Biochimica et Biophysica Acta 1832 (2013) 1969–1979

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Egr1 regulates lithium-induced transcription of the *Period 2* (*PER2*) gene

Se Hyun Kim [a], Hyun Sook Yu [b], Hong Guen Park [b], Yong Min Ahn [a,c], Yong Sik Kim [d], Young Han Lee [e,f], Kyooseob Ha [a,c,g,*], Soon Young Shin [e,f,*]

[a] Institute of Human Behavioral Medicine, Medical Research Center, Seoul National University, Seoul 110-744, Republic of Korea
[b] Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul 110-744, Republic of Korea
[c] Department of Psychiatry and Behavioral Sciences, Seoul National University College of Medicine, Seoul 110-744, Republic of Korea
[d] Department of Neuropsychiatry, Dongguk University International Hospital, Dongguk University Medical School, Goyang-si, Gyeonggi-do 410-773, Republic of Korea
[e] Department of Biological Sciences, College of Biological Science and Biotechnology, and Research Center for Transcription Control, Konkuk University, Seoul 143-701, Republic of Korea
[f] SMART-Institute of Advanced Biomedical Science, Institute of Clinical Medicine, Konkuk University Medical Center, Seoul 143-729, Republic of Korea
[g] Mood Disorders Clinic and Clinical Affective Neuroscience Laboratory, Department of Psychiatry, Seoul National University Bundang Hospital, Seongnam 463-707, Republic of Korea

ARTICLE INFO

Article history:
Received 9 December 2012
Received in revised form 22 May 2013
Accepted 19 June 2013
Available online 28 June 2013

Keywords:
Bipolar disorder
Mood stabilizer
Lithium
Circadian gene
Per2
Egr1

ABSTRACT

A growing body of evidence suggests that the circadian molecular system is involved in the pathogenic and therapeutic mechanisms underlying bipolar disorders. Lithium, a representative mood stabilizer, has been reported to induce the *Period2* (*PER2*) gene; however, the underlying molecular mechanisms require further study. We found that lithium upregulated *PER2* expression at the transcriptional level in neuronally differentiated SH-SY5Y human neuroblastoma cells. Promoter reporter analyses using serial deletions of the *PER2* promoter revealed that two early growth response 1 (Egr1)-binding sites (EBS) between positions −180 and −100 are required for maximal activation of the *PER2* promoter by lithium. Ectopic expression of Egr1 enhanced lithium-induced *PER2* promoter activity, while a point mutation in EBS abolished it. Electrophoretic mobility shift assays and chromatin immunoprecipitation indicated that Egr1 bound directly to the *PER2* promoter. Stimulation of the extracellular-signal regulated kinase (ERK)1/2/Elk1 pathway by lithium was functionally linked to *PER2* expression through Egr1 induction, and lithium-induced *PER2* expression was strongly attenuated by depletion of Egr1 by siRNA. Lithium also upregulated the expression of *Per2* and *Egr1* in mouse frontal cortex. Induction of *Per2* by lithium was attenuated in *Egr1*$^{-/-}$ mice. In conclusion, lithium stimulates *PER2* transcription through the ERK/Elk1/Egr1 pathway in neuronal cells, indicating a connection between the ERK-Egr1 pathway and a circadian gene system in the mechanism of action of lithium.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

The circadian clock is an intrinsic oscillation system that controls daily physiological and behavioral processes with ~24 h periodicity. A highly specialized master pacemaker of the mammalian circadian clock resides in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, which receives photic input from the retina and conveys timing information to the entire organism [1]. Outside the SCN, peripheral tissue clocks also exist and they are either synchronized with the SCN or independent from the master clock [2]. Peripheral circadian genes have been widely implicated in non-circadian roles including cell survival, proliferation, and metabolism [3,4]. Circadian genes are also widely expressed throughout the brain, and, in the forebrain regions, they are involved in the regulation of mood, anxiety, and cognition [5–11].

Oscillation of circadian gene expression is generated *via* interconnected transcriptional–translational feedback loops [1,12–14]. A positive feedback loop is formed by two bHLH-PAS domain proteins, brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1) and circadian locomotor output cycles kaput (CLOCK), that heterodimerize and activate a number of clock genes, including *period1* (*PER1*), *PER2*, *cryptochrome1* (*CRY1*), and *CRY2* in an E-box-dependent manner [15,16]. As a negative feedback loop, PER and CRY accumulate in the cytosol and then translocate to the nucleus and inhibit their own and other E-box-regulated promoters [16,17]. Various kinases, including glycogen synthase kinase-3β (GSK-3β), mitogen-activated protein kinase (MAPK), casein kinase (CK) I, and CKII, are involved in translational modifications of clock proteins regulating protein stability and translocation [18].

Involvement of the circadian system in bipolar disorder has been reported. Disturbances in the sleep–wake cycle are frequently associated with mood episodes, and bipolar patients may also have abnormal rhythms in body temperature, blood pressure, and melatonin secretion [9,10,19,20]. Genetic studies have examined variants in circadian genes including *PER2*, *PER3*, *BMAL1*, *CLOCK*, and retinoid *REV-ERBα* as potential

* Corresponding authors at: Department of Biological Sciences, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea. Tel.: +82 2 2030 7946; fax: +82 2 3437 9781.
E-mail addresses: kyooha@snu.ac.kr (K. Ha), shinsy@konkuk.ac.kr (S.Y. Shin).

0925-4439/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbadis.2013.06.010

susceptibility genes for bipolar disorder, although the findings have been inconclusive [21–24].

Lithium is still the first-line mood stabilizer for the treatment of bipolar disorder [25,26]. Research on the molecular-genetic mechanisms of lithium has been used to understand the therapeutic mechanisms of bipolar disorder. For example, the International Consortium on Lithium Genetics (ConLiGen) has been formed to investigate the pharmacogenetic underpinnings of bipolar disorder [27]. Lithium affects circadian systems, prolonging the circadian period of locomotor activity and body temperature rhythm in humans and mice [28,29]. The effects of lithium on circadian genes have been investigated, and PER2 has been suggested to be lithium-responsive. Lithium induces increased expression and a prolonged period of Per2 in mouse fibroblast NIH3T3 cells [30,31], and cells from PER2::LUC knock-in mice [29,32]. Microarray analysis revealed that chronic lithium treatment induces upregulation of *Per2* mRNA in mouse brain [33]. A recent report suggested that lithium upregulates *Per2* at the transcriptional level [29]. Although lithium-induced lengthening and upregulation of PER2 have been reported to be associated with changes in the phosphorylation of PER2 through GSK-3β [30], the detailed mechanisms of lithium's effects on Per2 transcription are not yet fully understood. For example, the lithium-induced increase in Per2 expression does not co-occur with a preceding increase in Clock and Bmal1, representative transcriptional activators of Per2 *via* the E-box [31].

In the present study, we found that Egr1-binding sites in the *PER2* promoter are required for lithium-responsive PER2 induction. An *in vitro* study revealed that lithium upregulated *PER2* transcription *via* an extracellular-signal regulated kinase 1/2 (ERK1/2)-Elk1-mediated Egr1 signaling pathway. We confirmed the role of Egr1 in lithium-induced Per2 expression using *Egr1*$^{-/-}$ mice. These findings reveal a novel mechanism regarding the effects of lithium on circadian genes.

2. Materials and methods

2.1. Materials and methods

Lithium chloride (LiCl) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). U0126, SB203580, and SP600125 were purchased from Calbiochem (San Diego, CA, USA). Antibodies against phospho-ERK1/2 (Thr202/Tyr204), phospho-JNK1/2 (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), and phospho-Elk1 (Ser383) (Cell Signaling Technology, Beverly, MA, USA), Egr1, β-actin, GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and PER2 (Abcam, Cambridge, UK) were used. The firefly and Renilla Dual-Glo Luciferase Assay System was purchased from Promega (Madison, WI, USA).

2.2. Neuronal differentiation of SH-SY5Y cells

SH-SY5Y cells (Cell Line Service, Eppelheim, Germany) were maintained in DMEM:Ham's F12 (1:1 mixture), supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). For differentiation, SH-SY5Y cells were treated with 50 μM all-*trans* retinoic acid (RA; Sigma-Aldrich) for 5 days as described previously [34].

2.3. Animals and drug treatment

Male C57BL6 mice and *Egr1*$^{-/-}$ mice (20–25 g) were grouped and maintained on a 12/12-h light/dark cycle with food and water freely available. *Egr1*$^{-/-}$ mice developed on a C57BL6 background were from Dr. Jeffrey Milbrandt (Washington University, St. Louis, MO, USA) and have been described elsewhere [35,36]. Animals were treated in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. Lithium was dissolved in vehicle (0.9% saline). Each mouse received an intraperitoneal (i.p.) injection of lithium or vehicle in a volume of 100 μL. Two hours after injection, mice were decapitated to acquire brain tissues. The dose of lithium (150 mg/kg) was determined based on previous studies [37,38]. A dose of 150 mg/kg has been reported to inhibit amphetamine-induced hyperlocomotion in mice [39–42], which mimics the therapeutic effects of lithium. Following systemic administration of lithium, serum and brain levels of the drug were found to be within the accepted therapeutic range for rats and mice [39,43].

2.4. Plasmids

A full-length *EGR1* promoter reporter plasmid, pEgr1-Luc (−780/+1), was provided by Dr. H. Eibel (Department of Orthopedic Surgery, University of Tübingen Medical Center, Germany) and is described elsewhere [44]. pFA2-Elk1, which encodes a fusion protein consisting of the yeast Gal4 DNA-binding domain (amino acids 1–147) and the activation domain of Elk1 (amino acids 307–427), and plasmid pFR-Luc, which contains five Gal4-binding element repeats upstream of the luciferase gene, were purchased from Stratagene (La Jolla, CA, USA). An Elk1 expression plasmid (pCMV/flagElk1) was a gift from Dr. A. Sharrocks (Faculty of Life Sciences, University of Manchester, UK). Plasmids expressing dominant negative (dn)-MEK1 (pCGN1/MEK DN), dn-Erk2 (pHA-Erk2 K52R), dn-JNK1 (pSRα/HA-JNK T183A/Y185F), and dn-p38 kinase (pCDNA3/flag-p38 T180A/Y182F) were provided by Dr. D.S. Min (Department of Molecular Biology, College of Natural Science, Pusan National University, Korea). The pCDNA3.1/Egr1 plasmid, which expresses dominant-active (da)-Egr1(I293F), was generated as described previously [45].

2.5. Western blot analysis

Total cell lysates were prepared, separated, and transferred to nitrocellulose filters as described previously [36]. Signals were developed using an enhanced chemiluminescence detection system (GE Life Sciences, Piscataway, NJ, USA). For brain tissue samples, the mice were decapitated at the designated time after intraperitoneal injection of lithium and/or SL327; the frontal cortex was dissected and homogenized, and the immunoblot analysis was performed as described previously [46].

