Figure 2A). Thereby TORC1 is tethered to the promoter and its own transcriptional activity can be examined independently of its binding to CREB. The GAL4 DNA-binding domain alone exhibited virtually no activity and did not show any response to the stimuli (data not shown). In contrast, GAL4-TORC1 conferred strong transcriptional activity (Figure 2), indicating the presence of a transactivation domain in TORC1 as suggested before [11, 12]. Treatment with 8Br-cAMP increased the activity of TORC1 3-fold (Figure 2B), LiCl affected the transcriptional activity of TORC1 neither alone nor in combination with 8Br-cAMP (Figure 2B). In view of these results, an influence of lithium salts on either the translocation of TORC1 into the nucleus or TORC1 transactivation potential seems to be unlikely.

Effect of lithium on the recruitment of TORC1 to the promoter

To examine whether lithium influences the recruitment of TORC1 to the promoter, we employed a chromatin immunoprecipitation (ChIP) assay. HIT cells were transfected with an expression plasmid containing a promoter controlled by four repeats of the rat somatostatin CRE. It contains the consensus sequence 5'-TGACGTCA-3' constituting a high-affinity binding site for endogenous CREB [18]. An expression vector coding for TORC1 tagged with

a FLAG epitope was co-transfected (Figure 3A). After treatment, DNA and proteins were cross-linked and precipitated with an antibody against the FLAG epitope. The amount of precipitated DNA was determined by quantitative real-time PCR. In control experiments, pBluescript as unspecific DNA was co-transfected with FLAG-TORC1 and did not result in a specific signal in real time PCR (data not shown). Treatment of HIT cells with LiCl alone or with 8Br-cAMP alone did not induce a detectable increase of promoter occupancy (Figure 3B). An increase has been shown before for TORC2 upon forskolin stimulation [13, 16]. This system might be not sensitive enough to the effect elicited by 8Br-cAMP alone. In fact, stimulation with forskolin is in general about 10-fold more potent compared to 8Br-cAMP [18, 32]. However, the combination of cAMP and lithium enhanced the promoter occupancy of TORC1 2-fold ($P < 0.025$, $n = 5$) (Figure 3B).

Effect of lithium on the interaction between CREB and TORC1: mammalian two-hybrid assay

A mammalian two-hybrid assay was employed to investigate the effect of lithium and cAMP directly on the interaction between CREB and TORC1. Only the interaction domains of the proteins of interest were used. HIT cells were transiently transfected with the luciferase reporter gene under control of five repeats of the GAL4-binding site and an expression vector coding for the first 44 amino acids of TORC1 as GAL4-fusion construct. Co-transfected was either the CREB bZip wild type (bZip-wt), or the mutant CREB bZip R300A (bZip-R300A), each fused to the potent viral protein VP16, or VP16 alone for control (Figure 4A). Compared to the VP16 control, VP16-bZip-wt showed a 2-fold higher basal activity (Figure 4B) indicating the interaction between bZip-wt and TORC1₁₋₄₄. LiCl strongly increased interaction between the wild type bZip and TORC1₁₋₄₄ (Figure 4B). 8Br-cAMP alone had no effect on luciferase activity, whereas treatment with LiCl plus 8Br-cAMP increased the interaction between TORC1₁₋₄₄ and bZip-wt to the same extent as LiCl alone (Figure 4B). The bZip R300A mutant (VP16-bZip-R300A) did not show significant differences from the VP16 control (Figure 4B), indicating lack of interaction between bZip-R300A and TORC1₁₋₄₄. This confirms previous reports in which this mutation disrupted complex formation [14] and the enhancement by lithium of cAMP-induced CREB target gene transcription [8]. These data show an enhancement by lithium of the protein-protein interaction between bZip and TORC1₁₋₄₄ that is independent of cAMP. The two-hybrid assay could not be applied to investigate the interaction of the full-length proteins due to very high basal activity of GAL4-TORC1 (data not shown).

Effect of lithium on the interaction between CREB and TORC1: in vitro GST pull-down assay

Recombinant GST-CREB-wt and GST-CREB-R300A mutant were expressed in bacteria and TORC1 was labeled by *in vitro* transcription and translation using [³⁵S]methionine. Specificity of the interaction was examined by use of GST alone which exhibited no remarkable interaction with [³⁵S]TORC1 (6.6% of GST-CREB-wt, $P < 0.002$) (Figure 5A). In the presence of 20 mM LiCl, the amount of [³⁵S]TORC1 recovered from GST-CREB-wt was clearly increased ($P < 0.035$) (Figure 5). In contrast, no specific binding of [³⁵S]TORC1 to the mutant GST-CREB-R300A was detectable, and there was no effect of lithium (Figure 5A). A concentration-response curve of LiCl revealed significantly increased binding of [³⁵S]TORC1 to GST-CREB-wt from concentrations of 5 mM LiCl (Figure 5B). These results indicate that lithium enhances directly the interaction between CREB and TORC1 also in this cell-free system.

