Effects of mood-stabilizing drugs on dendritic outgrowth and synaptic protein levels in primary hippocampal neurons


Objectives: Mood-stabilizing drugs, such as lithium (Li) and valproate (VPA), are widely used for the treatment of bipolar disorder, a disease marked by recurrent episodes of mania and depression. Growing evidence suggests that Li exerts neurotrophic and neuroprotective effects, leading to an increase in neural plasticity. The present study investigated whether other mood-stabilizing drugs produce similar effects in primary hippocampal neurons.

Methods: The effects of the mood-stabilizing drugs Li, VPA, carbamazepine (CBZ), and lamotrigine (LTG) on hippocampal dendritic outgrowth were examined. Western blotting analysis was used to measure the expression of synaptic proteins—that is, brain-derived neurotrophic factor (BDNF), postsynaptic density protein-95 (PSD-95), neurexin, and synaptophysin (SYP). To determine neuroprotective effects, we used a B27-deprivation cytotoxicity model which causes hippocampal cell death upon removal of B27 from the culture medium.

Results: Li (0.5–2.0 mM), VPA (0.5–2.0 mM), CBZ (0.01–0.10 mM), and LTG (0.01–0.10 mM) significantly increased dendritic outgrowth. The neurotrophic effect of Li and VPA was blocked by inhibition of phosphatidylinositol 3-kinase, extracellular signal-regulated kinase, and protein kinase A signaling; the effects of CBZ and LTG were not affected by inhibition of these signaling pathways. Li, VPA, and CBZ prevented B27 deprivation-induced decreases in BDNF, PSD-95, NLG1, β-neurexin, and SYP levels, whereas LTG did not.

Conclusions: These results suggest that Li, VPA, CBZ, and LTG exert neurotrophic effects by promoting dendritic outgrowth; however, the mechanism of action differs. Furthermore, certain mood-stabilizing drugs may exert neuroprotective effects by enhancing synaptic protein levels against cytotoxicity in hippocampal cultures.

Bipolar disorder is a chronic mental illness and its prevalence may be as high as 5% (1). Although it has been recognized as a major health problem, the underlying neurobiology of bipolar disorder remains largely unknown (2). Several studies have suggested that the pathophysiology of bipolar disorder is related to alterations in neural plasticity (3, 4). Neuroimaging and postmortem studies have revealed significant brain atrophy in patients with bipolar disorder. Decreased gray matter volume, reductions in the number, size, and density of neurons and glial cells, and decreased brain-derived neurotrophic factor (BDNF) levels have been observed (5–7). These findings may imply structural and functional impairments in neural plasticity in patients with bipolar disorder (8).
Although there are currently no ideal ‘mood-stabilizing’ drugs, lithium (Li), valproate (VPA), carbamazepine (CBZ), and lamotrigine (LTG) show mood-stabilizing effects and are used in clinical practice (9, 10). Up until recently, VPA has been the most commonly prescribed agent for the treatment of mania, whereas LTG has been preferred for the treatment of mild depressive episodes (11).

Li and VPA have neurotrophic and neuroprotective properties and can ameliorate the impairments in neural plasticity of patients with bipolar disorder (12). These mood-stabilizing drugs increase hippocampal neurogenesis, BDNF, and cytoprotective protein Bcl-2 levels in the brain and facilitate intracellular signaling pathways, including the glycogen synthase kinase-3β (GSK-3β), extracellular-regulated kinase (ERK), and Wnt/β-catenin signaling pathways (12). However, the mechanisms of action of CBZ and LTG are not well understood.

Neurite outgrowth is an important component of neural plasticity, and neural regeneration (13) is also essential for neural plasticity, especially for synaptic formation (14). Neuroimaging studies have demonstrated that long-term Li treatment increases gray matter volume in patients with bipolar disorder (15). These changes may be attributed to the increases in neurite outgrowth and synaptic connections as Li enhances neurite outgrowth and synapse formation. In neurons, important signaling pathways regulating neurite outgrowth include phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signaling-regulated kinase (ERK) signaling pathways via activation of neurotrophic receptors (16, 17). Additionally, the protein kinase A (PKA) signaling pathway is also involved in neurite outgrowth (18). A link between PKA activation and the stimulation of PI3K has been reported in cAMP-mediated neuritogenesis (19).