2.6. Immunofluorescence microscopy

RA-treated SH-SY5Y (RA-SH-SY5Y) cells plated on coverslips were either left untreated or treated with 20 mM LiCl for 12 h. The dose of 20 mM lithium used in the *in vitro* experiment was based on a previous study showing that lithium at that dose activated MAPK and inhibited GSK-3β in SH-SY5Y cells, with no evidence of cellular toxicity [47]. Then they were fixed and permeabilized as described previously [48]. The samples were co-stained with mouse anti-microtubule-associated protein 2 (MAP2, 1:100) for neuronal marker and rabbit anti-PER2 (1:200) antibodies for 90 min at room temperature and then incubated for 30 min with Alexa-Fluor 488-conjugated anti-mouse and Alexa-Fluor 555-conjugated anti-rabbit secondary antibodies, yielding green and red signals, respectively. Nuclear DNA was stained with 1 μg/mL Hoechst 33258 (blue signal) for 10 min. The labeled cells were examined using an EVOSf1 fluorescence microscope (Advance Microscopy Group, Bothell, WA, USA).

2.7. Northern blot analysis

For each sample, 10 μg total RNA was electrophoresed on a formaldehyde/agarose gel and transferred to a Hybond N^+ nylon membrane (Amersham Pharmacia Biotech). Northern blotting was performed with a [γ-^{32}P]dCTP-labeled human *PER2* cDNA probe, followed by hybridization with a *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) cDNA probe.

2.8. Quantitative real-time PCR and reverse transcription-PCR (RT-PCR)

Cells were treated with 20 mM LiCl for different periods of time. Total RNA was extracted using a Trizol RNA extraction kit (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR was performed using an icycler iQ system (Bio-Rad) with a TaqMan-iQ Supermix Kit (Bio-Rad). The TaqMan flurogenic probes and PCR primers for *PER2* and *GAPDH* were designed by Metabion Int. (Martinsried, Germany). The relative changes in *PER2* mRNA levels were normalized to *GAPDH* mRNA in the same samples. For RT-PCR analysis, the PCR conditions for all primers were as follows: hold for 4 min at 95 °C, followed by 30 cycles consisting of denaturation at 95 °C (30 s), annealing at 65 °C (30 s), and elongation at 72 °C (30 s). The amplified products were subjected to 1% agarose gel electrophoresis. RNA from the mouse frontal cortical tissue was detected using real-time PCR using an ABI PRISM 7000 instrument (Applied Biosystems, Foster City, CA, USA), and a SYBR Green PCR real-time PCR Master Mix (Toyobo, Osaka, Japan) was used according to the manufacturer's instructions. The reaction was first incubated at 50 °C for 2 min, then at 95 °C for 10 min, followed by 35 cycles of 95 °C for 15 s and 60 °C for 1 min. Each gene-specific PCR was performed in triplicate. The relative changes in *Per2* mRNA levels were normalized for *β-actin* mRNA in the same samples. The primers are summarized in Table 1.

2.9. Construction and site-directed mutagenesis of the human PER2 promoter reporter

A *PER2* promoter fragment spanning nucleotides −998 to +50 was synthesized from human genomic DNA (Promega) by PCR using the primers 5′-TCTCCCTAGTGATGCGCTTG-3′ (forward primer; −998F) and 5′-CAGCAGCCCAAGGAACTT-3′ (reverse primer; +50R). The products were ligated into the *Kpn*I and *Bgl*II sites of the pGL4-basic vector (Promega), yielding pPer2-Luc (−998/+50). A series of deletion constructs of human *PER2* promoter fragments was synthesized by PCR using the pPer2-Luc (−998/+50) plasmid as the template. Forward primer sequences were 5′-GGGCGTAGTGAATGGAAGGCG-3′ (−500/+50), 5′-CAGATGAGACGGAGTCGCGGC-3′ (−300/+50), 5′-ATACGTGCAGCTGTGGGCGGC-3′ (−180/+50), and 5′-GGCTGACGCG GGCGCGGC-3′ (−100/+50). One reverse primer, +50R, was used to generate all constructs. Amplified PCR products were ligated into the *Kpn*I and *Bgl*II sites of the pGL4-basic vector. Site-specific mutations in the Egr1-binding sites within the *PER2* promoter were made with the QuickChangeII Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), using pPer2-Luc (−180/+50) as template. Primer sequences used to generate point mutations were 5′-GTGGGCggCGG-3′ for disruption of the Egr1 binding site (lower-case letters are mutated bases). All mutations were verified by DNA sequencing.

Table 1
Primers for quantitative real-time PCR and reverse transcription-PCR (RT-PCR).

Real-time PCR (human, TaqMan)		
hPER2	Forward	5′-CCACTGCCATTACTA CTTG-3′
	Reverse	5′-GGACTTCAATTTTCTGTTCTTTC-3′
	TaqManTM fluorogenic probe	5′-FAM-AGCCAAGTGAACGAA CTG CCC-BHQ-3′
hGAPDH	Forward	5′-TCGACAGTCAGCCGCATCTTC-3′
	Reverse	5′-CGCCCAATACGACCAAATCCG-3′
	TaqManTM fluorogenic probe	5′-Yakima Yellow TM-CGTCGCCAGC CGAGCCACATCGC-BHQ-3′
RT-PCR (human)		
hPER2	Forward	5′-CCACGAGAATGAAATCCGCT-3′
	Reverse	5′-CCTCCCAATGATGAAGGAGA-3′
hGAPDH	Forward	5′-ACCCACTCCTCCACCTTTG-3′
	Reverse	5′-CTCTTGTGCTCTTGCTGGG-3′
Real-time PCR (mouse, SYBR green)		
mPer2	Forward	5′-GGCTTCACCATGCCTGTTGT-3′
	Reverse	5′-GGAGTTATTTCGGAGGCAAGTGT-3′
mEgr1	Forward	5′-AGCGAACAACCTATGAGCA-3′
	Reverse	5′-TCGTTTGGCTGGGATAACTC-3′
mActin	Forward	5′-CTTCCTCCCTGGAGAAGAGC-3′
	Reverse	5′-AAGGAAGGCTGGAAAAGAGC-3′

2.10. Luciferase reporter assay

SH-SY5Y cells were seeded into 12-well plates and transfected with 0.5 μg promoter reporter construct using Lipofectamine 2000 (Invitrogen). For promoter reporter assay, cells were transfected with 0.2 μg luciferase reporter constructs. For Elk1 *trans*-acting activity, cells were cotransfected with 50 ng *trans*-activator plasmid (pFA2-Elk1) and 0.5 μg reporter plasmid (pFR-Luc). To monitor transfection efficiency, 50 ng pRL-null plasmid encoding Renilla luciferase was included in all samples. Where indicated, 0.2 μg mammalian expression vector was also included. At 24 h post-transfection, the levels of firefly and Renilla luciferase activity were measured sequentially from a single sample using the Dual-Glo Luciferase Assay System with a luminometer (Centro LB960; Berthold Tech, Bad Wildbad, Germany).

2.11. Chromatin immunoprecipitation (ChIP)

RA-SH-SY5Y cells treated with 20 mM LiCl were cross-linked, lysed, and chromatin immunoprecipitated using rabbit anti-Egr1 antibody or normal rabbit IgG as described previously [36]. The precipitated DNA was analyzed by standard RT-PCR methods. The following promoter-specific primers were used for PCR: 5′-GCTGAGGGGATACGTGCA-3′ (target region; forward primer, −189/−172), 5′-GCGGTTACGTAAGC CGCA-3′ (target region; reverse primer, −53/−36), 5′-CGGTAAGGT GTGGATGAG-3′ (off-target region; forward primer, −1891/−1874), and 5′-GACACCTCGACACACTCC-3′ (off-target region; reverse primer, −1718/−1701).

2.12. Expression of siRNA

Short hairpin RNA (shRNA) plasmids expressing Egr1 siRNA or scrambled control siRNA have been described elsewhere [36,49].

2.13. Statistical analyses

Each experiment was repeated at least three times. Data are expressed as means ± standard errors. Statistical comparisons were performed using Student's *t*-test and a one-way or two-way analysis of variance (ANOVA) followed by a *post hoc* LSD test. *P*-values <0.05 were considered statistically significant. All tests were performed using the SPSS software (ver. 19.0 for Windows; IBM SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Lithium increases PER2 protein and mRNA in RA-SH-SY5Y cells

We first determined the effect of lithium on the amount of PER2 in neuronally differentiated SH-SY5Y (RA-SH-SY5Y) cells. The PER2 protein level increased in a dose-dependent manner following lithium stimulation for 12 h (Fig. 1A). Lithium is known to inhibit GSK-3β [50]. GSK-3β was reported to phosphorylate Per2 and promote the nuclear entry of Per2, which inhibits Bmal-Clock complex-mediated Per2 transcription in serum-shocked mouse NIH3T3 cells [30]. Although lithium-induced lengthening of the circadian period and upregulation of Per2 may be associated with changes in GSK-3β phosphorylation of Per2 [30], direct involvement of GSK-3β in the regulation of *PER2* transcription has not

been determined, and the detailed mechanisms that mediate the effect of lithium on *PER2* transcription are not understood. In RA-SH-SY5Y cells, to address whether lithium increases PER2 amount through the inhibition of GSK-3β, we tested the effect of a GSK-3β inhibitor on PER2 protein level. Treatment with the selective GSK-3β inhibitor, SB212763, alone did not affect the amount of PER2 (Fig. 1A), suggesting that lithium-induced increase in the amount of PER2 protein may be independent of the effect of GSK-3β in RA-SH-SY5Y cells. In a time-course analysis, an increase in PER2 protein was evident within 6 h and the maximal level was reached at 12 h, after which the level declined (Fig. 1B). In immunofluorescence staining, PER2 proteins were detected faintly in unstimulated cells, but stained strongly in the nuclei of cells 12 h after lithium treatment (Fig. 1C). To investigate whether increases in PER2 protein induced by lithium occur at the mRNA level, we analyzed *PER2* mRNA levels using RT-PCR (Fig. 1D) and Northern blot (Fig. 1E) analyses. Real-time PCR using the TaqMan flurogenic probes showed that lithium significantly increased *PER2* mRNA levels by 1.7 and 3.2-fold after 6 h ($P < 0.05$) and 9 h ($P < 0.05$), respectively compared with control cells (Fig. 1F). These data demonstrate that lithium stimulates *PER2* gene expression at the mRNA level in RA-SH-SY5Y cells.