Lithium is known to inhibit GSK3 by competition with magnesium at the non-Mg:ATP magnesium binding site [35, 36]. Therefore, the effect of increasing concentrations of magnesium on the interaction between CREB and TORC1 was investigated in the GST pull-

down assay. This analysis revealed that magnesium strongly inhibited the interaction in a concentration-dependent manner (Figure 6A): Already 0.5 mM MgCl₂ reduced the interaction by 40% ($P < 0.03$), and in the presence of 10 mM and 20 mM MgCl₂ only 25% and 14%, respectively, of the interaction between CREB and TORC1 remained (Figure 6A). This strong inhibition by high magnesium concentrations was attenuated by 5 mM LiCl (Figure 6B). LiCl thus enhanced the interaction about 3-fold in the presence of 10 mM or 20 mM MgCl₂ (Figure 6C). These data indicate an inhibitory effect of magnesium on the interaction between CREB and TORC1, which is reversed by lithium.

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DISCUSSION

CREB activity has been known for years to be induced by serine-119 phosphorylation and CBP/p300 cofactor recruitment [9, 37]. In contrast to CBP/p300, the more recently recognized other essential coactivator, TORC, binds to the CREB bZip domain [11, 12]. This interaction is per se independent of CREB serine-119 phosphorylation and extends the functional role of CREB bZip domain to include not only DNA binding and dimerization but also coactivator recruitment. The regulation of TORC by cAMP-activated and other signaling pathways is thus as important for CREB activity as the regulation of CREB serine-119 phosphorylation [10, 11, 14, 38]. This crucial role of the TORC family of coactivators was further emphasized by the recent identification of TORC as the target of a clinically important drug, when the mood stabilizer lithium was shown to stimulate CREB target gene expression through TORC [8]. The present study now defines what step in TORC function is influenced by lithium, and demonstrates that lithium does not affect TORC nuclear translocation and transcriptional transactivation, but increases as a cation the binding of TORC to the CREB bZip domain.

Cyclic AMP- and Ca^{2+} -activated signaling pathways control the nucleo-cytoplasmic shuttling of TORC [14, 39]. Under resting conditions, TORC1 and TORC2 are sequestered in the cytoplasm bound by 14-3-3 proteins [14]. This binding occurs in response to TORC phosphorylation by SIK. Elevated levels of cAMP inhibit SIK kinase activity via the protein kinase A-mediated phosphorylation of SIK at serine-587 (SIK2). In turn, the phosphorylation of TORC decreases and TORC accumulates in the nucleus [14, 39]. Our data confirm a previous report that TORC accumulates in the nucleus upon increased cAMP levels in HIT cells [14]. But no effect of lithium was detected on the translocation of TORC into the nucleus. Likewise, no effect of lithium on transactivation by TORC was found in the present study. The TORC proteins contain at their C-terminus a strong transactivation domain [11, 12]. This can be studied, when TORC is fused to the heterologous GAL4 DNA-binding domain and tested for induction of a promoter with GAL4-binding sites, as has been shown previously [12]. The transactivation potential of TORC may not entirely be constitutive, since cAMP stimulated GAL4-TORC1 transcriptional activity (this study). A possible explanation is offered by NONO (p54^{nrb}) as cAMP has been shown to stimulate the binding of TORC to NONO, which then acts as a bridge between the CREB/TORC complex and RNA polymerase II [40].

Several lines of evidence support the view that, once TORC has reached the nucleus, lithium enhances the binding of TORC to CREB. As revealed by the ChIP assay, the stimulation by lithium of cAMP-induced CREB target gene expression [8] is accompanied by an increase in CRE promoter occupancy by TORC. Since lithium does not alter the nuclear translocation of TORC (see above), the increase in promoter occupancy suggests that lithium stimulates TORC-CREB binding. This was then directly demonstrated by the results of the mammalian two-hybrid assay and the GST pull-down assay. Noteworthy, lithium stimulates TORC-CREB binding independently of cAMP and protein kinase A activity, since in the mammalian two-hybrid assay lithium induced a robust increase in the binding of the TORC N-terminus to the CREB bZip in the absence of cAMP and cAMP had no effect. This conclusion is further supported by the results of the GST pull-down. Lithium thus depends on cAMP to stimulate CRE/CREB-directed transcription [8], because cAMP provides CREB serine-119 phosphorylation / CBP recruitment as well as TORC nuclear translocation. Consistent with the previous finding that the stimulation by lithium of the CRE/CREB-directed transcription is not mediated via protein kinase A, GSK3 or IMPase [8], the effect of lithium in the cell-free GST pull-down assay indicates that lithium increases TORC-CREB binding independently not only of protein kinase A but also of other enzymes and cellular proteins. Therefore, when taken together, the results of the present study strongly suggest that, once TORC has entered the nucleus, lithium as a cation directly enhances the binding of the TORC N-terminus to the CREB bZip resulting in an increase in CREB target gene expression (Figure 7).