Postsynaptic BDNF, the most abundant neurotrophin in the brain, contributes to axonal branching, dendritic differentiation, and connectivity among neurons (20–22). Upregulation of BDNF expression requires the activation of transcription factor CREB, which activates the PKA, PI3K, and ERK signaling pathways (23).

Synaptic formation is induced by interactions between presynaptic and postsynaptic neurons that involve cell-adhesion molecules, scaffolding proteins, and proteins associated with synaptic vesicle machinery (24, 25). Postsynaptic density protein 95 (PSD-95), a postsynaptic scaffolding protein, is preferentially located in dendritic spines and plays a critical role in the regulation of dendritic spine size and shape (26, 27). Neuroligin 1 (NLG1), a postsynaptic adhesion molecule, binds with high affinity to the presynaptic adhesion molecule β-neurexin (28). This interaction promotes synapse assembly and pre- and postsynaptic differentiation (29). NLG1 binds to PSD-95 via its PSD-95/discs large/zona occludens 1 binding domain (27). In this way, the β-neurexin–NLG1–PSD-95 complex can establish a link between presynaptic and postsynaptic cells. Synaptophysin (SYP) is present in the presynaptic vesicles of almost all nerve cells. It represents a highly specific marker of nervous structure and is widely used for the marker of synaptic density (30).

We hypothesized that mood-stabilizing drugs would modify neural plasticity. Most previous studies have considered the effects of Li and VPA on the regulation of neural plasticity. The mechanisms underlying the neurotrophic effects of CBZ and LTG also remain largely elusive. In the present study, we examined the roles of Li, VPA, CBZ, and LTG in neurotrophic effects by investigating whether these drugs affected hippocampal dendritic outgrowth, and also whether the PI3K, ERK, and PKA signaling pathways were responsible for the neurotrophic effects of these drugs in rat hippocampal cells.

We used a B27-deprivation cytotoxicity model to determine whether these four mood-stabilizing drugs exhibited neuroprotective properties. B27 facilitates optimal growth and the long-term survival of rat embryonic hippocampal neurons. Deprivation of growth medium N2, which consists of constituents similar to those found in B27, has been reported to induce hippocampal cell death via activation of caspases 3, 8, and 9, enzymes that play a pivotal role in apoptosis-associated cell death (31). Moreover, our laboratory previously demonstrated a decrease in the expression of synaptic proteins following B27 deprivation in rat hippocampal cultures (32). To determine whether mood-stabilizing drugs exhibit neuroprotective properties in a toxic environment, we assessed the effect of the mood-stabilizing drugs on synaptic protein expression in hippocampal cells cultured with or without B27.

Material and methods

Drugs and reagents

The reagents used in the study included neurobasal medium, fetal bovine serum (FBS), horse serum (HS), B27 supplement, L-glutamine, penicillin–streptomycin, and trypsin (Invitrogen; Carlsbad, CA, USA). The mood-stabilizing...
drugs, Li, VPA, CBZ, and LTG, were obtained from Sigma-Aldrich (St. Louis, MO, USA). The antibodies used for Western blotting included anti-β-neurexin (sc-14334), anti-BDNF (sc-546), anti-goat and anti-rabbit IgG-horseradish–peroxide conjugates (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-α-tubulin and anti-mouse IgG peroxidase conjugates (Sigma-Aldrich), and anti-PSD-95 (AB9634; Millipore, Temecula, CA, USA), anti-neuroglial1 (ab56882), and anti-synaptophysin (ab52636) obtained from Abcam (Cambridge, UK). The antibodies used for immunostaining included anti-mitogen-activated protein kinase-2 (MAP-2) (Millipore), Alexa Fluor 568 goat anti-mouse IgG, and Hoechst 33258 (Invitrogen). Specific kinase inhibitors included the PI3K inhibitor LY294002 (Cell Signaling Technology, Beverly, MA, USA), the PKA inhibitor H-89, and the ERK inhibitor PD98059 (Calbiochem, San Diego, CA, USA).