3.2. Egr1-binding sequences within the PER2 promoter are critical for lithium-induced PER2 transcription

To investigate whether the lithium-induced expression of *PER2* mRNA is regulated by a transcriptional process, we isolated a 1047 bp genomic fragment encompassing the human *PER2* promoter (−938/+50), inserted it upstream of a luciferase reporter gene, and then tested responsiveness to lithium treatment. Lithium significantly increased *PER2* promoter activity (Fig. 2A). To identify the region responsible that mediates the responsiveness of the *PER2* gene to lithium, progressively deleted genome fragments encompassing the *PER2* promoter were constructed and transfected into SH-SY5Y cells. Basal promoter reporter activity was enhanced by deletion to −500, and further deletion resulted in progressive attenuation of basal reporter activity (Fig. 2A), suggesting the presence of a negative regulatory element between

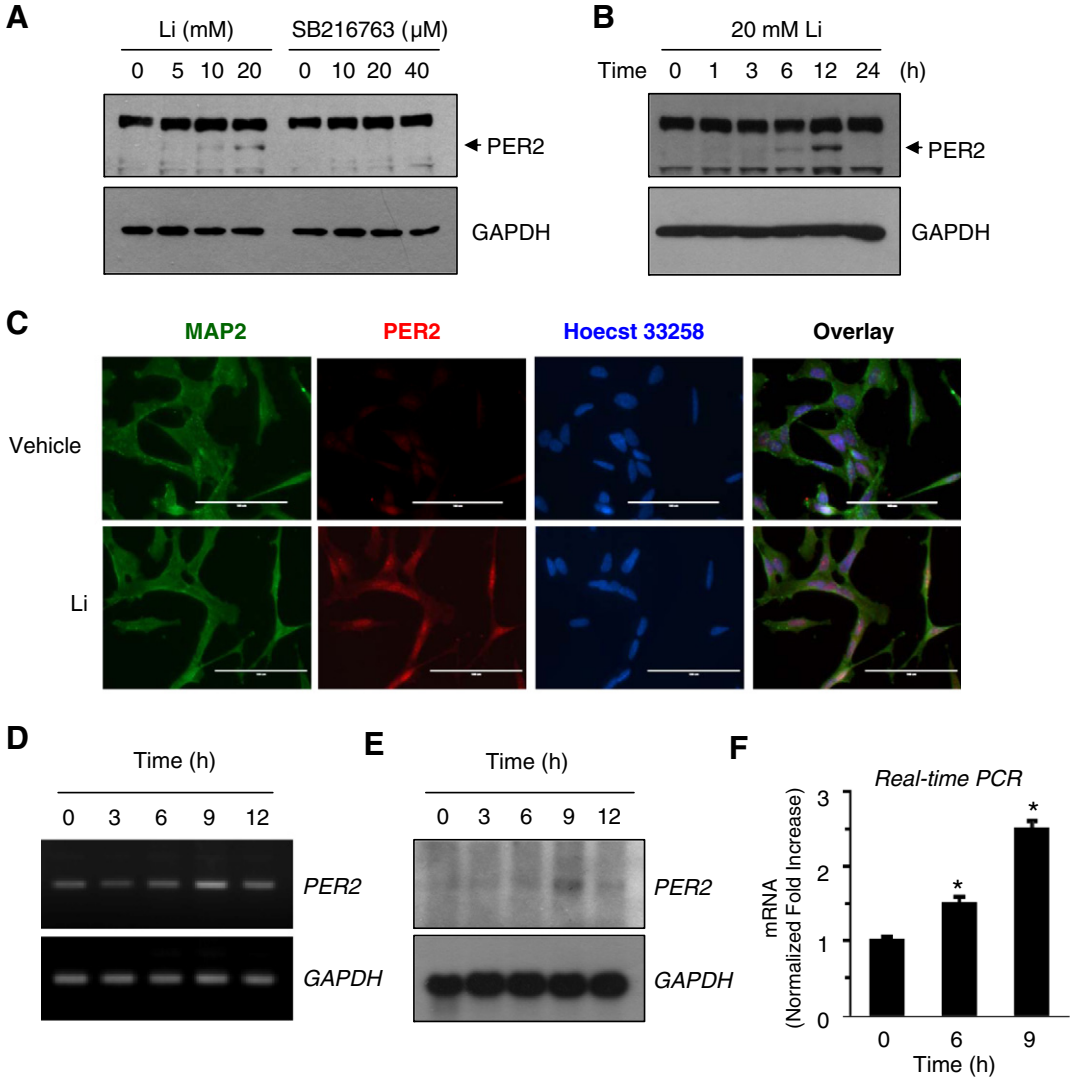


Fig. 1. Effect of lithium on PER2 expression. (A and B) RA-SH-SY5Y cells were treated with different concentrations of LiCl or SB216763 for 12 h (A) or 20 mM LiCl for various lengths of time (B). Whole cell lysates were prepared and Western blot analyses were performed with an anti-PER2 antibody. GAPDH was used as an internal control. (C) RA-SH-SY5Y cells were treated with vehicle or 20 mM LiCl for 12 h. MAP2, as a neuronal marker, was stained with Alexa-Fluor 488-conjugated anti-mouse antibody (*green*) and PER2 was stained with Alexa-Fluor 555-conjugated anti-rabbit secondary antibody (*red*). Nuclear DNA was stained with Hoechst 33258 (*blue*). Labeled cells were examined with an EVOSf1 fluorescence microscope. Bars indicate 100 μm. (D–F) RA-SH-SY5Y cells were treated with 20 mM LiCl for various lengths of time. Total RNA was isolated, and mRNA expression of PER2 and GAPDH was analyzed by regular RT-PCR (D), Northern blot (E) and quantitative real-time PCR (F). *, $P < 0.05$ ($n = 5$).

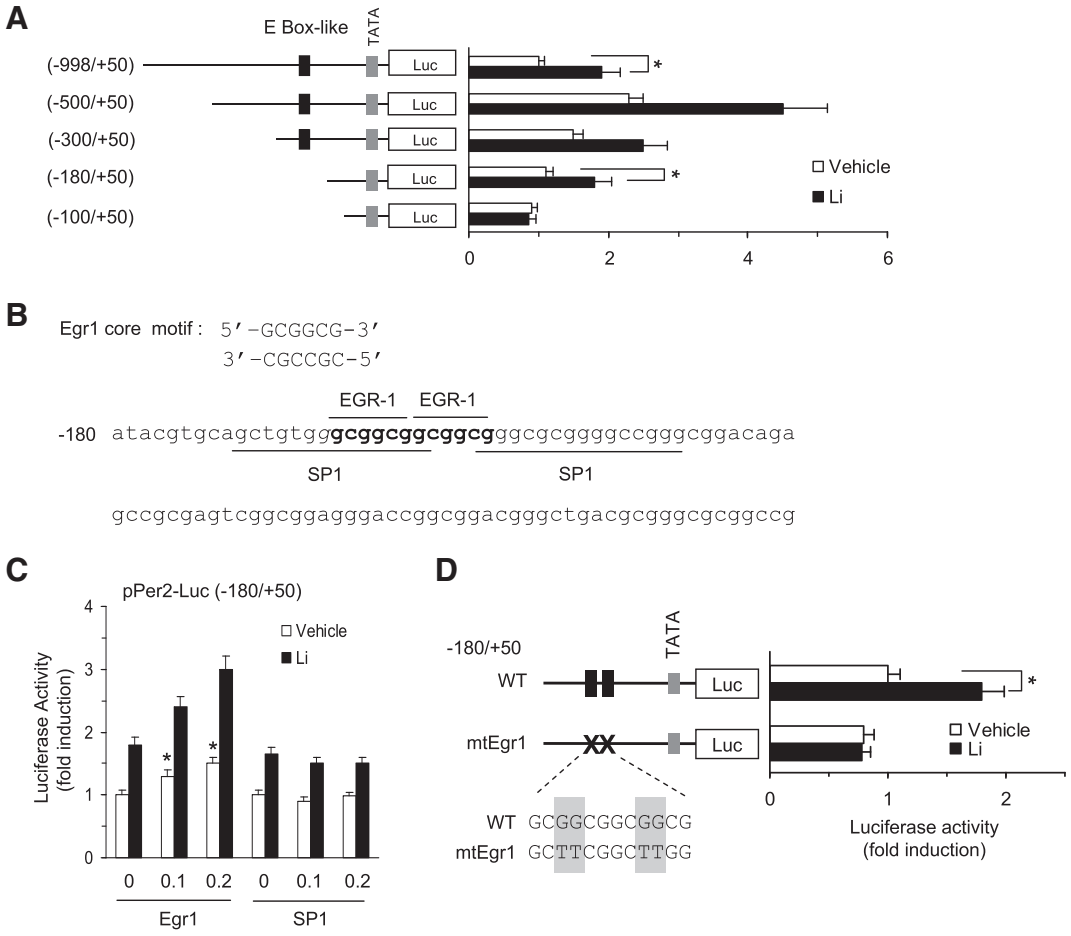


Fig. 2. Identification of EGR1-binding sequences required for lithium-induced PER2 promoter activation. (A) SH-SY5Y cells were transiently transfected with 0.2 μg of a series of 5′-deletion constructs of human *PER2* promoter reporter plasmids, along with 50 ng expression plasmid for Renilla luciferase (pRL-null) for normalization of transfection efficiency. Then, 48 h later, the cells were treated with 20 mM LiCl for 8 h, and luciferase activities were measured. The data shown are the means ± SD of three independent experiments performed in triplicate. *, $P < 0.05$ ($n = 9$). (B) Egr1 and SP1 binding motifs within the *PER2* promoter region. (C) SH-SY5Y cells were transiently cotransfected with 0.2 μg human *PER2* promoter reporter construct, pPer2-Luc (−180/+50), and different concentrations of expression plasmids for EGR1 or SP1, along with 50 ng expression plasmid for Renilla luciferase (pRL-null) to normalize transfection efficiency. The data shown are the means ± SD of three independent experiments performed in triplicate. *, $P < 0.05$ *vs.* control ($n = 9$). (D) SH-SY5Y cells were transiently cotransfected with 0.2 μg wild-type (WT) or site-specific mutant construct for EGR1 (mtEgr1) derived from the pPer2-Luc (−180/+50). Then, 48 h later, the cells were treated with 20 mM LiCl for 8 h, and luciferase activities were measured. The data shown are the means ± SD of three independent experiments performed in triplicate. *, $P < 0.05$ ($n = 9$).

nucleotides −998 and −500. An E-box-like sequence, a binding site for BMAL1 and CLOCK, is essential for *PER2* transcription [12,16]. Interestingly, lithium stimulated the −180/+50 construct lacking the E-box-like sequence; although basal promoter activity was suppressed, the increase (~1.6-fold) was similar compared to the −300/+50 construct. However, deletion of nucleotides −180 to −101 resulted in complete loss of lithium inducibility. Thus, we suggest that the proximal region between nucleotides −180 and −100 of the *PER2* promoter contains *cis*-acting regulatory element(s) responsive to lithium. To test this, we analyzed the human *PER2* promoter sequences spanning −180 to −100 using the program MatInspector (http://www.genomatix.de/) and found two overlapping binding motifs for EGR1 and specificity protein 1 (SP1) (Fig. 2B).

3.3. EGR1 transactivates the PER2 promoter

To assess whether EGR1 or SP1 activates the human *PER2* promoter, we cotransfected the pPer2-Luc (−182/+50) promoter construct and an expression plasmid for EGR1 or SP1 into SH-SY5Y cells. Ectopic expression of EGR1, but not SP1, enhanced lithium-induced promoter reporter activity (Fig. 2C). Notably, even in the absence of lithium treatment, EGR1 caused basal reporter activity to increase as the amount of transfected DNA increased.

To characterize whether the putative EGR1-binding sequences were responsible for the stimulation of the *PER2* gene promoter by lithium, two core sequences of the EGR1 binding site on the promoter were mutated (mtEgr1; GCGG → GCTT) within the context of the −180/+50 construct by site-directed mutagenesis. Disruption of EGR1 sites led to a complete loss in lithium-induced reporter activity (Fig. 2D), indicating that EGR1-binding sequences located at −180/−100 were key for lithium-induced *PER2* gene transcription.

3.4. EGR1 directly binds a consensus sequence within the PER2 promoter

To investigate whether EGR1 binds directly to the putative EGR1-binding sequence in the *PER2* promoter *in vivo*, a ChIP assay was performed. Cross-linked SH-SY5Y cells were immunoprecipitated with rabbit anti-Egr1 antibody or normal rabbit IgG. The resulting immunoprecipitates were analyzed by PCR assays using primers flanking the EGR1-binding sequences (−189 to −38) of the *PER2* promoter. A noticeable increase in the intensity of the DNA band was observed for the rabbit anti-Egr1 antibody but not for normal rabbit IgG (Fig. 3).