Both the CREB bZip and the TORC N-terminus are highly structured, forming a continuous α -helix and coiled-coil structure, respectively [12, 41]. Based on glutaraldehyde crosslinking experiments, it has been suggested that TORC binds as a tetramer to the CREB dimer [11]. The binding of a cation like lithium to such a composite structure, we speculate, may induce a conformational change that increases complex stability. The best-studied example of lithium acting as a cation is the competition of lithium with magnesium for one of the two magnesium binding sites of GSK3, the non-Mg:ATP magnesium binding site, leading to inhibition of GSK3 kinase activity [35, 36, 42]. Li^+ and Mg^{2+} have similar ionic radii [1], and it has been suggested that this enables lithium to physically invade the magnesium binding site within GSK3, where due to its lower charge density it disrupts catalytic function [36]. A similar model could hold true also for TORC-CREB binding, since magnesium was found in the present study to inhibit the binding of TORC to CREB over a similar range of concentrations as it stimulates GSK3 activity [36] and lithium antagonized the effect of magnesium, thus increasing TORC-CREB binding by release from magnesium inhibition. Interestingly, the crystal structure of the CREB bZip revealed the presence of a hexahydrated magnesium ion in the cavity between the bifurcating basic regions [41]. The role of this magnesium binding to the CREB bZip in the stimulation by lithium of TORC-CREB binding remains to be investigated.

Clinical studies have demonstrated neuronal atrophy, loss of glial cells/neurons, and impairments of neuroplasticity and resilience in the brain of patients that suffer from bipolar mood disorder and suggest that chronic treatment with lithium may attain mood stabilization via neuroprotective actions and induction of neuronal adaptation [43-46]. Since CREB has major roles in mediating adaptive responses and neuroprotection in the brain [47], the discovery of the lithium enhancement of TORC-CREB binding thus suggests its therapeutic relevance. In cortical neuronal, for example, the addition of glutamate with glycine increases the free intracellular magnesium ion concentration of approximately 300 μM to levels that are almost 10-fold higher [48] and contribute to glutamate-induced excitotoxicity [49]. In view of the results of the present study, it is not unreasonable to suggest that such fluxes in the concentration of magnesium ion could disrupt TORC-CREB interaction and that lithium could reverse this effect and increase the binding of TORC to CREB, thus increasing CREB activity and the expression of CREB target genes like BDNF and Bcl-2 [6, 46] that promote cell survival. Furthermore, TORC has been shown to be required for long-term synaptic plasticity in the hippocampus [26, 34]. Thus the stimulation by lithium of the binding of TORC to CREB, as demonstrated by the present study, represents a novel mechanism of lithium action that may contribute to the clinical mood stabilizing effect of lithium salts.

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FIGURE LEGENDS

Figure 1 Enhancement by cAMP but not LiCl of the nuclear accumulation of TORC in HIT cells as revealed by immunocytochemistry. (A) Co, controls without treatment; cAMP, treated with 2 mM 8Br-cAMP for 30 min; LiCl, treated with 20 mM LiCl for 90 min. (B) 300 cells per group were analyzed with respect to the localization of TORC, expressed as percentage of cells with nuclear TORC. Values are means \pm SEM. * $P < 0.05$, ** $P < 0.025$, two-way ANOVA and Student's *t*-test.

Figure 2 Lack of effect of LiCl on transactivation by TORC1. (A) Scheme of the GAL4-fusion constructs and the luciferase reporter gene used. GAL4 represents the DNA-binding domain of the yeast transcription factor GAL4 (aa1-147). GAL4-TORC1, full length TORC1 (aa1-651) fused to GAL4. (B) GAL4-TORC1 and the luciferase reporter gene (5xGal4E1B-Luc) were transiently co-transfected into HIT cells. Cells were treated as indicated with 20 mM LiCl 7 h prior to harvest and with 2 mM 8Br-cAMP 6 h prior to harvest. Luciferase activity is expressed relative to the mean value of the control (no treatment). Values are means \pm SEM of three independent experiments each performed in duplicate. *** $P < 0.001$, two-way ANOVA and Student's *t*-test.

Figure 3 Lithium increased the recruitment of TORC1 to the promoter in the presence of cAMP as revealed by ChIP assay in HIT cells. (A) Scheme of the FLAG-tagged TORC1 construct and the reporter plasmid used. FLAG-TORC1 represents TORC1 (aa1-651) containing a FLAG epitope (DYKDDDDK) between aa1 and aa2. The reporter plasmid contains 4 repeats of the CRE as high affinity binding site for endogenous CREB. TK, thymidine kinase promoter. (B) Expression vectors were transiently co-transfected into HIT cells. Treatment with 20 mM LiCl was for 90 min and treatment with 2 mM 8Br-cAMP was for 30 min. After treatment the proteins and DNA were cross-linked and DNA was precipitated using anti-FLAG antibody. The amount of precipitated DNA was determined by quantitative real-time PCR and is expressed relative to the mean value of the control (no treatment). Values are means \pm SEM of five independent experiments. * $P < 0.05$, ** $P < 0.025$, two-way ANOVA and Student's *t*-test.