Primary hippocampal cell cultures

All animal manipulations were performed in accordance with the animal care guidelines of the United States National Institute of Health [NIH publication no. 23-85, revised 1996]. The present experiment was approved by the Committee for Animal Experimentation and the Institutional Animal Laboratory Review Board of Inje Medical College (approval no. 2012-010).

Primary cultures of hippocampal neurons were prepared from fetal brains (embryonic day 17; E17) obtained from Sprague-Dawley rats (Orient Bio, Gyeonggi-Do, Korea) in a manner similar to that developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33). Briefly, the brains were exposed, the hippocampi were carefully developed by Kaech and Banker (33).
determined as the distance between the edge of the cell body and the tip of the growth cone using MetaMorph, an automated image-analysis program (Molecular Devices, Downingtown, PA, USA), performed by a researcher blinded to the treatment groups. These programs are routinely used for such analyses (37–39). At least 600–800 cells were analyzed using MetaMorph, an automated image-analysis program (Molecular Devices) (38).

Western blot analysis

The cells were washed twice with ice-cold PBS. Lysis buffer [20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 1% NonidetP-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 2 mM ethylenediaminetetraacetic acid (EDTA), and one tablet of complete protease inhibitor (Roche, Laval, Quebec, Canada)] was then added, and the lysates were centrifuged (1,000 g, 15 min, 4°C), after which the supernatants were boiled in lysis buffer. Equal amounts of protein (20 µg) derived from the cell extracts under each treatment condition were separated on SDS-polyacrylamide gels and transferred electrophoretically onto polyvinylidene fluoride membranes. The blots were blocked by incubation in 5% (w/v) non-fat milk in tris-buffered saline (TBS) with 0.15% tween 20 (TBS-T) for one hour. After incubation with a primary antibody (anti-PSD-95, 1:1,000; anti-β-neurexin, 1:1,000; anti-neuroligin 1, 1:1,000, anti-BDNF, 1:1,000; anti-synaptophysin, 1:1,000; or anti-α-tubulin, 1:2,000) in TBS-T at 4°C overnight, the membranes were washed three times in TBS-T for 10 min. The membranes were subsequently incubated for one hour in TBS-T containing the horseradish–peroxidase-conjugated secondary antibody (goat-anti-rabbit IgG for anti-PSD-95, 1:2,000, anti-BDNF, 1:2,000, and anti-synaptophysin, 1:2,000; donkey anti-goat IgG for anti-β-neurexin, 1:2,000; and anti-mouse IgG for anti-neuroligin 1, 1:2,000 and anti-α-tubulin, 1:10,000). Immunoreactive bands were visualized and quantified using ECL Western blotting reagents (Bio-Rad, Hercules, CA, USA) and chemiluminescence was detected using Las-3000 Image Reader (Fuji Film, Tokyo, Japan) software. The amount of a specific protein from total protein loaded to equal amounts per sample was assessed. α-tubulin was used to normalize protein loading to adjust for variations.

Statistical analysis

Samples for the neurite assay were obtained from two independent experiments, and the samples for Western blotting were obtained from three independent experiments. For the neurite assay, we analyzed 300–400 cells obtained from five randomly selected areas of each sample. In total, 600–800 cells from the ten fields were included. In the Western blotting analysis, values from all samples on an individual blot were expressed as the percent change in the average of control cells. Two or three individual blots were performed for each specific protein.

Changes in dendritic outgrowth were analyzed using a one-way analysis of variance (ANOVA). Changes in protein expression were analyzed using a two-way ANOVA to determine the individual effects of drug treatment or the B27 deprivation condition and the interactive effects between drug treatment and the B27 deprivation condition. Scheffe’s test was used for all post-hoc comparisons, and p-values < 0.05 were deemed to indicate statistical significance.