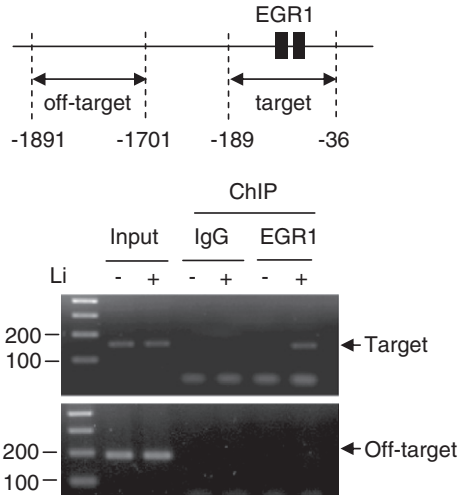


Fig. 3. Egr1 specifically interacts with the consensus EGR1-binding sequence within the PER2 promoter. SH-SY5Y cells treated with 20 mM LiCl for 12 h were cross-linked, lysed, and immunoprecipitated with anti-Egr1 antibody or normal rabbit IgG (negative control). The precipitated DNA was subjected to PCR with primers specific for the off-target region (−1891/−1701) or target region (−189/−36). One aliquot of input DNA was used as a positive control.

The off-target region (from −1891 to −1701) was not amplified, although positive results were obtained from input DNA. This result indicates that a physical interaction between EGR1 and the *PER2* promoter occurs upon lithium stimulation.

3.5. Lithium stimulates EGR1 expression in RA-SH-SY5Y cells

Next, we investigated whether lithium regulates EGR1 expression in RA-SH-SY5Y cells. Serum-starved cells were treated with 20 mM LiCl for different periods of time and the amount of *EGR1* mRNA was measured by Northern blotting. Upon lithium stimulation, accumulation of *EGR1* mRNA was detected within 0.5 h and the maximal level was reached at 1 h, after which there was a rapid decrease to control levels (Fig. 4A). To evaluate whether *EGR1* mRNA expression with lithium is caused by transcriptional activation, *EGR1* promoter activity was analyzed using a luciferase reporter. The *EGR1* promoter construct, pEgr1-Luc (−781/+1), was transfected into SH-SY5Y cells and luciferase activity was measured. Upon lithium exposure, *EGR1* promoter activities were significantly elevated in a dose-dependent manner (Fig. 4B), suggesting that lithium upregulates EGR1 expression at the transcriptional level. We also confirmed the amount of expressed EGR1 protein by Western blot analysis. The EGR1 protein level was reached a maximum at approximately 3 h followed by a gradual decrease, but readily detectable up to 12 h after lithium treatment (Fig. 4C).

3.6. Knockdown of EGR1 attenuates lithium-induced PER2 expression

To confirm the functional role of EGR1 in lithium-induced PER2 transcription, we depleted EGR1 using RNA interference. The knockdown of EGR1 expression in SH-SY5Y cells by transient transfection of shRNA plasmids generating EGR1-specific siRNA (pSilencer/siEgr1) was assessed using Western blotting (Fig. 4D). We found that knockdown of EGR1 expression reduced PER2 protein levels (Fig. 4E) and the promoter activity (Fig. 4F) induced by lithium.

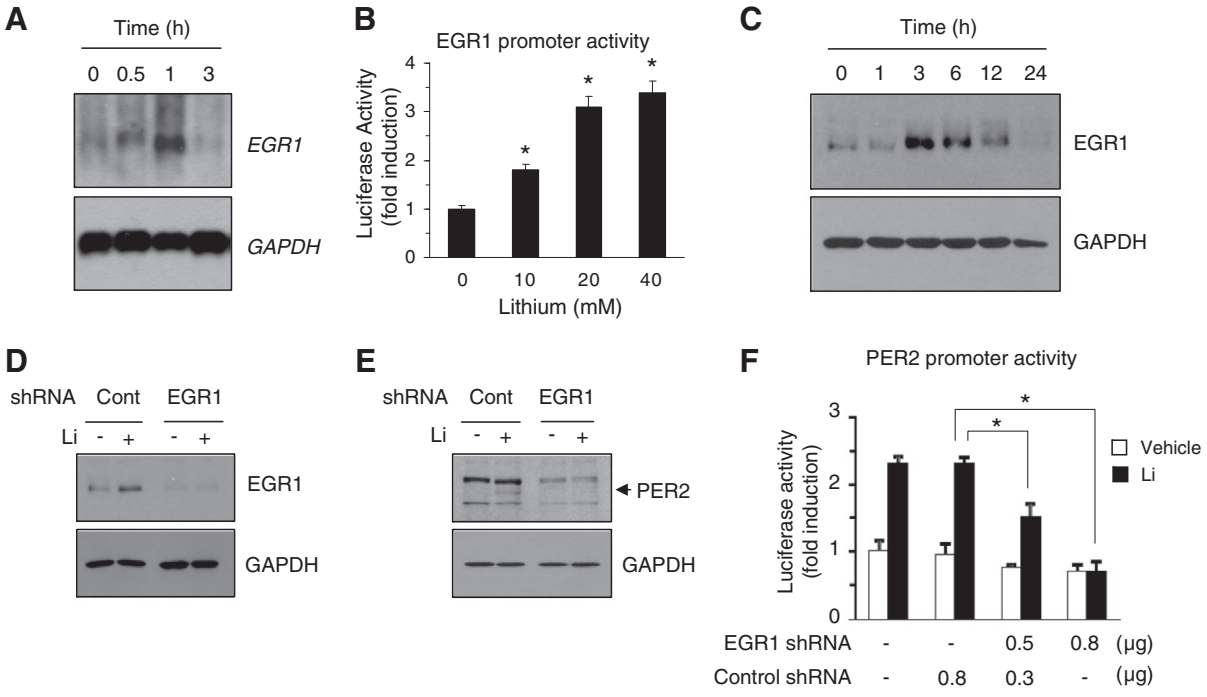


Fig. 4. Effect of EGR1 knockdown on the lithium-induced expression of PER2. (A) RA-SH-SY5Y cells were treated with 20 mM LiCl for various lengths of time and the amount of *EGR1* mRNA was detected by Northern blotting. (B) SH-SY5Y cells were transfected with 0.2 μg EGR1 promoter construct, pEgr1-Luc (−780/+1). Then, 48 h later, the cells were treated with varying concentrations of LiCl for 8 h, followed by dual luciferase assay. The data shown represent the means ± SD of three independent experiments performed in triplicate. *, $P < 0.05$ ($n = 9$), compared to the unstimulated control. (C) RA-SH-SY5Y cells were treated with 20 mM LiCl for various lengths of time and the amount of EGR1 protein was detected by Western blotting. GAPDH was used as an internal control. (D and E) RA-SH-SY5Y cells were transfected with shRNA plasmid expressing scrambled control (pSilencer/siCont) or EGR1 siRNA (pSilencer/siEgr1), as indicated. After 2 days, cells were treated with 20 mM LiCl for 3 h (D) or 12 h (E). Total cell lysates were prepared and Western blot analysis was performed with anti-EGR1 (D) or anti-PER2 antibodies (E). GAPDH was used as an internal control. (F) SH-SY5Y cells were cotransfected with 0.2 μg each of pPer2-Luc (−180/+50) and a shRNA plasmid expressing a scrambled control (pSilencer/Cont) or EGR1 siRNA (pSilencer/siEgr1), as indicated. Then, 24 h later, cells were left untreated or treated with 20 mM LiCl for 8 h and the luciferase activity was measured. Data are the means ± SD of three independent experiments, each performed in triplicate (*, $P < 0.05$, $n = 9$).

3.7. Lithium-induced EGR1 expression is mediated by MAPK/ELK1 pathway

In many cell systems, activation of MAPKs, including the ERK, JNK, and p38 MAPKs, mediates EGR1 expression [51–53]. To determine whether MAPK pathways are activated by lithium in RA-SH-SY5Y cells, serum-starved RA-SH-SY5Y cells were treated with lithium for various lengths of time, and the activation status of MAPKs was assessed using phospho-specific antibodies. The phosphorylation of ERK1/2 increased rapidly within 15 min, while JNK and p38 MAPK phosphorylation increased after 30 and 60 min, respectively, after lithium treatment (Fig. 5A), suggesting that lithium stimulated multiple MAPK signaling pathways. ELK1 is activated by ERK, JNK, and p38 MAPK [54], and complexed with the serum response factor (SRF) on the serum response element (SRE) within the *EGR1* gene [55]. Activated ELK1 plays a crucial role in extracellular-signal-induced transcription of the *EGR1* gene [56]. We also observed that ELK1 phosphorylation was increased in a time-dependent manner with lithium treatment (Fig. 5B).

To determine whether lithium stimulated ELK1 *trans*-acting activity, we used a luciferase reporter assay system. SH-SY5Y cells were cotransfected with the *trans*-acting plasmid Gal4-Elk1 and the pFR-Luc reporter plasmid containing Gal4-binding sequences. Treatment with lithium enhanced the *trans*-acting activity of Gal4-Elk1, which was strongly inhibited in the presence of dn-ERK2, dn-JNK1, and dn-p38 (Fig. 5C). Thus, ELK1 could be activated by all three major MAPKs in lithium-treated SH-SY5Y cells. To determine whether lithium-induced EGR1 expression is mediated by ELK1, we cotransfected an *EGR1* promoter construct and expression plasmid for ELK1. Transient transfection of ELK1 enhanced lithium-induced EGR1 promoter activity (Fig. 5D). Thus, lithium upregulates EGR1 expression *via* MAPK-dependent ELK1 transactivation.

