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ORIGINAL ARTICLE

The mood stabilizers lithium and valproate selectively activate the promoter IV of brain-derived neurotrophic factor in neurons

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Brain-derived neurotrophic factor (BDNF) has been strongly implicated in the synaptic plasticity, neuronal survival and pathophysiology of depression. Lithium and valproic acid (VPA) are two primary mood-stabilizing drugs used to treat bipolar disorder. Treatment of cultured rat cortical neurons with therapeutic concentrations of LiCl or VPA selectively increased the levels of exon IV (formerly rat exon III)-containing BDNF mRNA, and the activity of *BDNF* promoter IV. Surprisingly, lithium- or VPA-responsive element(s) in promoter IV resides in a region upstream from the calcium-responsive elements (CaREs) responsible for depolarization-induced BDNF induction. Moreover, activation of *BDNF* promoter IV by lithium or VPA occurred in cortical neurons depolarized with KCl, and deletion of these three CaREs did not abolish lithium- or VPA-induced activation. Lithium and VPA are direct inhibitors of glycogen synthase kinase-3 (GSK-3) and histone deacetylase (HDAC), respectively. We showed that lithium-induced activation of promoter IV was mimicked by pharmacological inhibition of GSK-3 or short interfering RNA (siRNA)-mediated gene silencing of GSK-3α or GSK-3β isoforms. Furthermore, treatment with other HDAC inhibitors, sodium butyrate and trichostatin A, or transfection with an HDAC1-specific siRNA also activated *BDNF* promoter IV. Our study demonstrates for the first time that GSK-3 and HDAC are respective initial targets for lithium and VPA to activate *BDNF* promoter IV, and that this BDNF induction involves a novel responsive region in promoter IV of the *BDNF* gene. Our results have strong implications for the therapeutic actions of these two mood stabilizers.
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Introduction

Brain-derived neurotrophic factor (BDNF), through activation of its receptor TrkB, has a central role in cortical development, synaptic plasticity and neural survival, and likely mediates the clinical effects of antidepressants.[1–4] BDNF is highly expressed in the cortex and hippocampus,[5] the brain areas thought to be critical for the control of mood, emotion and cognition. BDNF deficiency as well as other abnormalities have been linked to a variety of brain disorders including depression.[6,7] Treatment with various antidepressants induces the expression of BDNF and activation of its receptor TrkB in the rodent brain,[8,9] while administration of BDNF into the brain induces antidepressant-like behaviors.[10] It is known that the expression of BDNF is regulated by neuronal activity, as exemplified by the induction of BDNF following activation of voltage-sensitive Ca^{2+} channels.[11]

The genomic structure of *BDNF* in rodents is complex, containing multiple 5′-untranslated exons and one protein-coding 3′ exon.[12–14] Each of these untranslated exons is alternatively spliced to produce various BDNF mRNA species. Initially, only four untranslated exons were found to be in the rat *BDNF* gene.[12] More recent studies show that eight untranslated exons are present in the rodent (mouse and rat) *BDNF* gene [13,14] and thus these promoters have been renamed. Accordingly, former rat exon III, exon IV and exon V are now termed exon IV, VI and IX, respectively.[14] These rodent *BDNF*-splicing variants display distinct expression profiles in the brain regions and show differential regulation following treatment of rats with cocaine[13] or kainic acid.[14] Exon IV (formerly rat exon III[12])-containing transcripts of *BDNF* are robustly expressed in response to KCl-induced membrane depolarization in primary cultures of rat cortical neurons.[15] This transcriptional activation requires the promoter region 80 bp upstream from the transcription initiation site of exon IV-containing three calcium-responsive elements,

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CaRE1, CaRE2 and CaRE3/CRE, to which their respective stimulating factors, CaRF, USF1/2 and cyclicAMP responsive element binding protein (CREB), are bound.[16,17]

Lithium and valproic acid (VPA) are two mainstream drugs used for treatment and prophylaxis of bipolar mood disorder. Increasing evidence supports the hypothesis that both drugs exhibit neuroprotective and neurotrophic properties, which may increase cellular resilience and contribute to their clinical efficacy in treating bipolar disorder.[4] Both mood stabilizers also show robust neuroprotection against diverse apoptotic stimuli in cultured primary neurons, neurally related cell types and animal models of brain disorders.[18] It has also been reported that BDNF protein levels are increased in the brain of rats chronically treated with lithium or VPA,[18–20] and in cultured rat cortical neurons exposed to lithium or VPA.[21,22] In the case of lithium-induced protection against glutamate-elicited neurotoxicity in cortical neurons, induction of BDNF is an obligatory event, as BDNF knockout or its receptor blockade prevents the neuroprotective effects of lithium.[21]

Recent studies demonstrate that glycogen synthase kinase-3 (GSK-3), a serine/threonine kinase consisting of two isoforms and regulating an array of transcription factors, is a direct target of lithium,[23,24] while VPA inhibits histone deacetylase (HDAC), which has a prominent role in the regulation of gene expression.[25,26] Emerging evidence suggests that inhibition of GSK-3 and HDAC are responsible, at least in part, for the neuroprotective effects of lithium and VPA, respectively.[27,28] The present study was undertaken to investigate whether *BDNF* exon IV-containing mRNA and promoter IV activity are increased by lithium or VPA treatment in rat cortical neurons, and to determine the *BDNF* promoter region that confers the sensitivity to either drug. We also investigated whether inhibiting GSK-3 or HDAC mimics the ability of mood stabilizers to induce *BDNF* transcription in cortical neurons.