Results

Effect of mood-stabilizing drugs on dendritic outgrowth

A neurite outgrowth assay was performed to determine whether mood-stabilizing drugs regulate dendritic morphology in hippocampal cells cultured with B27 (control condition). To obtain images of a single neuron, hippocampal cells were plated at low density, thereby causing greater cell death under B27 deprivation. The neurotrophic effects of mood-stabilizing drugs were investigated in the control condition accordingly. Hippocampal cells were analyzed using fluorescence microscopic images (Fig. 1A) and scored to quantify dendritic outgrowth induced by application of therapeutically relevant concentrations of mood-stabilizing drugs for five days (Fig. 1B) (8, 35, 36). Hippocampal cells without mood-stabilizing drugs exhibited modest dendritic differentiation, with an average dendrite length of approximately 51.6 µm. All mood-stabilizing drugs significantly increased dendritic outgrowth in hippocampal cells in a concentration-dependent manner (Li: $F = 10.453, df = 3, p < 0.01$; VPA: $F = 43.120, df = 3, p < 0.01$; CBZ: $F = 3.397, df = 3, p < 0.01$; LTG: $F = 4.932, df = 3, p < 0.01$).

We examined the effects of selective PI3K (LY294002), ERK (PD98059), and PKA (H-89) inhibitors on hippocampal morphology to determine whether the PI3K/Akt, ERK, and PKA signaling pathways played a role in the enhancement of dendritic outgrowth induced by mood-stabilizing drugs.
LY294002 concentrations of 0.1–10 μM and PD98059 and H-89 concentrations of 0.01–1.0 μM did not significantly affect dendritic outgrowth and cell viability; we found that higher concentrations (> 10 μM for LY294002 and > 1 μM for PD98059 and H-89) reduced cell viability (data not shown). Thus, we used the highest concentrations that did not affect dendritic outgrowth and cell viability (10 μM for LY294002 and 1 μM for PD98059 and H-89). The effects of LY294002, PD98059, and H-89 on mood-stabilizing drug-induced dendritic outgrowth are shown in Figure 2A–C (Li and VPA: 2 mM; CBZ and LTG: 0.1 mM).

LY294002, PD98059, and H-89 blocked the neurotrophic effects induced by Li and VPA [LY294002: F = 24.379, df = 3, p < 0.01 for Li; F = 47.137, df = 3, p < 0.01 for VPA (Fig. 2A); PD98059: F = 13.957, df = 3, p < 0.01 for Li; F = 39.107, df = 3, p < 0.01 for VPA (Fig. 2B); H-89: F = 22.81, df = 3, p < 0.01 for Li; F = 53.553, df = 3, p < 0.01 for VPA (Fig. 2C)]. By contrast, these inhibitors did not alter the neurotrophic effects of CBZ and LTG.

Effect of mood-stabilizing drugs on the expression of postsynaptic proteins BDNF, PSD-95, and neuroolin 1 and presynaptic proteins β-neurexin and synaptophysin

We used B27 deprivation-induced toxicity, which causes cell death in primary hippocampal cells (31), to investigate the neuroprotective properties of the four mood-stabilizing drugs. Mood-stabilizing drug-induced changes in the expression of the postsynaptic proteins BDNF, PSD-95, and NLG1 and the presynaptic proteins β-neurexin

Fig. 1. Effects of mood-stabilizing drugs on dendritic outgrowth in hippocampal neurons. Cells were treated with three doses of mood-stabilizing drugs or cultured in no-drug control conditions for five days. Two independent experiments were performed for each sample (two samples/drug/given concentration, n = 2). Cells were photographed (A) ([a] = CON; [b] = Li; [c] = VPA; [d] = CBZ; [e] = LTG) and scored (B) according to the methods described in the text. In total, 600–800 cells from each sample were analyzed. Data (mean ± standard error) are expressed as values of the control cells (no drug treatment). CBZ = carbamazepine; CON = control; Li = lithium; LTG = lamotrigine; VPA = valproate. *p < 0.05; **p < 0.01 versus control.
and SYP were measured in rat hippocampal neurons maintained in media with or without B27. In a preliminary experiment, we evaluated the effects of mood-stabilizing drugs on hippocampal cell viabilities in culture with and without B27 (Supplementary Fig. 1). B27 deprivation reduced cell viability to approximately 53% of the level of control cells \( (p < 0.01) \). However, no changes induced by the mood-stabilizing drugs were observed between culture conditions with and without B27.