3.8. The ERK pathway is necessary for lithium-induced EGR1 and PER2 expression

To determine which MAPK was responsible for lithium-induced EGR1 expression, SH-SY5Y cells were cotransfected with an *EGR1* promoter construct and expression plasmids with various mutant MAPK constructs. Lithium-induced *EGR1* promoter activity was reduced significantly by transient transfection with dn-ERK2 or dn-JNK1, but not by dn-p38 (Fig. 6A). To confirm the role of MAPKs in lithium-induced EGR1 expression, we used chemical inhibitors of the MAPKs. Similar to the promoter assay, Western blot analysis showed inhibition of ERK (U0126) and JNK (SP600125) inhibited lithium-induced accumulation of EGR1 protein, whereas the p38 MAPK inhibitor SB203580 had no effect (Fig. 6B). Meanwhile, the lithium-induced increase in *PER2* promoter activity (Fig. 6C) and PER2 protein level were blocked only by

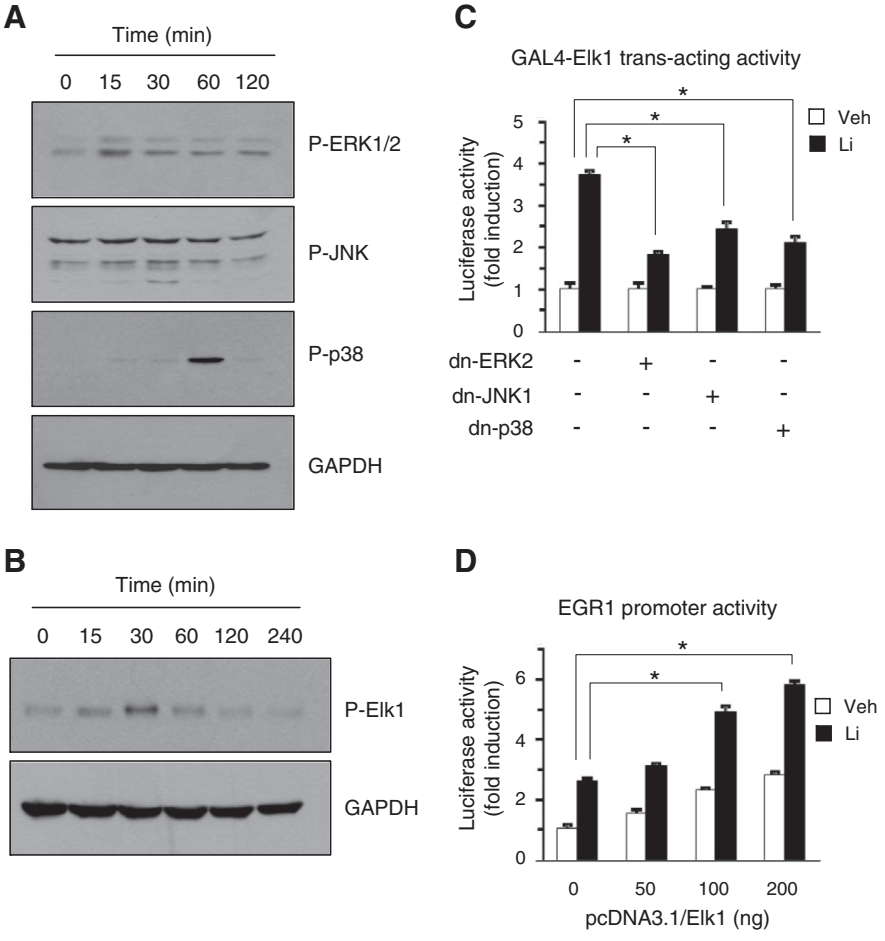


Fig. 5. Effect of forced expression of dn-ERK, -JNK or -p38 kinase in lithium-induced Elk1 activation and Egr1 expression. (A and B) RA-SH-SY5Y cells were treated with 20 mM LiCl for various lengths of time. Total protein extracts were analyzed by Western blotting using antibodies raised against phospho-ERK1/2 (Thr202/Tyr204), phospho-JNK1/2 (Thr183/Tyr185), or phospho-p38 kinase (Thr180/Tyr182) antibody (A), or phospho-Elk1 (Ser383) antibody (B). GAPDH was used as an internal control. (C) SH-SY5Y cells were cotransfected with 50 ng Elk1 *trans*-activator plasmid pFA/Gal4-Elk1 and 0.5 μg pFR-Luc, along with an expression plasmid encoding dn-ERK2, dn-JNK1, or dn-p38 (0.2 μg each), as indicated. Then, 48 h later, cells were treated with 20 mM LiCl for 8 h and their luciferase activities were measured. Data are the means ± SD of three independent experiments, each performed in triplicate (*, $P < 0.05$, $n = 9$). (D) SH-SY5Y cells were cotransfected with 0.2 μg EGR1 promoter reporter, pEgr1-Luc (−780/+1) and different concentrations of expression plasmid for Elk1, pCMV/flagElk1. After 48 h, cells were treated with 20 mM LiCl for 8 h and their luciferase activities were measured. Data are the means ± SD of three independent experiments, each performed in triplicate (*, $P < 0.05$, $n = 9$).

inhibition of the ERK MAPK pathway (Fig. 6D). These data suggest that although all three major MAPK pathways are stimulated and involved in ELK1 stimulation by lithium treatment, only the ERK/ELK1/EGR1 cascade is functionally linked to PER2 expression.

3.9. Lithium-induced PER2 expression is impaired in the *Egr1*$^{-/-}$ mouse brain

To evaluate the involvement of EGR1 in lithium-induced PER2 expression *in vivo*, *Egr1*$^{+/+}$ and *Egr1*$^{-/-}$ mice were i.p. injected with lithium and *Per2* expression in the frontal cortex was analyzed. Real-time PCR analysis showed that administration of lithium significantly increased *Egr1* and *Per2* mRNA levels in the frontal cortex 2 h after drug delivery in *Egr1*$^{+/+}$ mice, but not in *Egr1*$^{-/-}$ mice ($P < 0.05$; Fig. 7A). The Western blot analysis showed similar results ($P < 0.05$; Fig. 7B). These findings demonstrate that lithium-induced Per2 expression is mediated by Egr1 in the brain *in vivo*.

4. Discussion

We demonstrated that the mood stabilizer lithium induced *PER2* expression *via* EGR1, activated by the ERK/ELK1 signaling pathway. Lithium increased mRNA and protein levels of *PER2* in differentiated neuronal cells, and stimulated the transcription of the *PER2* gene through EGR1 *cis*-acting response elements within the proximal region of the *PER2* promoter. The induction of *PER2* through EGR1 was mediated through the ERK/ELK1 signal pathway. This was also found in the brain *in vivo*. Lithium increased Per2 in the mouse frontal cortex, which was attenuated in *Egr1*$^{-/-}$ mice, compared to *Egr1*$^{+/+}$ mice. Taken together, these results indicate that the mood stabilizer lithium regulates the circadian gene PER2 *via* the ERK/ELK1/EGR1 signaling pathway in neuronal cells.

Induction of PER2 by lithium has been reported previously under several different experimental conditions. For example, lithium has been shown to significantly increase the expression of *Per2* and *Cry1* and prolong the period of *Per2* in NIH3T3 cells [31], to lengthen the period of *Per2* in fibroblasts and SCN tissues from PER2::LUC knock-in mice [29,30,32], and to restore to neural Per2 expression in the mouse brain that was blunted by chronic restraint stress [57]. In the present study, we also found that lithium upregulates PER2 expression in differentiated SH-SY5Y cells and mouse brain. Taken together, these results suggest that *PER2* could be a lithium-responsive target clock gene.

Although evidence about the effect of lithium on PER2 induction has accumulated, the underlying mechanisms are not yet understood. BMAL1 and CLOCK, or NPAS2, a forebrain analog of CLOCK [58], positively regulate the transcription of *PER2* through the E-box in the promoter region [16]. However, in previous studies, a lithium-related increase in *Per2* expression was reported to occur without a preceding increase in *Clock* or *Bmal1* in serum-shocked NIH3T3 cells [31], and lithium did not affect the *Bmal1* promoter in a Bmal1::LUC reporter cell line [29]. Lithium has been reported to induce a phase delay in *Per2* expression and increase Per2 protein levels in serum-shocked NIH3T3 cells and PER2::LUC cells. Furthermore, the inhibitory action of lithium on GSK-3β has been suggested to stimulate *Per2* mRNA expression [29,30]. In the present study, GSK-3β was not involved in lithium-induced *PER2* expression in differentiated SH-SY5Y cells. In addition, activating

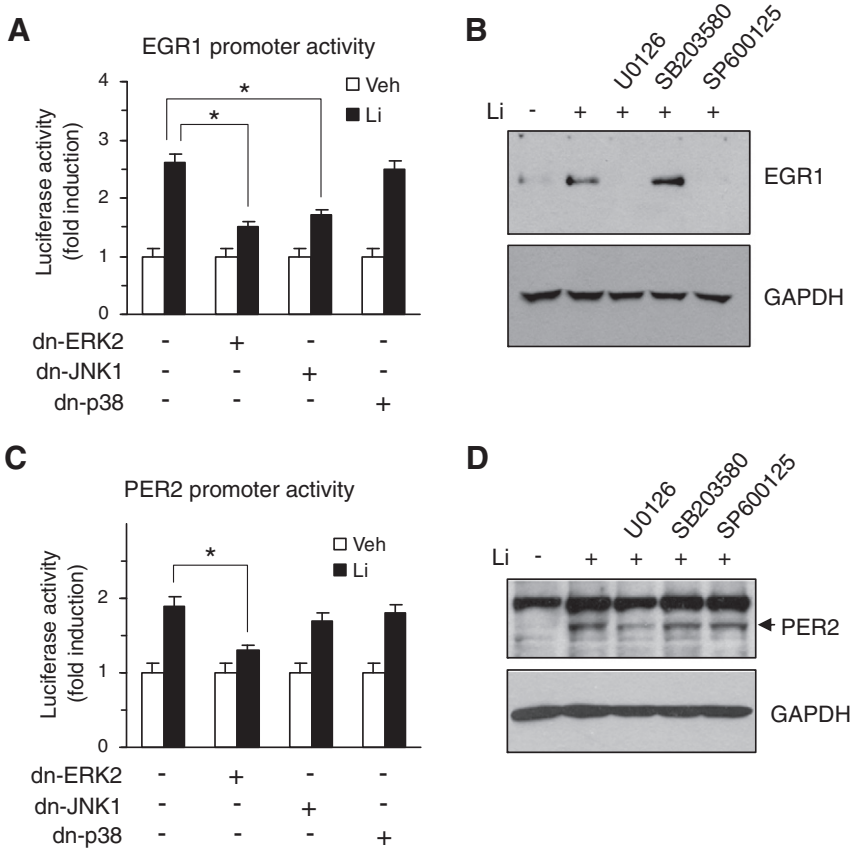


Fig. 6. Requirement of ERK MAPK for lithium-induced EGR1 and PER2 expression. (A and C) SH-SY5Y cells were transfected with 0.2 μg pEgr1-Luc(−780/+1) (A) or 0.2 μg pPer2-Luc (−180/+50) (C), along with expression plasmid encoding dn-ERK2, dn-JNK1, or dn-p38 (0.2 μg each), as indicated. Then, 48 h later, cells were treated with 20 mM LiCl for 8 h and their luciferase activities were measured. Data are the means ± SD of three independent experiments, each performed in triplicate (*, $P < 0.05$, $n = 9$). (B and D) RA-SH-SY5Y cells were pretreated with 5 μM U0126, 10 μM SP600125, or 10 μM SB203580 for 30 min, and then treated with 20 mM LiCl for 3 h (B) or 12 h (D). Total protein extracts were analyzed by Western blotting using an antibody against EGR1 (B) or PER2 antibody (D). GAPDH was used as an internal control.

transcription factor 4 (ATF4) and ATF2 positively regulate Per2 expression by binding to a cAMP-responsive element (CRE) [59,60]. In contrast, the bZIP transcription factor E4BP4 represses the transcription of *Per2* by binding to its promoter [61]. In the present study, we are the first to demonstrate that ERK pathway-mediated EGR1 positively regulates *PER2* transcription in response to lithium in differentiated SH-SY5Y cells by directly binding to the promoter. Further studies regarding the interactive mechanisms of *PER2* transcription through ERK-EGR1, cAMP-ATF, GSK-3β, and E4BP4 (as well as possible differential effects depending on cell type) are required.