Materials and methods

Cortical neuronal cultures

Cortical neurons were prepared from day-18 embryonic (E18) rats as described previously[28] with slight modifications. Briefly, cortical cells were cultured in Neurobasal medium supplemented with B27 (Invitrogen; Carlsbad, CA, USA) in the presence of 0.5 mM L-glutamine. The cells were seeded at a density of 3.0×10^5 cells per well in 48-well plates precoated with poly-D-lysine (Sigma; St Louis, MO, USA) and incubated at 37°C in a humidified CO_2 (5%) incubator.

Total RNA extraction, first-strand cDNA synthesis and real-time PCR

One microgram of RNA extracted with Trizol Reagent (Invitrogen; Carlsbad, CA, USA) was used for reverse transcription with a First Strand Superscript II kit (Invitrogen). The cDNA was amplified by PCR with the forward primer and reverse primer specific for exon I, II, IV or VI: exon I forward, 5′-CAGGACAGC AAAGCCACAA-3′; exon I reverse, 5′-CCAGCAGAAA GAGCAGAGGA-3′; exon II forward, 5′-GGAGAGCC AGCCCCAGTT-3′; exon II reverse, 5′-CAGCAGAAAG AGCAGAGGAG-3′; exon IV forward, 5′-GCTGCC TTGATGTTTACTTTG-3′; exon IV reverse, 5′-ATGGG ATTACACTTGGTCTCGT-3′; exon VI forward, 5′-TTGGGGCAGACGAGAAAG-3′; exon VI reverse, 5′-CAGCAGAAAGAGCAGAGGAG-3′. The amounts of the cDNAs were measured using a Light Cycler (Roche Applied Science; Indianapolis, IN, USA) with DNA Master SYBER Green I. The real-time PCR was performed at 95°C for 1 s, 56°C for 5 s and 72°C for 10 s, with a maximum of 40 cycles. The mRNA levels of BDNF were normalized to those of cyclophilin, which were measured in parallel using its specific primers: cyclophilin forward, 5′-AGATCGAAGTGG AGAAACCCTTTG-3′; cyclophilin reverse, 5′-TAAA AATCAGGCCTGTGGAATGTG-3′.

Enzyme-linked immunosorbent assay

Cells were collected in lysis buffer (137 mM NaCl, 20 mM Tris HCl (pH 8.0), 1% NP-40, 10% glycerol, 1 mM PMSF, 10 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin and 0.5 mM sodium vanadate). Protein levels of BDNF were determined with a BDNF Emax ImmunoAssay System (Promega; Madison, WI, USA) according to the manufacturer's instructions.

Construction of BDNF promoter IV-exon IV plasmids

Rat genomic DNA was used as the template for PCR to amplify the fragment of *BDNF* promoter IV-exon IV, by using the forward primer linked with *Xho*I sequence and the reverse primer linked with *Hin*dIII sequence. Various lengths of the PCR product containing sequences of the promoter IV and exon IV from +341 bp (downstream from the transcription initiation site) to −80, −170, −710 or −2000 bp (upstream from the initiation site) were produced, cut with both *Xho*I and *Hin*dIII restriction enzymes and then purified by agarose electrophoresis. The respective fragments were ligated into *Xho*I and *Hin*dIII sites of pGL3-Basic luciferase reporter vector (Promega). To generate the pGL3-80 luc, the forward primer used was 3-80*Xho* (5′-CTCGAGATTCGTGCACTAGAGTGT CT-3′) and the reverse primer was *Rev3* + 341*Hind* (5′-AAGCTTCCTTCAGTCACTACTTGTCAA-3′). For pGL3-170 luc, the forward primer was 3-170*Xho* (5′-CTCGAGAAAGCATGCAATGCCCTGGAA-3′) and the reverse primer was *Rev3* + 341*Hind*. For pGL3-710 luc, the forward primer was 3-710*Xho* (5′-CTCGA GCCTCAGCCATACTAAAGGCTAG-3′) and the reverse primer was *Rev3* + 341*Hind*. For pGL3-2000 luc, the forward primer was 3-2000*Xho* (5′-CTCGAG ACTTCTTCTTTTTCTCAGTAAAAA-3′) and the reverse primer was *Rev3* + 341*Hind*. The internal deletion plasmid, pGL3-710ΔCaRE luc, was generated by ligating two fragments into pGL3-710 luc after cutting with *Sac*I, *Xho*I and *Hin*dIII. One fragment

was generated by PCR with a forward primer, *SacI*-710 (5′-GAGCTCGCCTCAGCCATACTAAAG-3′) and a reverse primer, 74-*XhoI* (5′-CTCGAGACACTCTAG TGCACGAAT-3′). The other was generated with a forward primer, *XhoI*-31(5′-CTCGAGAGGCAGCGTG GAGCCCTC-3′) and a reverse primer, *Rev*3 + 341*Hind*.

Transfection of BDNF plasmid into cortical neurons
Two methods were used for the transfection of *BDNF* promoter IV-exon IV plasmid into cortical neurons. The cultures at 8 days *in vitro* (DIV) were transfected with Lipofectamine 2000 (Invitrogen), while the cultures at 0-DIV were transfected with an Amaxa Nucleofector (Amaxa; Cologne, Germany). In the former method, 100 μl of OptiMEM I per well were mixed with 0.5 μg plasmid DNA and 1 μl Lipofectamine 2000 (Invitrogen) in 48-well culture plates. After the complex of DNA and Lipofectamine 2000 was formed, the mixture was added directly to cells in each well. In the latter method, freshly prepared cells were electroporated before plating with 3 μg plasmid conjugated with Rat Neuron Nucleofector Solution (Amaxa) using the Amaxa Nucleofector. The electroporation technique produced a high transfection efficiency of approximately 50%.