Synaptic protein expression was not affected by the mood-stabilizing drugs when B27 was present in the culture medium (control condition) (Table 1). However, B27 deprivation significantly reduced the expression of BDNF, PSD-95, NLG1, β-neurexin, and SYP (all \( p < 0.001 \)) (Table 1). Li, VPA, and CBZ significantly increased BDNF,
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PSD-95, NLG1, β-neurexin, and SYP levels, in a concentration-dependent manner, under B27 deprivation conditions (p < 0.05 or p < 0.01, Fig. 3A–E). By contrast, LTG had no effect on protein levels (Fig. 3A–E).

The results of the two-way ANOVA are shown in Table 2. Li, VPA, and CBZ, but not LTG (Drug), and B27 deprivation (B27) had significant individual effects on the levels of BDNF, PSD-95, β-neurexin, and SYP (all p < 0.05); VPA, but no other drug, had a significant effect on NLG1 levels (p < 0.05). The analysis revealed a significant Drug × B27 interaction, demonstrating significant effects of Li, VPA, and CBZ, but not LTG, on BDNF, PSD-95, β-neurexin, and SYP levels (all p < 0.05) under B27 deprivation conditions; VPA, but no other drug, was found significantly to affect NLG1 levels under B27 deprivation conditions (p < 0.05).

Discussion

We demonstrated that Li, VPA, CBZ, and LTG increased dendritic outgrowth in primary hippocampal neurons and that the neurotrophic effects of Li and VPA, but not CBZ and LTG, were mediated by PI3K, ERK, and PKA signaling. Moreover, Li, VPA, and CBZ, but not LTG, prevented a B27 deprivation-induced decrease in the expression of BDNF, PSD-95, NGL1, β-neurexin, and SYP in the hippocampal cultures. Thus, we provide evidence that the mechanisms underlying the neurotrophic and neuroprotective effects of the drugs may differ.

Several previous studies have shown that Li and VPA promote neurite outgrowth in human neuroblastoma, SH-SY5Y, N2a, and mouse N1E-115 neuroblastoma cells (35, 40, 41). Our results showed that Li and VPA treatment also promoted neurite outgrowth in human hippocampal cultures. Little is known about the effects of CBZ and LTG on neurite outgrowth. Two previous studies found that CBZ (30 μM) did not affect neurite outgrowth in rat forebrain stem cells (42), and CBZ (50 and 100 μM) and LTG (10 and 50 μM) did not promote neurite extension in SH-SY5Y cells (21). By contrast, we found that therapeutic, and slightly higher, doses of CBZ (50 and 100 μM) and LTG (100 μM) enhanced hippocampal dendritic outgrowth. The therapeutic concentrations of CBZ and LTG are 20–50 μM and 19–50 μM, respectively (8, 35, 36). The discrepancy between the present results and those of previous studies may be attributed to differences in cell type, drug concentration, and duration of treatment.

Our findings suggest that PI3K, ERK, and PKA signaling are required for Li- and VPA-induced enhancement of dendritic outgrowth in rat primary

Table 1. Effects of mood-stabilizing drugs and B27 deprivation on the expression of BDNF, PSD-95, neurogin 1, β-neurexin, and synaptophysin in primary hippocampal cultures