Many previous reports have demonstrated phase advances of circadian rhythms in mood disorders, including depressive disorder and bipolar disorder [9,62], although the evidence regarding the phase advance hypothesis of depressive disorder has not been confirmatory [63]. In addition, several reports have shown that lithium lengthens the circadian period of behavioral rhythms [28,29,64]. The lithium-induced lengthening of behavioral rhythms is accompanied by a prolonged circadian period and increased oscillation amplitude in Per2 [29] that is mediated by transcriptional activation [29,32]. These findings suggest that the upregulation of *Per2* transcription may be related to the phase-delay effects of lithium on circadian rhythm; however, this hypothesis requires further clarification.

EGR1 is abundantly expressed in the nervous system and activated rapidly in response to various stimuli as an immediate early gene (IEG) [65]. In the present study, lithium rapidly induced EGR1 in differentiated SH-SY5Y cells and in the frontal cortex of the mouse brain. EGR1 encodes several functionally different products, such as transcription factors, cytoskeletal proteins, kinases, phosphatases, and components of the proteasomal and ubiquitination machinery [65–67]. Previous studies have indicated that Egr1 also affects the transcription of circadian genes. The Cry1 gene has an Egr1 response element in the promoter, and Egr1 binds to its promoter and inhibits the transcription of Cry1 in the pars tuberalis region of the anterior pituitary [68]. Egr1 is involved in the induction of Per1 by GnRH in pituitary gonadotrope cells [69]. In the present study, the *PER2* gene was also found to have EGR1 binding sequences in its promoter region, and EGR1 positively regulated *PER2* transcription *via* the ERK/Elk1 signaling pathway.

IEGs including Egr1 are induced by photic stimuli in the SCN of rats and mice [70–73] suggesting a role for Egr1 in phase shifting in response to light stimuli. However, *Egr1*$^{-/-}$ mice entrain normally to a 12-h light–dark cycle and have been shown to re-entrain locomotor activity to phase advances and phase delays in the light cycle [74]. Although phase shifting of locomotor activity rhythm was not affected, other physiological variables can be altered in *Egr1*$^{-/-}$ mice. As discussed earlier, Egr1 has been reported to be involved in the transcription of *Cry1* [68], *Per1* [69], and, as demonstrated in our study, *Per2*. Egr1 is involved in the regulation of melatonin-sensitive genes, including *Cry1* and melatonin receptor 1 (*mt1*) [68]. An investigation of the circadian parameters in *Egr1*$^{-/-}$ mice is necessary to extend the current findings to various other circadian functions.

ERK1/2 is a major upstream kinase that induces EGR1 [53,65]. Lithium is known to increase ERK1/2 phosphorylation [37,47,75], and the present findings were consistent with this. The *PER2* gene was shown to co-localize with p-ERK immunoreactivity in SCN cells in response to gastrin-releasing peptide (GRP) [76], and Per2 induction also co-occurred with expression of c-Fos, another IEG, in response to various stimuli, including photic stimuli and GRP [76–78]. ERK1/2 mediates the expression of IEGs, including Egr1, c-Fos, and JunB, in the SCN in response to photic stimuli as the first step leading to the transcription of various genes [79]. These data suggest a possible relationship between the ERK pathway and the rapid inducibility of PER2. ERK is involved in the regulation of circadian genes as well as circadian behaviors. The activity of ERK itself oscillates in the SCN as well as forebrain regions, including the hippocampus [80,81]. The expressions of Per1 and Cry1 are also related to Erk1/2 activity [82–84]. The present study demonstrated that the ERK/ELK1 signal pathway upregulates PER2 expression by inducing IEG EGR1 in differentiated SH-SY5Y cells and *in vivo* in the brain. Lithium, as well as light, alters circadian genes *via* ERK pathway-dependent EGR1 induction.

Findings regarding the transcriptional regulation of Per2 *via* the Erk/Elk1/Egr1 pathway were also confirmed *in vivo*. The *Per2* gene is widely expressed in extra-hypothalamic areas of the brain [7,8,11]. Per2 is abundantly expressed in the frontal cortex and shows circadian changes in gene expression [7,11]. Furthermore, in the frontal cortex, Per2 has been localized in neurons [7]. Per2 plays important roles in the initiation and resetting of the circadian clock system in the SCN of the hypothalamus and other brain regions, including the frontal cortex [7,85]. There has been a recent increase in reports of novel functions of peripheral Per2 in behavioral regulation. For example, Per2 in forebrain regions has been shown to be involved in behavioral responses such as stress responses and fear memory [57,86,87]. The *Per2* gene in cortical regions of the brain has been shown to be involved in the manifestation of locomotor activity rhythm in rats [6]. Furthermore, antidepressants, including fluoxetine and agomelatine [88,89], and addictive agents such as morphine, alcohol, and cocaine [90–92] affect Per2 protein levels in forebrain regions. Per2 is also known to affect cell proliferation, acting as a tumor suppressor gene [3,93] and affecting neurogenesis in

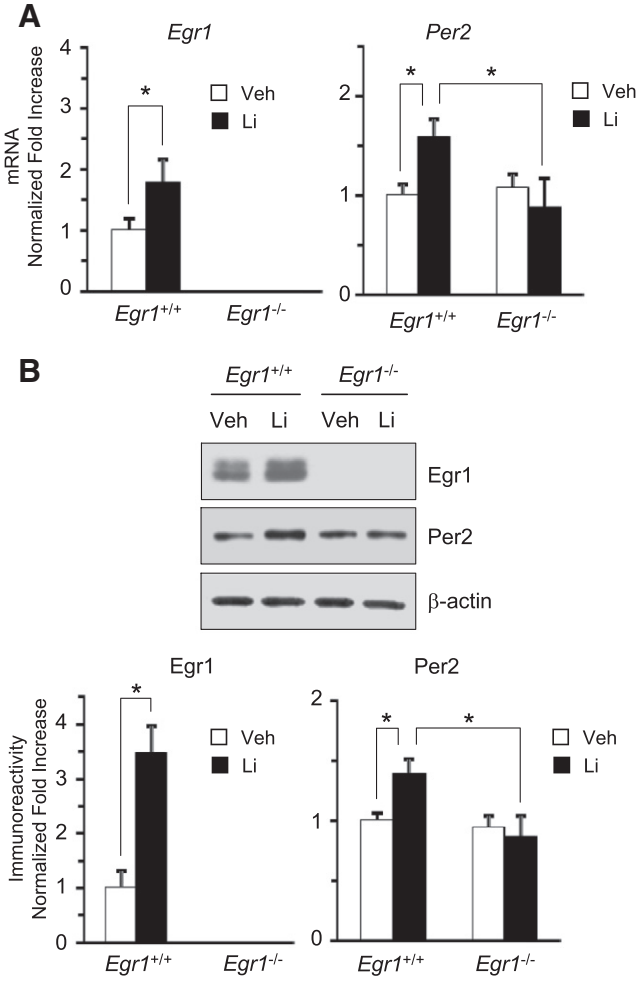


Fig. 7. Effect of lithium on mRNA and protein expression of Egr1 and Per2 in the frontal cortex of *Egr1*$^{-/-}$ mice. (A and B) Lithium (150 mg/kg) was injected intraperitoneally to control wild-type (*Egr1*$^{+/+}$) or *Egr1* KO mice (*Egr1*$^{-/-}$). At 2 h after lithium injection, mRNA (A) and protein (B) levels of Egr1 and Per2 in the frontal cortex were measured with real-time PCR (A) and immunoblot analysis (B). mRNA levels are presented as relative values to each control level. The immunoreactivity value was quantified and presented as relative optical density (OD) to that of the control. Values are presented as means ± SD. *, $P < 0.05$ ($n = 4$–6).

the hippocampus [94]. However, its function in the brain outside of the SCN, including the frontal cortex, requires further investigation. The frontal cortex is a core region related to pathogenic and therapeutic mechanisms of mood disorders [95,96]. Behavioral and cellular implications of lithium-induced PER2 in neuronal cells and in frontal cortex need to be investigated in future studies.

In summary, lithium induces *PER2* gene expression in neuronally differentiated SH-SY5Y cells and *in vivo* in the mouse brain through EGR1, activated *via* the ERK signaling pathway. PER2 is not only a core component of a circadian clock system but is also involved in the regulation of behavioral manifestations, such as mood, anxiety, locomotor activity, and cell proliferation. The present findings demonstrate the mechanisms of lithium's effects on PER2 *via* EGR1, indicating a connection between the MAPK-EGR1 pathway and the circadian gene system in the mechanism of action of lithium.

Acknowledgement

This study was supported by a grant (A101915) from the Korea Healthcare Technology R&D Project, Ministry for Health & Welfare, Republic of Korea. This paper was supported by the KU Research Professor Program of Konkuk University.