Luciferase assays
Luciferase assays were carried out after transfection using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Briefly, cells grown on 48-well plates were lysed with 1 × Passive Lysis Buffer and transferred onto a 96-well opaque plate, followed by addition of 100 μl of Luciferase Assays Reagent II to each well. The firefly luciferase activities were measured by a LumiCount microplate luminometer (Packard BioScience Company; Meriden, CT, USA). Renilla luciferase pRL-TK was co-transfected to normalize for the transfection efficiency and sample handling. Relative firefly luciferase activity is expressed as a ratio of firefly to renilla value.

Inhibition of GSK-3 or HDAC, and siRNA for GSK-3 or HDAC
Two ATP-competitive GSK-3 inhibitors, SB216763 and SB415286, were purchased from Tocris (Ellisville, MO, USA), and two ATP-non-competitive inhibitors, inhibitor I and inhibitor VII from Calbiochem (La Jolla, CA, USA). HDAC inhibitors, sodium butyrate (SB) and trichostatin A (TSA) were products of Sigma. Four short interfering RNAs (siRNAs) used for gene silencing of either GSK-3α or GSK-3β were designed and prepared as described previously.[27] Rat HDAC1-targeted siRNA and non-targeting control siRNA were purchased from Dharmacon (Lafayette, CO, USA). Rat primary cortical cultures were cultured for 8-DIV and transfected for 48 h with GSK-3 or HDAC1 siRNA using 8 μl siPORT Amine transfection reagent (Ambion; Austin, TX, USA) in 1 ml of the transfection mixture.

Immunoblotting analysis
Rat cortical neurons were harvested and immunoblots were prepared as described previously.[27] Polyclonal antibodies against yeast acetylated histones 3 and 4 (AcH3 and AcH4), mouse HDAC1 and human HDAC3 were obtained from Upstate (Charlottesville, VA, USA). Polyclonal antibody against phospho-GSK-3α/β (Ser21/9) was obtained from Cell Signaling Technology (Beverly, MA, USA). Polyclonal antibody against HDAC4 (ab186) was a kind gift from Dr Tso-Pang Yao (Duke University, Durham, NC). Polyclonal antibody against HDAC5 and monoclonal antibody against β-actin were purchased from Santa Cruz (Santa Cruz, CA, USA). ECL Plus western blotting detection system (Amersham Biosciences, Little Chalfont, UK) was used to obtain western blot signals and autoradiographs were scanned. Intensity of immunoblot signals was analyzed using Scion Image software (Scion Image for Windows, NIH).

Statistical analysis
The results are presented as means ± s.e.m. from three to six independent experiments, with three replicates for each data point. The results were analyzed for statistical significance in GraphPad Prism (GraphPad, San Diego, CA, USA) by using two-sample *t*-test, or one-way analysis of variance and Bonferroni multiple comparison test.

Results

Lithium and VPA treatments increase the levels of exon IV-containing BDNF mRNA and the activity of BDNF promoter IV in rat cortical neurons
Since the rodent *BDNF* promoter contains eight exons that are alternatively spliced to join the structure-encoding exon IX (Figure 1a), we first attempted to study the effects of treatment with lithium and VPA on the levels of each exon-containing BDNF mRNA. Cortical neuronal cultures at 8-DIV were treated with 1 mM LiCl, 0.5 mM VPA or vehicle for 48 h. These drug concentrations are within the therapeutic plasma levels for treating bipolar mood disorder. Total RNA was then extracted and real-time PCR was performed using each exon-specific forward and reverse primers. Lithium or VPA treatment caused a significant increase in the level of exon IV-containing mRNA, while levels of exon I, II or VI-containing BDNF mRNA were not significantly altered by lithium or VPA treatment (Figure 1b). Treatment with lithium and VPA also enhanced the protein levels of BDNF as determined by enzyme-linked immunosorbent assay, with a significant increase at 5 and 7 days, respectively (Figure 1c).

Brain-derived neurotrophic factor promoter IV has been shown to contain three Ca^{2+}-responsive elements, CaRE1, CaRE2 and CaRE3/CRE, located between −32 and −73 bp from the transcription initiation site[16,17] (Figure 2a). We next investigated whether lithium or VPA treatment causes an increase in *BDNF* promoter IV and, if so, which region of *BDNF*