Figure 4 Enhancement by lithium of the interaction between CREB-bZip and TORC1₁₋₄₄ as revealed by the mammalian two-hybrid assay. (A) Scheme of expression vectors and the luciferase reporter gene used. VP16 comprises the transactivation domain (aa411-490) of the viral protein VP16. VP16-bZip-wt, CREB-bZip encompassing aa269-327 C-terminally fused to VP16. VP16-bZip-R300A, CREB-bZip mutant Arg300Ala fused to VP16. HIT cells were transiently transfected with the luciferase reporter gene 5xGal4E1B-Luc and GAL4-TORC1₁₋₄₄ representing the CREB-interaction domain of TORC1 (aa1-44) fused to the DNA-binding domain of GAL4 (aa1-147). Co-transfected was either VP16 as control, VP16-bZip-wt, or VP16-bZip-R300A. (B) HIT cells were transfected as indicated and treated with 20 mM LiCl 30 h prior to harvest, and with 1 mM 8Br-cAMP 6 h prior to harvest. Luciferase activity is expressed relative to the control VP16. Values are means \pm SEM of three independent experiments each performed in duplicate. *** $P < 0.001$, two-way ANOVA and Student's *t*-test.

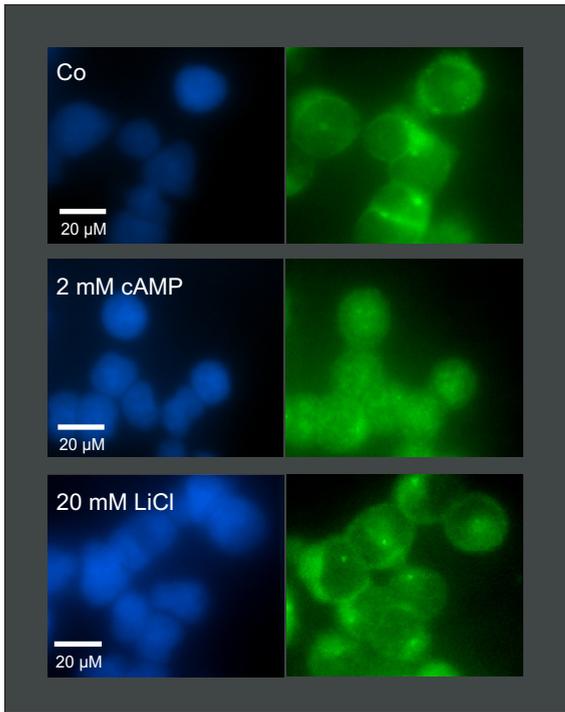
Figure 5 Enhancement by lithium of the protein-protein interaction between CREB and TORC1 in the *in vitro* GST pull-down assays. (A) Recombinant GST-CREB wild type or Arg300Ala mutant (GST-CREB-wt or GST-CREB-R300A, respectively) were employed in pull down assays with [³⁵S]-labeled TORC1. To control for specificity of the interaction, GST alone was also used. The samples were analyzed by SDS-PAGE and densitometry of the bands representing [³⁵S]TORC1. A typical gel is shown at the bottom. Values are expressed relative to the control in each experiment and are means ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.025$, *** $P < 0.001$, two-way ANOVA and Student's *t*-test. (B) Concentration-response curve. GST-CREB-wt and [³⁵S]TORC1 were incubated with increasing concentrations of LiCl. A typical gel is shown at the bottom. Values are means ± SEM of seven independent experiments. * $P < 0.05$, ** $P < 0.025$, two-way ANOVA and Student's *t*-test.

Figure 6 Effect of magnesium on the interaction between CREB and TORC1 in the *in vitro* GST pull-down assays. (A) Recombinant GST-CREB and [³⁵S]-labeled TORC1 were incubated with increasing concentrations of MgCl₂. To control for specificity, GST alone was used. The samples were analyzed by SDS-PAGE and the amount of [³⁵S]TORC1 was determined by densitometry. A typical gel is shown at the bottom. Values are expressed relative to the GST-CREB control (no MgCl₂) in each experiment and are means ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.025$, *** $P < 0.001$, two-way ANOVA and Student's *t*-test. (B) GST-CREB and [³⁵S]TORC1 were incubated with increasing concentrations of MgCl₂ in the presence or absence of a constant concentration of 5 mM LiCl. Values are expressed relative to the control of each group (no MgCl₂) and are means ± SEM of four independent experiments. * $P < 0.05$, two-way ANOVA and Student's *t*-test. (C) To demonstrate the effect of lithium on top of magnesium, the values from Figure 6B without lithium at each MgCl₂ concentration were set as 100% and the values with 5 mM LiCl were expressed relative to these.