References

[1] S.M. Reppert, D.R. Weaver, Coordination of circadian timing in mammals, Nature 418 (2002) 935–941.
[2] A. Balsalobre, Clock genes in mammalian peripheral tissues, Cell Tissue Res. 309 (2002) 193–199.
[3] S. Gery, H.P. Koeffler, Circadian rhythms and cancer, Cell Cycle 9 (2010) 1097–1103.
[4] W. Huang, K.M. Ramsey, B. Marcheva, J. Bass, Circadian rhythms, sleep, and metabolism, J. Clin. Invest. 121 (2011) 2133–2141.
[5] A.M. Rosenwasser, Circadian clock genes: non-circadian roles in sleep, addiction, and psychiatric disorders? Neurosci. Biobehav. Rev. 34 (2010) 1249–1255.
[6] S. Masubuchi, S. Honma, H. Abe, K. Ishizaki, M. Namihira, M. Ikeda, K. Honma, Clock genes outside the suprachiasmatic nucleus involved in manifestation of locomotor activity rhythm in rats, Eur. J. Neurosci. 12 (2000) 4206–4214.
[7] M.F. Rath, K. Rohde, J. Fahrenkrug, M. Moller, Circadian clock components in the rat neocortex: daily dynamics, localization and regulation, Brain Struct. Funct. 218 (2013) 551–562.
[8] K.R. Shieh, Distribution of the rhythm-related genes rPERIOD1, rPERIOD2, and rCLOCK, in the rat brain, Neuroscience 118 (2003) 831–843.
[9] E.W. Lamont, D. Legault-Coutu, N. Cermakian, D.B. Boivin, The role of circadian clock genes in mental disorders, Dialogues Clin. Neurosci. 9 (2007) 333–342.
[10] C.A. McClung, Circadian genes, rhythms and the biology of mood disorders, Pharmacol. Ther. 114 (2007) 222–232.
[11] D. Matsui, S. Takekida, H. Okamura, Molecular oscillation of Per1 and Per2 genes in the rodent brain: an in situ hybridization and molecular biological study, Kobe J. Med. Sci. 51 (2005) 85–93.
[12] E.E. Zhang, S.A. Kay, Clocks not winding down: unravelling circadian networks, Nat. Rev. Mol. Cell Biol. 11 (2010) 764–776.
[13] M.H. Hastings, A.B. Reddy, E.S. Maywood, A clockwork web: circadian timing in brain and periphery, in health and disease, Nat. Rev. Neurosci. 4 (2003) 649–661.
[14] J.C. Dunlap, Molecular bases for circadian clocks, Cell 96 (1999) 271–290.
[15] N. Gekakis, D. Staknis, H.B. Nguyen, F.C. Davis, L.D. Wilsbacher, D.P. King, J.S. Takahashi, C.J. Weitz, Role of the CLOCK protein in the mammalian circadian mechanism, Science 280 (1998) 1564–1569.
[16] X. Jin, L.P. Shearman, D.R. Weaver, M.J. Zylka, G.J. de Vries, S.M. Reppert, A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock, Cell 96 (1999) 57–68.
[17] K. Kume, M.J. Zylka, S. Sriram, L.P. Shearman, D.R. Weaver, X. Jin, E.S. Maywood, M.H. Hastings, S.M. Reppert, mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop, Cell 98 (1999) 193–205.
[18] M. Gallego, D.M. Virshup, Post-translational modifications regulate the ticking of the circadian clock, Nat. Rev. Mol. Cell Biol. 8 (2007) 139–148.
[19] D.F. Kripke, D.J. Mullaney, M. Atkinson, S. Wolf, Circadian rhythm disorders in manic-depressives, Biol. Psychiatry 13 (1978) 335–351.
[20] H.A. Mansour, J. Wood, K.V. Chowdari, M. Dayal, M.E. Thase, D.J. Kupfer, T.H. Monk, B. Devlin, V.L. Nimgaonkar, Circadian phase variation in bipolar I disorder, Chronobiol. Int. 22 (2005) 571–584.
[21] D.F. Kripke, C.M. Nievergelt, E. Joo, T. Shekhtman, J.R. Kelsoe, Circadian polymorphisms associated with affective disorders, J. Circadian Rhythms 7 (2009) 2.
[22] H.A. Mansour, M.E. Talkowski, J. Wood, K.V. Chowdari, L. McClain, K. Prasad, D. Montrose, A. Fagiolini, E.S. Friedman, M.H. Allen, C.L. Bowden, J. Calabrese, R.S. El-Mallakh, M. Escamilla, S.V. Faraone, M.D. Fossey, L. Gyulai, J.M. Loftis, P. Hauser, T.A. Ketter, L.B. Marangell, D.J. Miklowitz, A.A. Nierenberg, J. Patel, G.S. Sachs, P. Sklar, J.W. Smoller, N. Laird, M. Keshavan, M.E. Thase, D. Axelson, B. Birmaher, D. Lewis, T. Monk, E. Frank, D.J. Kupfer, B. Devlin, V.L. Nimgaonkar, Association study of 21 circadian genes with bipolar I disorder, schizoaffective disorder, and schizophrenia, Bipolar Disord. 11 (2009) 701–710.
[23] H.A. Mansour, J. Wood, T. Logue, K.V. Chowdari, M. Dayal, D.J. Kupfer, T.H. Monk, B. Devlin, V.L. Nimgaonkar, Association study of eight circadian genes with bipolar I disorder, schizoaffective disorder and schizophrenia, Genes Brain Behav. 5 (2006) 150–157.
[24] C.M. Nievergelt, D.F. Kripke, T.B. Barrett, E. Burg, R.A. Remick, A.D. Sadovnick, S.L. McElroy, P.E. Keck Jr., N.J. Schork, J.R. Kelsoe, Suggestive evidence for association of the circadian genes PERIOD3 and ARNTL with bipolar disorder, Am. J. Med. Genet. B Neuropsychiatr. Genet. 141B (2006) 234–241.
[25] A. Podawiltz, A review of current bipolar disorder treatment guidelines, J. Clin. Psychiatry 73 (2012) e12.
[26] L.N. Yatham, S.H. Kennedy, S.V. Parikh, A. Schaffer, S. Beaulieu, M. Alda, C. O'Donovan, G. Macqueen, R.S. McIntyre, V. Sharma, A. Ravindran, L.T. Young, R. Milev, D.J. Bond, B.N. Frey, B.I. Goldstein, B. Lafer, B. Birmaher, K. Ha, W.A. Nolen, M. Berk, Canadian Network for Mood and Anxiety Treatments (CANMAT) and International Society for Bipolar Disorders (ISBD) collaborative update of CANMAT guidelines for the management of patients with bipolar disorder: update 2013, Bipolar Disord. 15 (2013) 1–44.
[27] T.G. Schulze, M. Alda, M. Adli, N. Akula, R. Ardau, E.T. Bui, C. Chillotti, S. Cichon, P. Czerski, M. Del Zompo, S.D. Detera-Wadleigh, P. Grof, O. Gruber, R. Hashimoto, J. Hauser, R. Hoban, N. Iwata, L. Kassem, T. Kato, S. Kittel-Schneider, S. Kliwicki, J.R. Kelsoe, I. Kusumi, G. Laje, S.G. Leckband, M. Manchia, G. Macqueen, T. Masui, N. Ozaki, R.H. Perlis, A. Pfennig, P. Piccardi, S. Richardson, G. Rouleau, A. Reif, J.K. Rybakowski, J. Sasse, J. Schumacher, G. Severino, J.W. Smoller, A. Squassina, G. Turecki, L.T. Young, T. Yoshikawa, M. Bauer, F.J. McMahon, The International Consortium on Lithium Genetics (ConLiGen): an initiative by the NIMH and IGSLI to study the genetic basis of response to lithium treatment, Neuropsychobiology 62 (2010) 72–78.
[28] A. Johnsson, W. Engelmann, B. Pflug, W. Klemke, Period lengthening of human circadian rhythms by lithium carbonate, a prophylactic for depressive disorders, Int. J. Chronobiol. 8 (1983) 129–147.
[29] J. Li, W.Q. Lu, S. Beesley, A.S. Loudon, Q.J. Meng, Lithium impacts on the amplitude and period of the molecular circadian clockwork, PLoS One 7 (2012) e33292.
[30] C. Iitaka, K. Miyazaki, T. Akaike, N. Ishida, A role for glycogen synthase kinase-3beta in the mammalian circadian clock, J. Biol. Chem. 280 (2005) 29397–29402.
[31] T.M. Osland, J. Ferno, B. Havik, I. Heuch, P. Ruoff, O.D. Laerum, V.M. Steen, Lithium differentially affects clock gene expression in serum-shocked NIH-3T3 cells, J. Psychopharmacol. 25 (2011) 924–933.
[32] A.S. Johansson, J. Brask, B. Owe-Larsson, J. Hetta, G.B. Lundkvist, Valproic acid phase shifts the rhythmic expression of Period2::Luciferase, J. Biol. Rhythms 26 (2011) 541–551.
[33] A. McQuillin, M. Rizig, H.M. Gurling, A microarray gene expression study of the molecular pharmacology of lithium carbonate on mouse brain mRNA to understand the neurobiology of mood stabilization and treatment of bipolar affective disorder, Pharmacogenet. Genomics 17 (2007) 605–617.
[34] H.J. Lee, F. Khoshaghideh, S. Patel, S.J. Lee, Clearance of alpha-synuclein oligomeric intermediates *via* the lysosomal degradation pathway, J. Neurosci. 24 (2004) 1888–1896.
[35] S.L. Lee, Y. Wang, J. Milbrandt, Unimpaired macrophage differentiation and activation in mice lacking the zinc finger transplantation factor NGFI-A (EGR1), Mol. Cell. Biol. 16 (1996) 4566–4572.
[36] S.Y. Shin, H. Song, C.G. Kim, Y.K. Choi, K.S. Lee, S.J. Lee, H.J. Lee, Y. Lim, Y.H. Lee, Egr-1 is necessary for fibroblast growth factor-2-induced transcriptional activation of the glial cell line-derived neurotrophic factor in murine astrocytes, J. Biol. Chem. 284 (2009) 30583–30593.
[37] R. Longoni, L. Spina, S. Vinci, E. Acquas, The MEK inhibitor SL327 blocks acquisition but not expression of lithium-induced conditioned place aversion: a behavioral and immunohistochemical study, Psychopharmacology (Berl) 216 (2011) 63–73.
[38] F. Armani, M.L. Andersen, R. Andreatini, R. Frussa-Filho, S. Tufik, J.C. Galduroz, Successful combined therapy with tamoxifen and lithium in a paradoxical sleep deprivation-induced mania model, CNS Neurosci. Ther. 18 (2012) 119–125.
[39] T.D. Gould, K.C. O'Donnell, A.M. Picchini, H.K. Manji, Strain differences in lithium attenuation of D-amphetamine-induced hyperlocomotion: a mouse model for the genetics of clinical response to lithium, Neuropsychopharmacology 32 (2007) 1321–1333.
[40] T.J. Gould, R.A. Keith, R.V. Bhat, Differential sensitivity to lithium's reversal of amphetamine-induced open-field activity in two inbred strains of mice, Behav. Brain Res. 118 (2001) 95–105.
[41] P. Sabioni, I.P. Baretta, E.M. Ninomiya, L. Gustafson, A.L. Rodrigues, R. Andreatini, The antimanic-like effect of tamoxifen: Behavioural comparison with other PKC-inhibiting and antiestrogenic drugs, Prog. Neuropsychopharmacol. Biol. Psychiatry 32 (2008) 1927–1931.
[42] U. Berggren, L. Tallstedt, S. Ahlenius, J. Engel, The effect of lithium on amphetamine-induced locomotor stimulation, Psychopharmacology (Berl) 59 (1978) 41–45.
[43] J.M. Morrison Jr., H.D. Pritchard, M.C. Braude, W. D'Aguanno, Plasma and brain lithium levels after lithium carbonate and lithium chloride administration by different routes in rats, Proc. Soc. Exp. Biol. Med. 137 (1971) 889–892.
[44] W.K. Aicher, K.M. Sakamoto, A. Hack, H. Eibel, Analysis of functional elements in the human Egr-1 gene promoter, Rheumatol. Int. 18 (1999) 207–214.
[45] M.W. Russo, C. Matheny, J. Milbrandt, Transcriptional activity of the zinc finger protein NGFI-A is influenced by its interaction with a cellular factor, Mol. Cell. Biol. 13 (1993) 6858–6865.
[46] S.H. Kim, M.S. Seo, W.J. Jeon, H.S. Yu, H.G. Park, G.A. Jung, H.Y. Lee, U.G. Kang, Y.S. Kim, Haloperidol regulates the phosphorylation level of the MEK-ERK-p90RSK