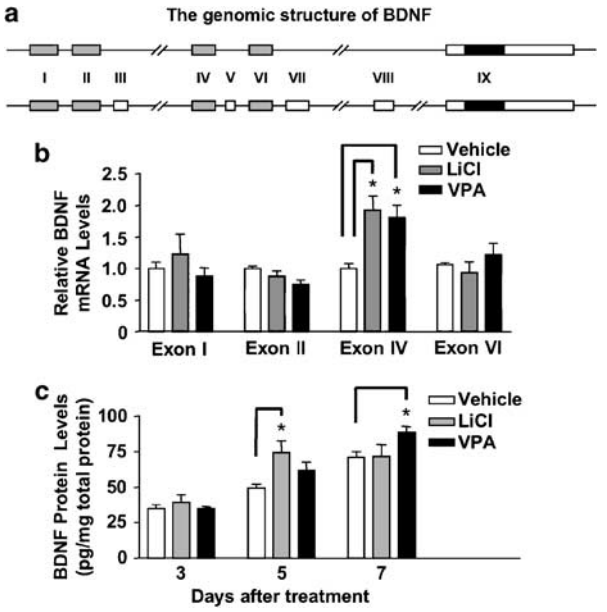

Figure 1 Treatment with lithium or valproic acid (VPA) increases the levels of brain-derived neurotrophic factor (*BDNF*) exon IV-containing transcripts and total BDNF protein in cortical neurons. (**a**) A schematic representation of the structure of the *BDNF* gene. The upper genomic structure is as described by Timmusk *et al*.[12] and the lower structure is the new arrangement and nomenclature made by Aid *et al*.[14] The *BDNF* gene consists of nine exons as shown by boxes, and each exon, I, II, IV or VI, indicated by a gray box, encodes a 5′-UTR of the transcript. The coding region of exon IX is indicated by the black box. (**b**) Effects of lithium or VPA on the levels of *BDNF* exon IV-containing mRNA. Rat cortical neuronal cultures were treated with LiCl (1.0 mM) or VPA (0.5 mM) for 48 h starting from 8 days *in vitro* (DIV). Total RNA was extracted from cells, and the first-strand cDNA synthesis was made from the total RNA. Real-time PCR was performed to amplify exons I-, II-, IV- or VI-containing transcripts of *BDNF*, using cyclophilin as an internal control. The values shown are relative to each vehicle (normal saline) control and expressed as means ± s.e.m. from three independent cultures. (**c**) Lithium and VPA enhanced BDNF protein levels in cortical neurons. LiCl (1 mM), VPA (0.5 mM) or its vehicle was added into the cultures at 8-DIV. The levels of BDNF protein were measured by enzyme-linked immunosorbent assay after treatment for 3, 5 and 7 days. The values are expressed as means ± s.e.m. of pg mg^{-1} total protein from six independent experiments. $^{*}P<0.05$ compared with respective vehicle control.

promoter IV confers the sensitivity to lithium or VPA-induced exon IV-containing BDNF mRNA upregulation. Accordingly, we constructed reporter plasmids bearing various lengths of *BDNF* exon IV 5-flanking region and then fused exon IV to the firefly luciferase reporter gene. Cortical neurons were treated with LiCl, VPA or vehicle at 7-DIV followed by lipofectamine-mediated transfection with specific *BDNF* promoter IV plasmids at 8-DIV, using Renilla luciferase reporter plasmid pRL-TK as the internal control (Figure 2b). Lithium or VPA treatment failed to elicit

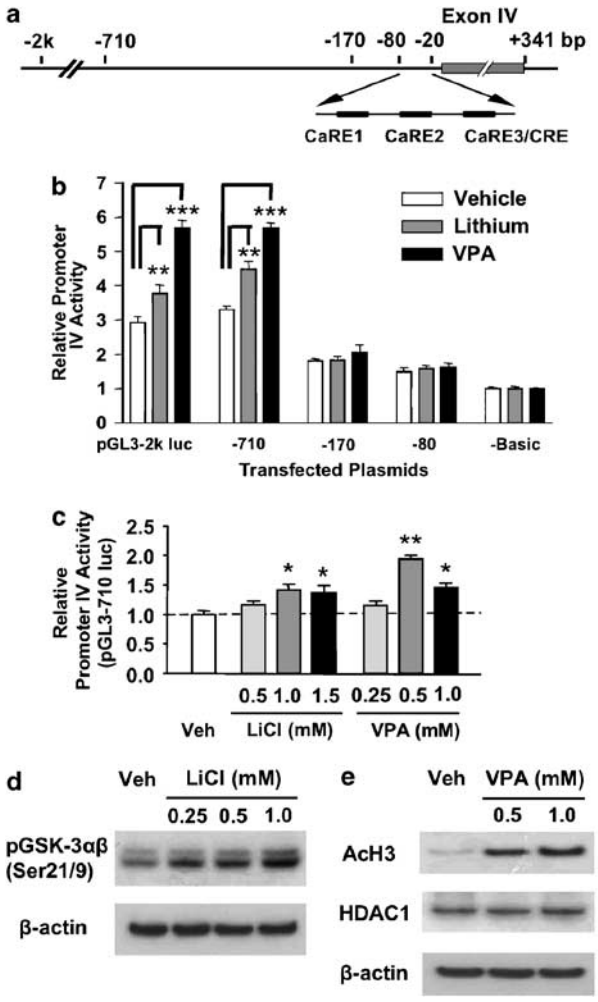

Figure 2 Lithium and valproic acid (VPA) induce activation of brain-derived neurotrophic factor promoter IV. (**a**) The scheme delineates the promoter IV region and each number indicates bp position from the transcriptional initiation site of the exon. The enlarged promoter region between −20 and −80 bp contains three calcium-responsive elements (CaREs). (**b**) Firefly luciferase reporter vectors, pGL3 basic, were constructed to certain specific lengths of the promoter, namely, −2000, −710, −170 or −80 bp. 'Basic' refers to pGL3 basic without promoter. Cortical neurons at 7 days *in vitro* (DIV) were treated with 1 mM LiCl or 0.5 mM VPA for 3 days. At 8-DIV, neurons were transfected with each reporter vector (pGL3-2000 luc, −710 luc, −170 luc, −80 luc or −Basic) and pRL-TK using Lipofectamine 2000. Cell lysates were prepared at 10-DIV and assayed for luciferase activity. The values were normalized to those of pGL3 basic. (**c**) Cortical neurons were treated with indicated concentrations of LiCl, VPA, or their vehicle (normal saline), and transfected with pGL3-710 luc. The values were normalized to those of vehicle-treated cells. Quantified data are expressed as means ± s.e.m. from four experiments. $^{*}P<0.05$; $^{**}P<0.01$, $^{***}P<0.001$ compared with respective vehicle control. (**d**) Cortical neurons at 8-DIV were treated with LiCl at indicated concentrations for 48 h. Cell lysates were then prepared for western blotting of p-GSK-3αβ (Ser21/9) and β-actin. (**e**) Cortical neurons at 8-DIV were treated with indicated concentrations of VPA for 48 h prior to western blotting analysis of acetylated histone H3 (AcH3), HDAC1 and β-actin.