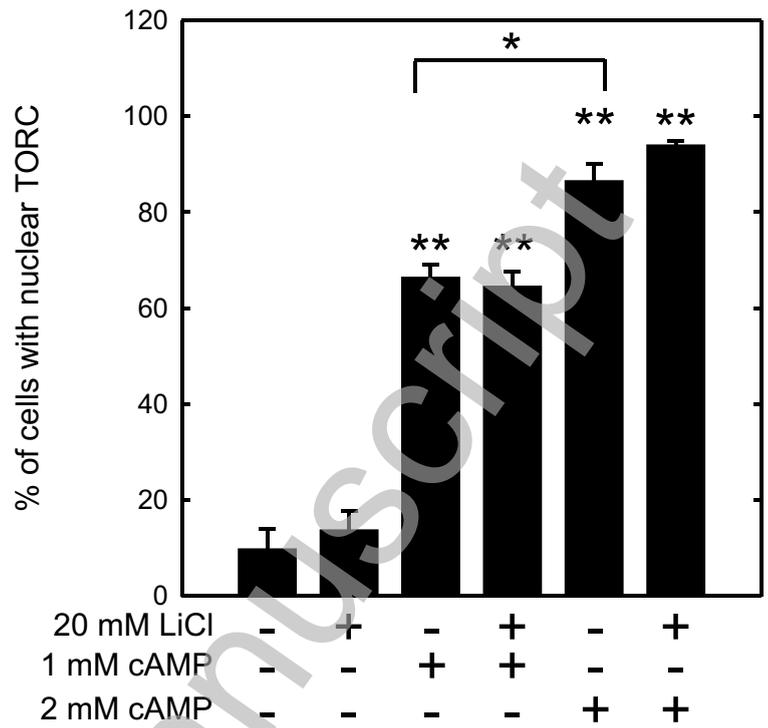
Figure 7 A new mechanism of action of lithium. Taken together, the data of the present study suggest that, once TORC has entered the nucleus, lithium stimulates the binding of TORC1 to the CREB bZip, thereby increasing cAMP-induced CREB target gene transcription. See discussion for details.