<table>
<thead>
<tr>
<th>Drug</th>
<th>± B27</th>
<th>Dose (mM)</th>
<th>BDNF</th>
<th>PSD-95</th>
<th>NLG1</th>
<th>β-neurexin</th>
<th>SYP</th>
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<tr>
<td>Li</td>
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<td>0</td>
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<td></td>
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<td>0.5</td>
<td>97 ± 8</td>
<td>105 ± 7</td>
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<td></td>
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<td>1.0</td>
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<td>108 ± 7</td>
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<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>106 ± 5</td>
<td>107 ± 8</td>
<td>101 ± 8</td>
<td>113 ± 9</td>
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<tr>
<td></td>
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<td>0</td>
<td>46 ± 6a</td>
<td>43 ± 6a</td>
<td>55 ± 15a</td>
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<td>46 ± 8a</td>
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<td>43 ± 5a</td>
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<td>98 ± 9</td>
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<td>44 ± 11a</td>
<td>48 ± 9a</td>
<td>43 ± 7a</td>
<td>45 ± 10a</td>
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</table>

Values are means ± standard error expressed as percentage of values of the control cells (+B27, no drug treatment).

BDNF = brain-derived neurotrophic factor; CBZ = carbamazepine; Li = lithium; LTG = lamotrigine; NLG = neuregin; PSD = postsynaptic density protein; SYP = synaptophysin; VPA = valproate.

*p < 0.01.
hippocampal neurons, whereas activation of these pathways is not necessary for CBZ and LTG neurotrophic effects.

GSK-3β is a downstream target of PI3K/Akt and is inactivated by phosphorylation of serine-9 by Akt (43). PI3K/Akt/GSK-3β signaling is an important pathway for neural plasticity. Li and VPA have well-established direct links with the PI3K/Akt pathway (44). The finding that Li and VPA inhibit GSK-3β supports an association between these drugs and the PI3K/Akt/GSK-3β signaling pathway (45). On the other hand, CBZ and LTG had no effects on Akt/GSK-3β in SH-SY5Y cells (46). Thus, the present finding that PI3K signaling is necessary for Li- and VPA-induced enhancement of dendritic outgrowth, but is not related to the neurotrophic activity of CBZ and LTG, is consistent with the findings of these previous studies.

Activation of the MEK/ERK signaling pathway is necessary for Li-induced neuritic outgrowth in N2a cells (40). Furthermore, therapeutic concentrations of VPA have been shown to produce effects similar to those of neurotrophic factors, such as the activation of ERK signaling and promotion of neurite outgrowth in SH-SY5Y cells (47). However, it is not clear whether the neurotrophic effects of mood-stabilizing drugs involve ERK signaling. CBZ, but not VPA, has been reported to activate ERK in differentiated SH-SY5Y cells (48). Moreover, we found that inhibition of ERK signaling suppressed Li- and VPA-induced dendritic outgrowth, whereas no effect was observed for CBZ- and LTG-induced outgrowth.

The present study is the first to show that Li- and VPA-induced dendritic outgrowth requires activation of PKA signaling, whereas inhibition of the PKA signaling does not significantly affect CBZ- and LTG-induced neurotrophic activity. PKA signaling is involved in the regulation of CREB. Moreover, activation of the transcription factor CREB via the cAMP/PKA signaling pathway induces the expression of cAMP-responsive genes, including BDNF, which plays a role in neural plasticity (23). Li has been shown to stabilize cAMP fluctuation by suppressing stimulatory and inhibitory G-proteins or by regulating adenyl cyclase (49). Furthermore, Li and VPA activate CREB (50, 51). CBZ modulates PKA signaling via different mechanisms. CBZ decreases cAMP production by inhibiting adenyl cyclase (52) and the DNA binding activity of CREB (53). At present, little is known about the relationship between LTG and PKA signaling. We did not investigate whether CBZ or LTG negatively regulate PKA signaling. Further study is needed to address this issue.