any significant change in the *BDNF* promoter activity measured 48 h after transfection with pGL3-80 luc or pGL3-170 luc, which contains 80 or 170 nucleotides of exon IV 5′-flanking sequence, respectively. However, a significant increase in *BDNF* promoter activity by lithium or VPA treatment was observed when neurons were transfected with pGL3-710 luc (Figure 2b). A similar extent of increase in *BDNF* promoter activity was observed when pGL3-2000 luc was used for transfection. Thus, the lithium or VPA-responsive element(s) resides between −170 and −710 bp and excludes three CaREs. The effects of lithium and VPA on the promoter IV activity were concentration dependent with the maximal activation at 1 and 0.5 mM for LiCl and VPA, respectively (Figure 2c). These concentrations are within the therapeutic range of both drugs for treating bipolar disorder. Under these treatment conditions, lithium induced a concentration-dependent increase in the levels of phospho-GSK-3α^{Ser21}/β^{Ser9} (Figure 2d), thus indicating an inhibition of GSK-3 activity.[23,24,27] VPA treatment also markedly increased the levels of histone 3 acetylation with no apparent effects on the levels of HDAC1 isoform (Figure 2e), suggesting the inhibition of HDAC activity.

Lithium and VPA enhance BDNF promoter IV activity in neurons depolarized with KCl or transfected with a plasmid devoid of CaRE sites

It is known that KCl-induced depolarization increases the activity of *BDNF* promoter IV through transcription factor binding to three CaREs located between −32 and −73 bp on the promoter IV.[15] To obtain further evidence that lithium/VPA acts on an element(s) outside the CaRE-containing region, cortical cells were pretreated with lithium or VPA at 8-DIV followed by plasmid transfection and then depolarization with KCl for 8 h. KCl (50 mM) alone elicited an approximately 5-fold increase in *BDNF* promoter IV activity when neurons were transfected with pGL3-80 luc, pGL3-170 luc or pGL3-710 luc (Figure 3a), but not with pGL3-20 luc (data not shown). Pretreatment with LiCl or VPA markedly augmented KCl-induced promoter IV activity when neurons were transfected with pGL3-710 luc, but not pGL3-170 luc or pGL3-80 luc. We have also generated a *BDNF* promoter IV internal deletion plasmid, pGL3-710ΔCaRE luc, which was a pGL3-710 luc devoid of the three CaRE sites (Figure 3b). Cortical neurons were transfected with pGL3-710ΔCaRE luc by electroporation using the Amaxa Nucleofector before plating to achieve high transfection efficiency (approximately 50%). Lithium or VPA treatment was still able to elicit a significant increase in *BDNF* promoter IV activity in neurons transfected with pGL3-710ΔCaRE luc, while KCl-induced depolarization was ineffective (Figure 3c). These results further support that the promoter sequences that confer the responsiveness to mood stabilizers and neuronal depolarization are distinct.

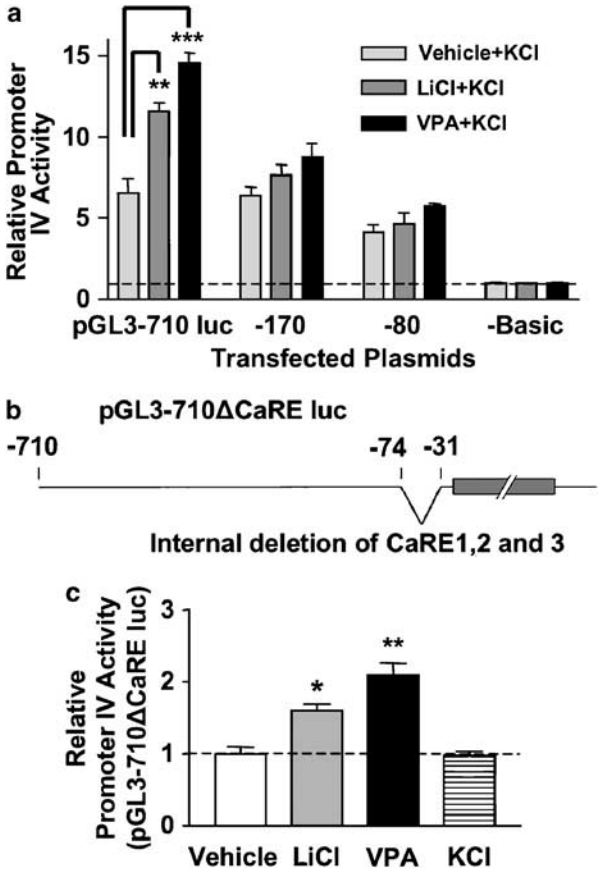

Figure 3 Lithium and valproic acid (VPA) potentiate KCl-induced brain-derived neurotrophic factor promoter IV activation and increase the promoter activity in a construct devoid of calcium-responsive elements (CaREs). (**a**) Cortical neurons at 7 days *in vitro* (DIV) were treated with 1 mM LiCl or 0.5 mM VPA for 3 days, and at 8-DIV were transfected with each reporter vector and pRL-TK. Forty hours after transfection, cells were stimulated with 50 mM KCl, and 8 h later, luciferase activity was measured. The values were normalized to those of pGL3 basic and shown as means ± s.e.m. from four experiments. $^{**}P<0.01$, $^{***}P<0.001$ compared with KCl alone. (**b** and **c**) pGL3-710ΔCaRE luc, which is devoid of the region of −74 to −31 bp, as shown in (**b**), was transfected into cortical neurons at 0-DIV by electroporation. The cultures at 8-DIV were then treated with LiCl (1 mM) and VPA (0.5 mM) for 48 h or exposed to KCl (50 mM) for 8 h. Luciferase activity from cell lysates was measured at 10-DIV (**c**). The values shown are relative to the vehicle control and expressed as means ± s.e.m. from four independent cultures. $^{*}P<0.05$, $^{**}P<0.01$ compared with vehicle control.