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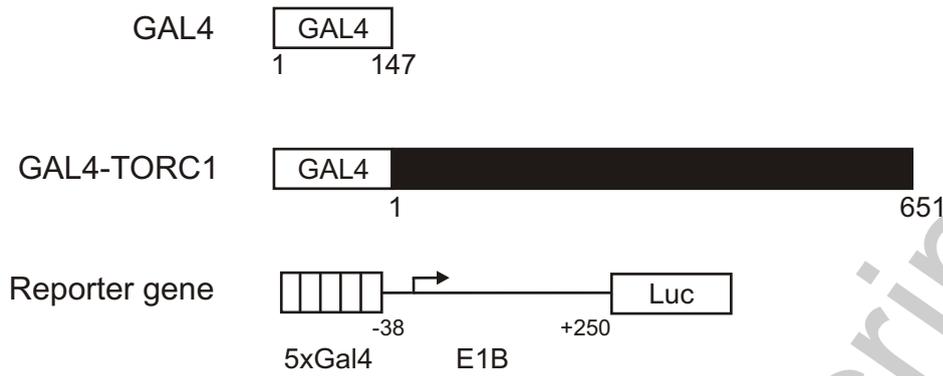
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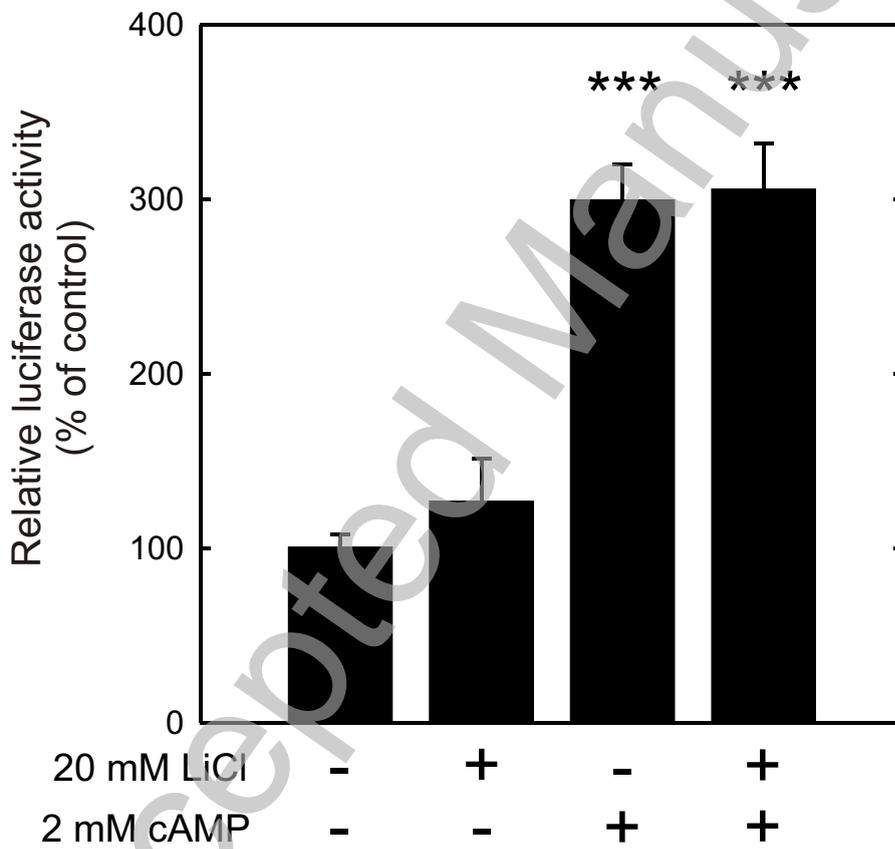
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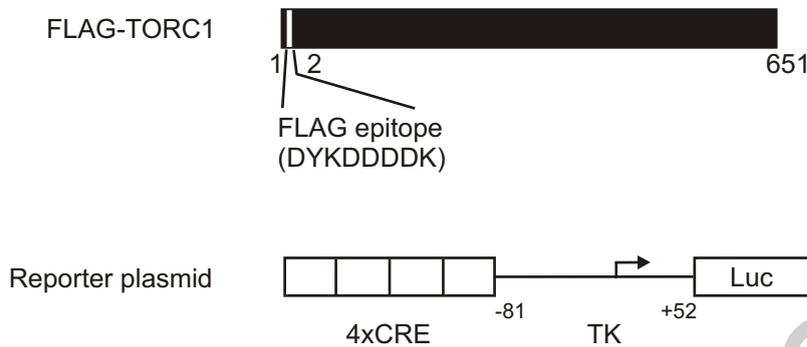