signal pathway *via* protein phosphatase 2A in the rat frontal cortex, Int. J. Neuropsychopharmacol. 11 (2008) 509–517.
[47] L. Mai, R.S. Jope, X. Li, BDNF-mediated signal transduction is modulated by GSK3beta and mood stabilizing agents, J. Neurochem. 82 (2002) 75–83.
[48] S.Y. Shin, J.H. Kim, J.H. Lee, Y. Lim, Y.H. Lee, 2′-Hydroxyflavanone induces apoptosis through Egr-1 involving expression of Bax, p21, and NAG-1 in colon cancer cells, Mol. Nutr. Food Res. 56 (2012) 761–774.
[49] B.H. Choi, C.G. Kim, Y.S. Bae, Y. Lim, Y.H. Lee, S.Y. Shin, p21 Waf1/Cip1 expression by curcumin in U-87MG human glioma cells: role of early growth response-1 expression, Cancer Res. 68 (2008) 1369–1377.
[50] R.S. Williams, A.J. Harwood, Lithium therapy and signal transduction, Trends Pharmacol. Sci. 21 (2000) 61–64.
[51] C.G. Kim, B.H. Choi, S.W. Son, S.J. Yi, S.Y. Shin, Y.H. Lee, Tamoxifen-induced activation of p21Waf1/Cip1 gene transcription is mediated by Early Growth Response-1 protein through the JNK and p38 MAP kinase/Elk-1 cascades in MDA-MB-361 breast carcinoma cells, Cell. Signal. 19 (2007) 1290–1300.
[52] C.P. Lim, N. Jain, X. Cao, Stress-induced immediate-early gene, egr-1, involves activation of p38/JNK1, Oncogene 16 (1998) 2915–2926.
[53] D.M. Cohen, Urea-inducible Egr-1 transcription in renal inner medullary collecting duct (mIMCD3) cells is mediated by extracellular signal-regulated kinase activation, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 11242–11247.
[54] S.H. Yang, A.D. Sharrocks, Convergence of the SUMO and MAPK pathways on the ETS-domain transcription factor Elk-1, Biochem. Soc. Symp. (2006) 121–129.
[55] Y. Ling, J.H. Lakey, C.E. Roberts, A.D. Sharrocks, Molecular characterization of the B-box protein–protein interaction motif of the ETS-domain transcription factor Elk-1, EMBO J. 16 (1997) 2431–2440.
[56] D.K. Watson, L. Robinson, D.R. Hodge, I. Kola, T.S. Papas, A. Seth, FLI1 and EWS-FLI1 function as ternary complex factors and ELK1 and SAP1a function as ternary and quaternary complex factors on the Egr1 promoter serum response elements, Oncogene 14 (1997) 213–221.
[57] C. Kinoshita, K. Miyazaki, N. Ishida, Chronic stress affects PERIOD2 expression through glycogen synthase kinase-3beta phosphorylation in the central clock, Neuroreport 23 (2012) 98–102.
[58] M. Reick, J.A. Garcia, C. Dudley, S.L. McKnight, NPAS2: an analog of clock operative in the mammalian forebrain, Science 293 (2001) 506–509.
[59] S. Koyanagi, A.M. Hamdan, M. Horiguchi, N. Kusunose, A. Okamoto, N. Matsunaga, S. Ohdo, cAMP-response element (CRE)-mediated transcription by activating transcription factor-4 (ATF4) is essential for circadian expression of the Period2 gene, J. Biol. Chem. 286 (2011) 32416–32423.
[60] F. Shimizu, Y. Fukada, Circadian phosphorylation of ATF-2, a potential activator of Period2 gene transcription in the chick pineal gland, J. Neurochem. 103 (2007) 1834–1842.
[61] T. Ohno, Y. Onishi, N. Ishida, The negative transcription factor E4BP4 is associated with circadian clock protein PERIOD2, Biochem. Biophys. Res. Commun. 354 (2007) 1010–1015.
[62] A. Wirz-Justice, Biological rhythm disturbances in mood disorders, Int. Clin. Psychopharmacol. 21 (Suppl. 1) (2006) S11–S15.
[63] A.J. Lewy, Depressive disorders may more commonly be related to circadian phase delays rather than advances: time will tell, Sleep Med. 11 (2010) 117–118.
[64] H. Klemfuss, Rhythms and the pharmacology of lithium, Pharmacol. Ther. 56 (1992) 53–78.
[65] E. Knapska, L. Kaczmarek, A gene for neuronal plasticity in the mammalian brain: Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK? Prog. Neurobiol. 74 (2004) 183–211.
[66] A.B. James, A.M. Conway, B.J. Morris, Regulation of the neuronal proteasome by Zif268 (Egr1), J. Neurosci. 26 (2006) 1624–1634.
[67] K. Baumgartel, D. Genoux, H. Welzl, R.Y. Tweedie-Cullen, K. Koshibu, M. Livingstone-Zatchej, C. Mamie, I.M. Mansuy, Control of the establishment of aversive memory by calcineurin and Zif268, Nat. Neurosci. 11 (2008) 572–578.
[68] J.M. Fustin, H. Dardente, G.C. Wagner, D.A. Carter, J.D. Johnston, G.A. Lincoln, D.G. Hazlerigg, Egr1 involvement in evening gene regulation by melatonin, FASEB J. 23 (2009) 764–773.
[69] H.E. Resuehr, D. Resuehr, J. Olcese, Induction of mPer1 expression by GnRH in pituitary gonadotrope cells involves EGR-1, Mol. Cell. Endocrinol. 311 (2009) 120–125.
[70] N. Aronin, S.M. Sagar, F.R. Sharp, W.J. Schwartz, Light regulates expression of a Fos-related protein in rat suprachiasmatic nuclei, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 5959–5962.
[71] J.M. Kornhauser, D.E. Nelson, K.E. Mayo, J.S. Takahashi, Photic and circadian regulation of c-fos gene expression in the hamster suprachiasmatic nucleus, Neuron 5 (1990) 127–134.
[72] J.M. Kornhauser, D.E. Nelson, K.E. Mayo, J.S. Takahashi, Regulation of jun-B messenger RNA and AP-1 activity by light and a circadian clock, Science 255 (1992) 1581–1584.
[73] B. Rusak, H.A. Robertson, W. Wisden, S.P. Hunt, Light pulses that shift rhythms induce gene expression in the suprachiasmatic nucleus, Science 248 (1990) 1237–1240.
[74] T.S. Kilduff, C. Vugrinic, S.L. Lee, J.D. Milbrandt, J.D. Mikkelsen, B.F. O'Hara, H.C. Heller, Characterization of the circadian system of NGFI-A and NGFI-A/NGFI-B deficient mice, J. Biol. Rhythms 13 (1998) 347–357.
[75] H. Einat, P. Yuan, T.D. Gould, J. Li, J. Du, L. Zhang, H.K. Manji, G. Chen, The role of the extracellular signal-regulated kinase signaling pathway in mood modulation, J. Neurosci. 23 (2003) 7311–7316.
[76] M.C. Antle, L.J. Kriegsfeld, R. Silver, Signaling within the master clock of the brain: localized activation of mitogen-activated protein kinase by gastrin-releasing peptide, J. Neurosci. 25 (2005) 2447–2454.
[77] K. Mateju, Z. Bendova, R. El-Hennamy, M. Sladek, S. Sosniyenko, A. Sumova, Development of the light sensitivity of the clock genes Period1 and Period2, and immediate-early gene c-fos within the rat suprachiasmatic nucleus, Eur. J. Neurosci. 29 (2009) 490–501.
[78] G. Antoun, P.B. Cannon, H.Y. Cheng, Regulation of MAPK/ERK signaling and photic entrainment of the suprachiasmatic nucleus circadian clock by Raf kinase inhibitor protein, J. Neurosci. 32 (2012) 4867–4877.
[79] H. Dziema, B. Oatis, G.Q. Butcher, R. Yates, K.R. Hoyt, K. Obrietan, The ERK/MAP kinase pathway couples light to immediate-early gene expression in the suprachiasmatic nucleus, Eur. J. Neurosci. 17 (2003) 1617–1627.
[80] K.L. Eckel-Mahan, T. Phan, S. Han, H. Wang, G.C. Chan, Z.S. Scheiner, D.R. Storm, Circadian oscillation of hippocampal MAPK activity and cAmp: implications for memory persistence, Nat. Neurosci. 11 (2008) 1074–1082.
[81] T.X. Phan, G.C. Chan, C.B. Sindreu, K.L. Eckel-Mahan, D.R. Storm, The diurnal oscillation of MAP (mitogen-activated protein) kinase and adenylyl cyclase activities in the hippocampus depends on the suprachiasmatic nucleus, J. Neurosci. 31 (2011) 10640–10647.
[82] K. Nomura, Y. Takeuchi, K. Fukunaga, MAP kinase additively activates the mouse Per1 gene promoter with CaM kinase II, Brain Res. 1118 (2006) 25–33.
[83] N. Miyamura, J. Hirayama, K. Sawanobori, T. Tamaru, Y. Asaoka, R. Honda, T. Yamamoto, H. Uno, K. Takamatsu, H. Nishina, CLOCK:BMAL-independent circadian oscillation of zebrafish Cryptochrome1a gene, Biol. Pharm. Bull. 32 (2009) 1183–1187.
[84] T. Sugimoto, N. Morioka, K. Sato, K. Hisaoka, Y. Nakata, Noradrenergic regulation of period1 expression in spinal astrocytes is involved in protein kinase A, c-Jun N-terminal kinase and extracellular signal-regulated kinase activation mediated by alpha1- and beta2-adrenoceptors, Neuroscience 185 (2011) 1–13.
[85] K. Bae, X. Jin, E.S. Maywood, M.H. Hastings, S.M. Reppert, D.R. Weaver, Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock, Neuron 30 (2001) 525–536.
[86] H. Pantazopoulos, H. Dolatshad, F.C. Davis, A fear-inducing odor alters PER2 and c-Fos expression in brain regions involved in fear memory, PLoS One 6 (2011) e20658.
[87] L.M. Wang, J.M. Dragich, T. Kudo, I.H. Odom, D.K. Welsh, T.J. O'Dell, C.S. Colwell, Expression of the circadian clock gene Period2 in the hippocampus: possible implications for synaptic plasticity and learned behaviour, ASN Neuro 1 (2009).
[88] O. Koresh, N. Kozlovsky, Z. Kaplan, J. Zohar, M.A. Matar, H. Cohen, The long-term abnormalities in circadian expression of Period 1 and Period 2 genes in response to stress is normalized by agomelatine administered immediately after exposure, Eur. Neuropsychopharmacol. 22 (2012) 205–221.
[89] T. Uz, R. Ahmed, M. Akhisaroglu, M. Kurtuncu, M. Imbesi, A. Dirim Arslan, H. Manev, Effect of fluoxetine and cocaine on the expression of clock genes in the mouse hippocampus and striatum, Neuroscience 134 (2005) 1309–1316.
[90] S. Hood, P. Cassidy, S. Mathewson, J. Stewart, S. Amir, Daily morphine injection and withdrawal disrupt 24-h wheel running and PERIOD2 expression patterns in the rat limbic forebrain, Neuroscience 186 (2011) 65–75.
[91] R. Spanagel, G. Pendyala, C. Abarca, T. Zghoul, C. Sanchis-Segura, M.C. Magnone, J. Lascorz, M. Depner, D. Holzberg, M. Soyka, S. Schreiber, F. Matsuda, M. Lathrop, G. Schumann, U. Albrecht, The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption, Nat. Med. 11 (2005) 35–42.
[92] C. Abarca, U. Albrecht, R. Spanagel, Cocaine sensitization and reward are under the influence of circadian genes and rhythm, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 9026–9030.
[93] L. Fu, H. Pelicano, J. Liu, P. Huang, C. Lee, The circadian gene Period2 plays an important role in tumor suppression and DNA damage response *in vivo*, Cell 111 (2002) 41–50.
[94] L. Borgs, P. Beukelaers, R. Vandenbosch, L. Nguyen, G. Moonen, P. Maquet, U. Albrecht, S. Belachew, B. Malgrange, Period 2 regulates neural stem/progenitor cell proliferation in the adult hippocampus, BMC Neurosci. 10 (2009) 30.
[95] J.L. Price, W.C. Drevets, Neural circuits underlying the pathophysiology of mood disorders, Trends Cogn. Sci. 16 (2012) 61–71.
[96] B. Myers-Schulz, M. Koenigs, Functional anatomy of ventromedial prefrontal cortex: implications for mood and anxiety disorders, Mol. Psychiatry 17 (2012) 132–141.