Pharmacological inhibition or gene silencing of GSK-3 mimics lithium's ability to upregulate BDNF promoter IV activity

Lithium is a direct and indirect inhibitor of GSK-3, which phosphorylates a variety of transcription factors and thereby regulates their activity.[29,30] We thus investigated whether other GSK-3 inhibitors are capable of upregulating *BDNF* promoter IV activity in cortical neurons transfected with pGL3-710 luc by

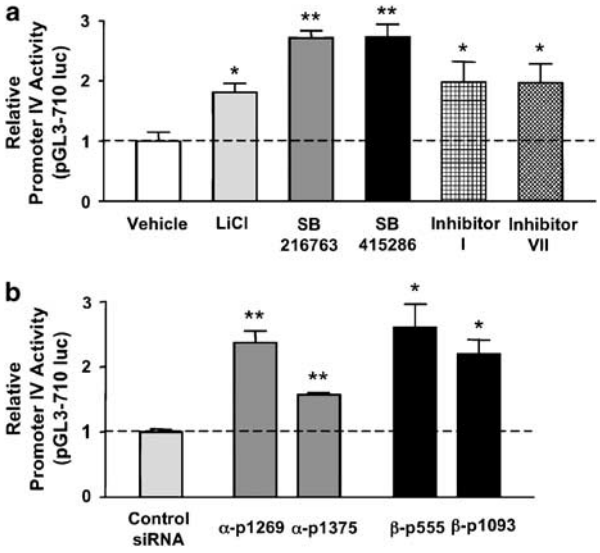

Figure 4 Treatment with glycogen synthase kinase (GSK) inhibitors or short interfering RNA (siRNA) silencing of GSK-3 isoforms increases brain-derived neurotrophic factor (*BDNF*) promoter IV activity. (**a**) Cortical cultures at 0 day *in vitro* (DIV) were transfected with pGL3-710 luc by electroporation. LiCl (1 mM), SB216763 (5 μM), SB415286 (20 μM), inhibitor I (10 μM) or inhibitor VII (2 μM) was added at 8-DIV. Cell lysates were prepared at 10-DIV for the measurement of *BDNF* promoter IV activity. (**b**) Each isoform-specific siRNA of GSK-3α (α-p1269, α-p1375) or -3β (β-p555, β-p1093) at 80 nM was transfected into cortical neurons at 8-DIV with siPORT Amine reagent. Forty-eight hours later, *BDNF* promoter IV activity in the cell lysates was measured using pGL3-710 luc. Non-targeting siRNA was used as the control siRNA. The values are relative to each control and expressed as means ± s.e.m. from four independent cultures. $^{*}P<0.05$, $^{**}P<0.01$ compared with respective control.

electroporation. Treatment with two GSK-3 ATP-competitive inhibitors, SB216763 (5 μM) and SB415286 (20 μM), or two ATP-non-competitive inhibitors, inhibitor I (10 μM) and inhibitor VII (2 μM), at 8-DIV for 48 h resulted in an approximately 2-fold increase in the promoter IV activity, similar to the effects of lithium (Figure 4a).

Glycogen synthase kinase-3 consists of two isoforms, α and β, whose roles in transcriptional modulation are not always identical.[27,31] We next examined the effects of RNAi-mediated GSK-3 gene silencing using siRNAs targeting GSK-3α or -3β isoforms. Cortical neurons at 8-DIV were transfected with 80 nM of a GSK-3 siRNA, specific for either GSK-3α (α-p1269 and α-p1375) or -3β (β-p555 and β-p1093), using siPORT Amine reagent. Transfection with each of these GSK-3 isoform-specific siRNAs induced a significant increase in *BDNF* promoter IV activity measured 48 h later (Figure 4b). Under these treatment conditions, siRNA transfection caused a 70–85% reduction in protein levels of the targeted GSK-3 isoform, with little or no effect on the protein levels of the non-targeted GSK-3 isoform.[31]

HDAC inhibitors and HDAC1 gene silencing also upregulate BDNF promoter IV activity