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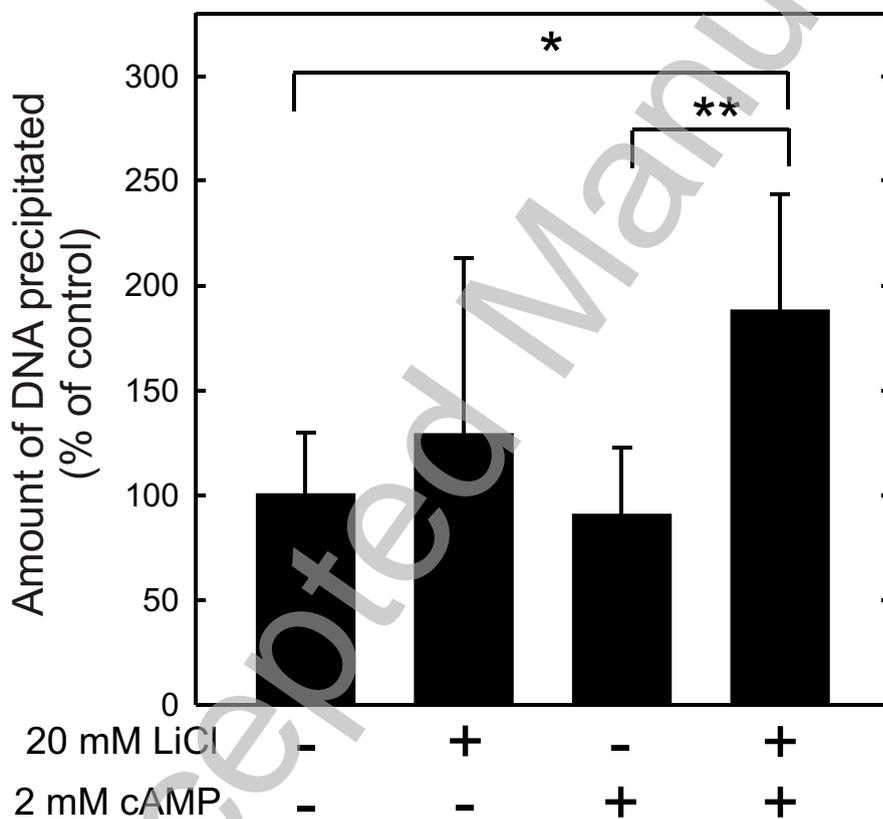
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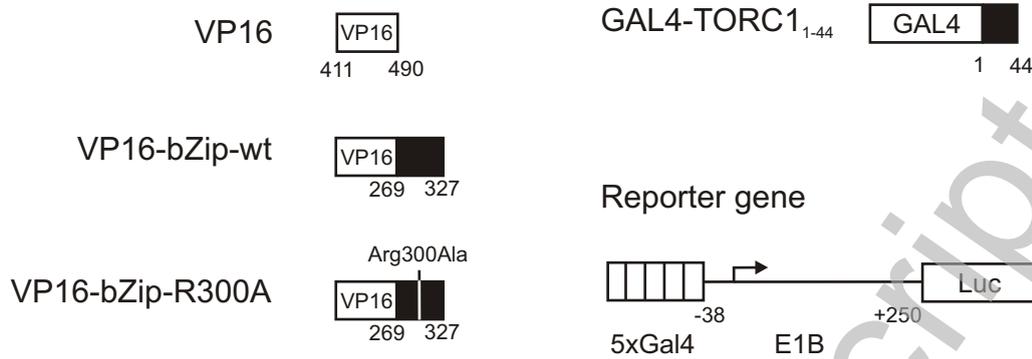
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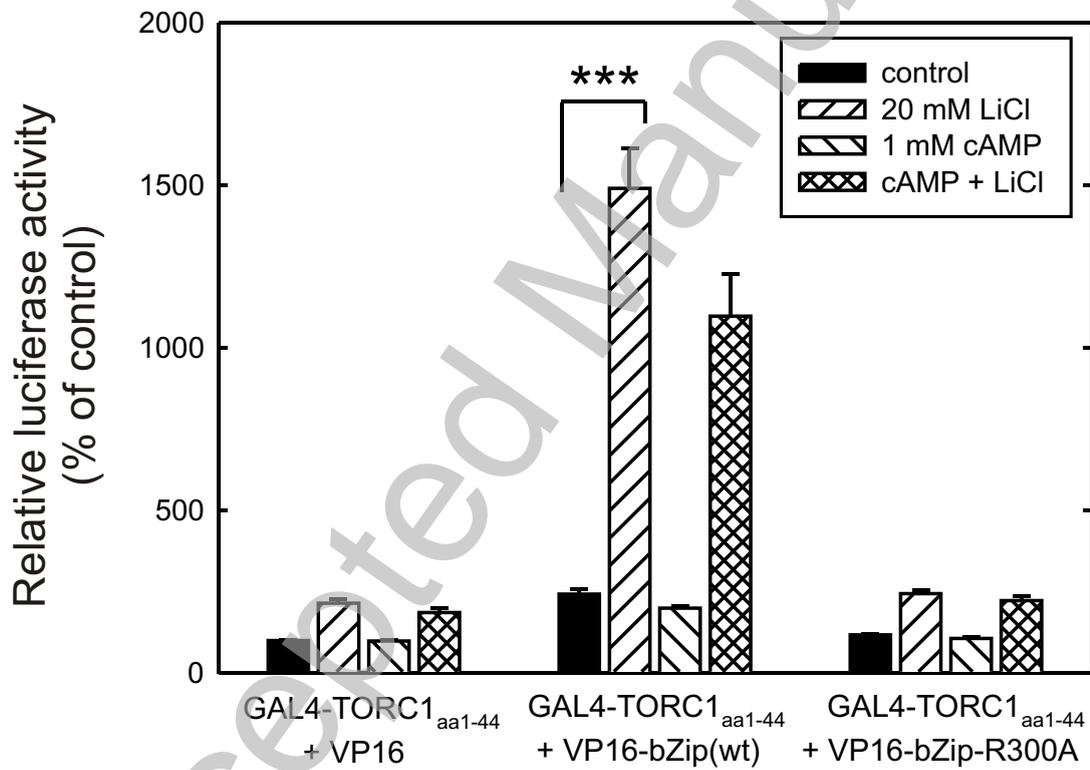
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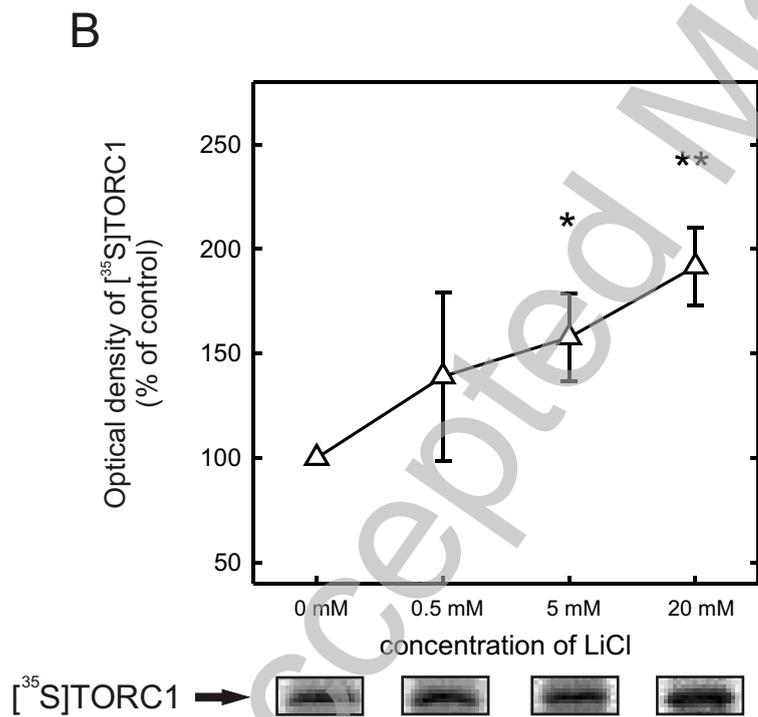
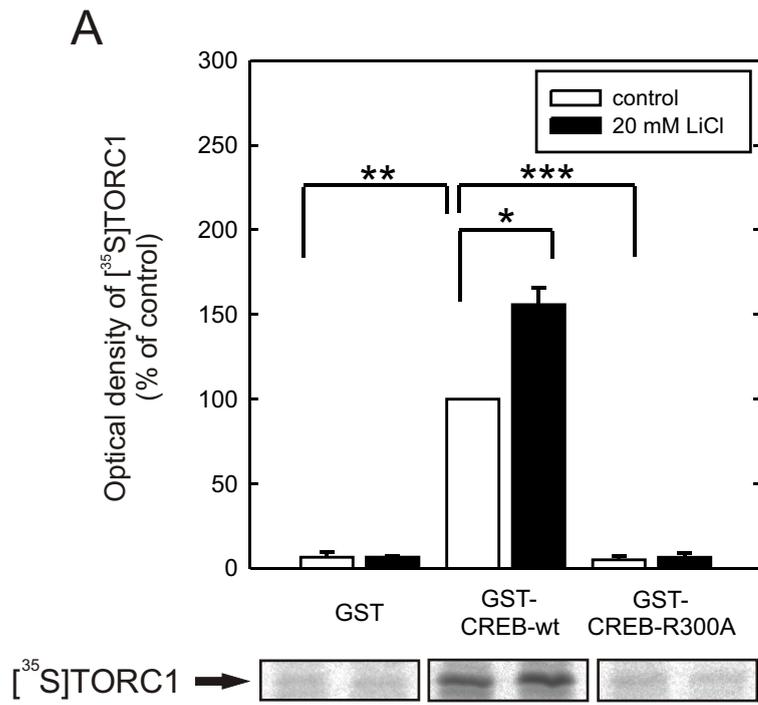
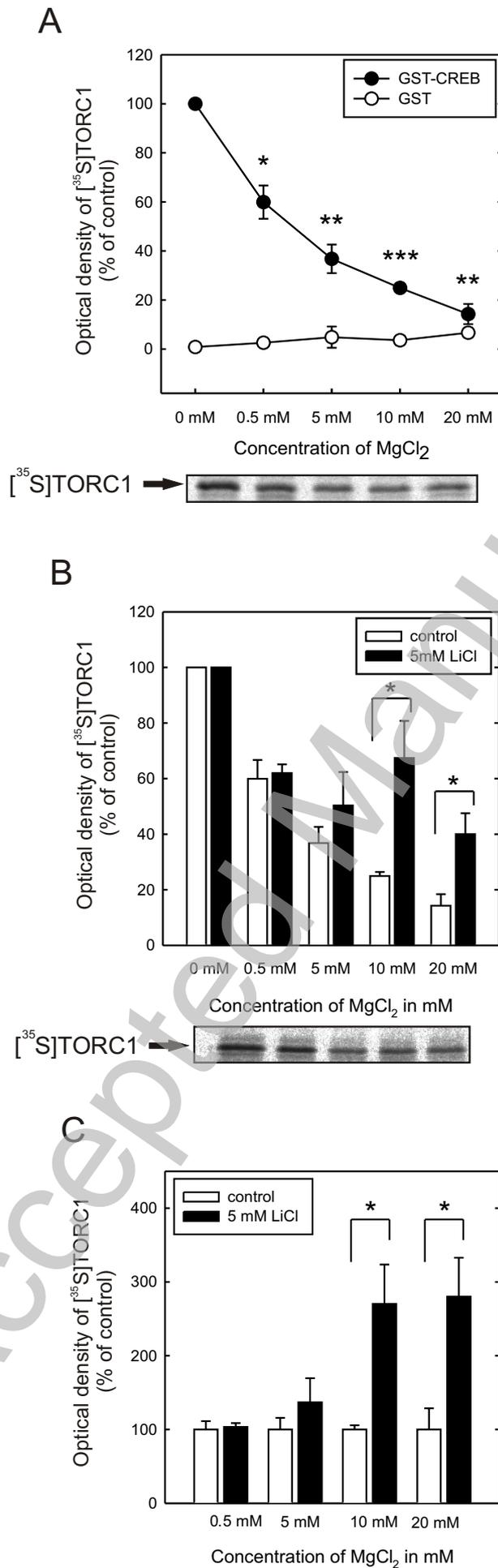
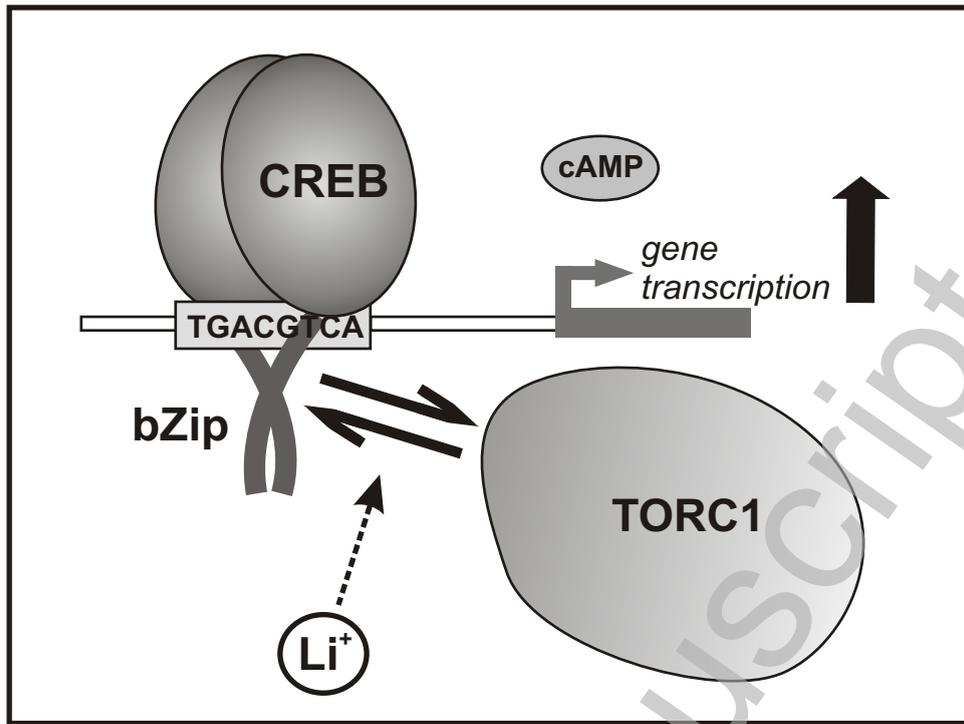


Figure 6





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