Valproic acid is an inhibitor of HDAC, which is a robust regulator of gene expression via multiple mechanisms such as chromatin remodeling through histone hyperacetylation.[32] To examine whether HDAC inhibition is related to VPA-induced activation of *BDNF* promoter IV, we treated neuronal cultures at 8-DIV with a structurally related short-chain fatty acid, SB (0.3 mM) and a structurally dissimilar hexamic acid, TSA (30 nM), for 48 h. Treatment with either SB or TSA caused an increase in the promoter IV activity by more than 2-fold, similar to the extent elicited by VPA treatment (Figure 5a). Under these treatment conditions, acetylation levels of histone H3 and histone H4 were markedly increased (data not shown), suggesting the occurrence of HDAC inhibition. It has been reported that VPA inhibits class I HDAC,[33] which includes the HDAC1 isoform. Therefore, we next examined the effects of RNAi-mediated HDAC1 silencing using HDAC1 isoform-specific siRNA on *BDNF* promoter IV activity. Cortical neurons transfected with HDAC1-specific siRNA (80 nM) showed approximately a 2.5-fold increase in promoter IV activity, while transfection with the non-targeted siRNA control had no effect (Figure 5b). HDAC1 siRNA transfection caused a concentration-dependent reduction in the protein levels of HDAC1 with an almost complete depletion at 80 nM (Figure 5c). In contrast, the protein levels of HDAC3 or β-actin were essentially unchanged, supporting the selectivity of this gene silencing. The levels of histone H3 acetylation were also robustly elevated after transfection with 40 and 80 nM of HDAC1 siRNA. HDAC4 has been implicated in neuronal cell death[34] and HDAC5 has been suggested to be a mediator of the action of an antidepressant.[35] However, neither the level of HDAC4 nor that of HDAC5 was affected by treatment with HDAC1 siRNA (Figure 5d), further indicating the specificity of the gene silencing. In neurons transfected with the promoter IV internal deletion plasmid, pGL3-710ΔCaRE luc, TSA, but not KCl, was able to significantly increase promoter IV activity (Figure 5e). Treatment with siRNA for GSK-3α, GSK-3β or HDAC1 markedly increased the levels of exon IV-containing mRNA (Figure 5f), similar to their effects on the promoter IV activity. In HDAC1 siRNA-treated neurons, lithium, but not VPA, was able to further enhance promoter IV activity (Figure 5g).

Discussion

We studied the regulation of BDNF expression by two gold standard mood-stabilizing drugs, lithium and VPA, in rat cortical neurons. The principal findings of this study are: (a) treatment with either lithium or VPA upregulates BDNF protein with a selective increase in the levels of exon IV-containing BDNF mRNA; (b) the effects are associated with an enhancement of *BDNF* promoter IV activity; (c) the lithium- and VPA-sensitive element(s) resides in the promoter

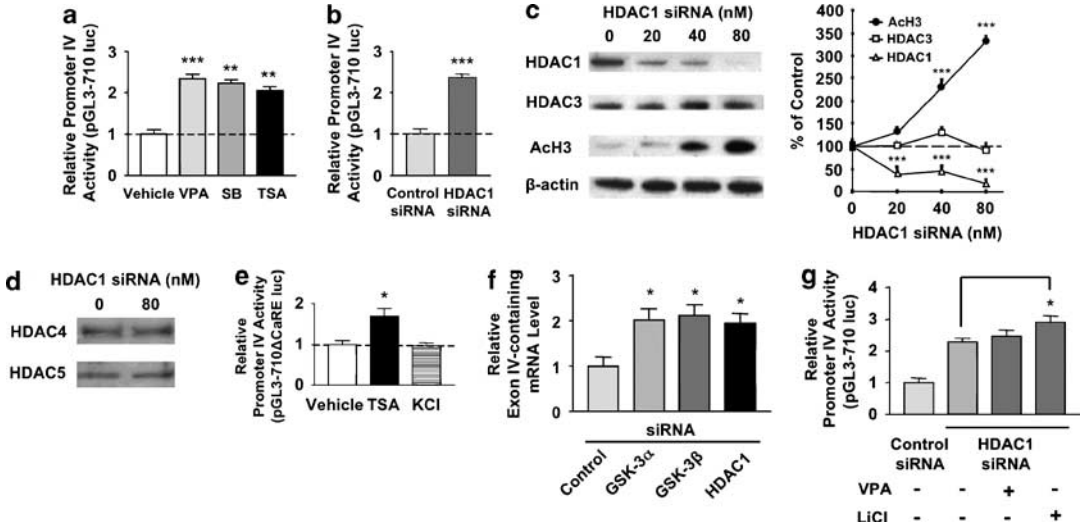

Figure 5 Treatment with histone deacetylase (HDAC) inhibitors or HDAC1 gene silencing increases brain-derived neurotrophic factor (*BDNF*) promoter IV activity. (**a**) pGL3-710 luc was transfected into cortical neurons at 0 day *in vitro* (DIV) by electroporation. The cultures at 8-DIV were then treated with valproic acid (VPA) (0.5 mM), sodium butyrate (SB) (0.3 mM) and trichostatin A (TSA) (30 nM) for 48 h prior to the measurement of *BDNF* promoter IV activity. (**b**) Cortical cultures at 0-DIV were transfected with pGL3-710 luc and at 8-DIV were either transfected with short interfering RNA (siRNA) (80 nM) specific for HDAC1 or non-targeting control using siPORT Amine reagent. Two days later, *BDNF* promoter IV activities were measured in cell lysates. (**c**) Representative western blots of levels of HDAC1, HDAC3, acetylated histone H3 (AH3) and β-actin. Cortical neurons at 9-DIV were transfected with HDAC1 siRNA at indicated concentrations for 48 h. Control group represents cortical neurons transfected with non-targeting siRNA. Cell lysates were collected at 11-DIV for immunoblotting. The immunoblots on the left are from a representative experiment, and the quantified results are shown on the right. (**d**) Western blotting of HDAC4 and HDAC5 after neurons at 9-DIV were transfected with HDAC1-targeting siRNA for 48 h. (**e**) The internal deletion plasmid, pGL3-710ΔCaRE luc, was transfected by electroporation at 0-DIV. The cultures at 8-DIV were then treated with TSA (30 nM) for 48 h or exposed to KCl (50 mM) for 8 h before the measurement of promoter IV activity at 10-DIV. (**f**) BDNF exon IV-containing mRNA levels in neurons treated with siRNA for GSK-3α, GSK-3β or HDAC1 siRNA. Neurons at 8-DIV were transfected with indicated siRNA (80 nM) for 48 h. Real-time PCR was performed to amplify exon IV-containing transcripts of BDNF. The values shown are relative to each vehicle control. (**g**) Lithium, but not VPA, enhanced promoter IV activity in HDAC1 siRNA-treated neurons. Cortical neurons at 8-DIV were transfected with HDAC1 siRNA, and simultaneously treated with LiCl (1 mM) or VPA (0.5 mM) for 48 h. The *BDNF* promoter IV activity was measured in cell lysates at 10-DIV. All quantified values are relative to respective control and expressed as means ± s.e.m. from four independent cultures. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared with respective control.

region between −170 and −710 bp from the initiation site of the promoter IV; and (d) lithium and VPA may elicit these effects by inhibiting their direct targets, GSK-3 and HDAC, respectively. This is the first demonstration of the activation of *BDNF* promoter IV by mood stabilizers and their molecular targets underlying this promoter activation.

The mechanisms underlying depolarization-induced activation of *BDNF* promoter IV are known to involve binding of transcription factors to three CaREs located between −32 and −73 bp of the promoter.[15–17] That lithium- and VPA-sensitive site(s) resides in a region upstream from the CaREs is supported by our observations that (a) lithium or VPA activates the promoter IV only when pGL3-710 luc, but not pGL3-170 luc or pGL3-80 luc is used as the reporter plasmid (Figure 2), (b) either drug is able to activate promoter IV in the internal deletion mutant lacking the three CaREs (Figure 3c) and (c) either mood stabilizer upregulates the promoter IV activity in neurons depolarized with KCl, and thus potentiates the depolarization-induced promoter activation (Figure 3a). The precise genomic sequences in the promoter IV that define the responsiveness to lithium and VPA, and the transcription factors bound to these nucleotides warrant further investigation.

The ability of various GSK-3 inhibitors to activate *BDNF* promoter IV (Figure 4) suggests that lithium-induced promoter IV activation is related to GSK-3 inhibition. This notion is strengthened by our observation of promoter IV activation via RNAi-mediated gene silencing of either GSK-3α or -3β, suggesting the involvement of both GSK-3 isoforms in the BDNF induction. CREB, one of the transcription factors mediating activity-dependent BDNF induction,[15] is negatively regulated by GSK-3β-mediated phosphorylation at Ser129 following CREB phosphorylation at Ser133.[36–38] Thus, inhibition or depletion of GSK-3 could result in disinhibition of CREB, which could contribute to the promoter IV activation. However, our results show that the lithium- or VPA-sensitive promoter region resides upstream from the

three CaRE sites. Thus, it is possible that other mechanisms involving other transcription factors regulated by GSK-3 are the downstream molecular targets. GSK-3 inhibition-mediated BDNF expression likely participates in the antidepressant or even antimanic effects of lithium. For example, lithium, GSK-3β knockout and other GSK-3 inhibitors show antidepressant effects in rodents.[39–41] Lithium and other GSK-3 inhibitors also attenuate the hyperactivity induced by amphetamine in rats[40] or dopamine transporter knockout in mice.[42]

Valproic acid-induced promoter IV activation is mimicked by treatment with other HDAC inhibitors, SB and TSA, or by gene knockdown with HDAC1-specific siRNA (Figure 5), suggesting the involvement of HDAC inhibition. In HDAC1 siRNA-treated neurons, VPA failed to further enhance *BDNF* promoter IV activity (Figure 5g), suggesting that HDAC1 is likely the predominant isoform involved. A large volume of literature supports the view that histone hyperacetylation, resulting from HDAC inhibition, causes chromatin remodeling, which results in a more relaxed physical state of the nucleoprotein and allows the transcription factors and RNA polymerase to interact with specific promoters. Thus, *BDNF* promoter may be activated by enhanced histone acetylation at promoter IV following VPA treatment. In this context, it has been reported that status epilepticus-induced BDNF upregulation in the brain of rats is accompanied by hyperacetylation of histone H4 of the *BDNF* promoters.[43] Moreover, chronic cocaine-induced activation of *BDNF* promoters in rat brain is associated with histone H3 hyperacetylation.[44] On the other hand, *BDNF* promoter IV binds the transcriptional repressor, MeCP2 (a methyl-CpG-binding domain protein), in complex with the corepressor sin3a and HDACs.[45–47] Inhibition of HDAC by VPA or other inhibitors could cause dissociation of the repressor–corepressor complex from the promoter, resulting in transcriptional disinhibition. It is noteworthy that the antidepressant effect of imipramine is largely reversed by HDAC5 overexpression in the hippocampus of mice subjected to chronic defeat stress, which *per se* induces down-regulation of *BDNF* III and IV mRNA.[35] In addition, antidepressant-like effects of SB have been reported in mice following chronic treatment.[48]

In conclusion, our studies have demonstrated that two mainstay mood stabilizers, lithium and VPA, selectively increase the activity of *BDNF* promoter IV and the level of exon IV-containing BDNF mRNA. Evidence is also presented that the effects of lithium and VPA on the activity of promoter IV are likely mediated though inhibition of GSK-3 and HDAC, respectively. Our results also suggest strongly that the site(s) that confers the responsiveness to both mood stabilizers resides upstream from the three CaREs responsible for depolarization-induced promoter IV activation. Lithium- and VPA-induced *BDNF* promoter IV activation could be part of the molecular mechanisms underlying their therapeutic effects in bipolar patients.

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