Our findings indicate that Li and VPA have a neuroprotective function. Induction of BDNF expression is essential for neuroprotective effects. Several studies have shown that Li and VPA elevate BDNF levels in the hippocampus (54, 55). A previous study found that CBZ increased mRNA and BND protein levels in the rat frontal cortex (56). LTG has been shown to have a positive effect on BDNF protein levels in the amygdala, but not in the prefrontal cortex and hippocampus, of maternally deprived rats (57). Furthermore, LTG reversed a stress-induced decrease in BDNF expression in the frontal cortex and hippocampus (58). The effect of LTG on BDNF levels may vary depending on brain region, cell type, and duration of treatment. These results are supported by our finding that LTG exerts a neurotrophic effect by promoting dendritic outgrowth, although BDNF expression was unchanged following treatment with LTG.

Phosphorylation of GSK-3β (inactive form) by activation of Akt promotes cell survival by inhibiting apoptosis, whereas dephosphorylated GSK-3β (active form), via inhibition of Akt, initiates the apoptosis pathway (59). BDNF inhibits GSK-3β activity via increased phosphorylation in cerebellar granule cells and human neuroblastoma SH-SY5Y cells (48, 60). Therefore, elevation of BDNF levels by Li and VPA may exert a neuroprotective effect due to GSK-3β inhibition.

Dendrites are the primary site of synaptic connections. Thus, dendritic outgrowth is essential for synapse formation. PSD-95 and SYP are widely used as synaptic markers (30, 61). An association between PSD-95 and the postsynaptic adhesion molecule NLG1 is preferentially manifested in the dendritic spine (62). Interactions between the presynaptic adhesion molecule β-neurexin and the NLGs promote synapse assembly (29). Taken together, these findings suggest that increases in the levels of these synaptic proteins may reflect an increase in the number of synapses.

We found that Li increased the expression of these synaptic proteins in B27-deprived hippocampal cultures. This observation is consistent with a Li-induced increase in the formation of new synapses and increased SYP levels in hippocampal neurons (63) and Li-induced axonal sprouting in cerebellar granule cells (64). A recent study showed that histone deacetylase (HDAC) inhibitors promoted neuroprotection and neurite outgrowth via an increase in BDNF expression in primary rat cortical neurons following ischemic insult caused by oxygen and glucose deprivation (OGD) (65).
VPA action is linked to the direct inhibition of HDAC, leading to histone hyperacetylation (66). In the study by Hasan et al. (65), VPA and trichostatin A were used as HDAC inhibitors. These drugs were associated with significant increases in the synaptic markers PSD-95 and SYP and in acetylated histone H3 under OGD conditions. The findings of this study are consistent with our data showing that VPA exerts a neuroprotective effect under toxic conditions induced by B27 deprivation. The present study revealed that CBZ, similar to Li and VPA, exerts a neuroprotective effect by increasing synaptic protein levels under cytotoxic conditions. However, few studies have investigated the neuroprotective effects of CBZ, nor the evidence for such effects (67) or their absence (68). Although LTG has neuroprotective effects in several brain injury conditions (68, 69), the effect of LTG on synaptic protein levels has not been investigated previously. Our results suggest that the mechanisms underlying the neurotrophic or neuroprotective effects of Li and VPA are distinct from those of CBZ and LTG. These differences are yet to be fully investigated. CBZ and LTG are sodium-channel blockers that reduce neuronal excitability, and this action is a proposed mechanism underlying their anticonvulsant action (70). However, no evidence suggests that the efficacy of CBZ and LTG as mood-stabilizing drugs is related to sodium-channel inhibition.

In our preliminary experiments, B27 deprivation reduced cell death, and the mood-stabilizing drugs evaluated had no protective effect on cell viability under these conditions (Supplementary Information). Our present data demonstrate that clinically relevant concentrations of Li, VPA, and CBZ, but not LTG, significantly attenuated B27 deprivation-induced decreases in BDNF, PSD-95, NLG1, PSD-95, and -neurexin, and synaptophysin expression following B27 deprivation. Cells were treated with three doses of mood-stabilizing drugs for four days in the absence of B27. (A) BDNF, (B) PSD-95, (C) neuroligin 1 (NLG 1), (D) -neurexin, and (E) synaptophysin (SYP). Cell lysates were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with each primary antibody. Western blot analyses were repeated two or three times for each protein, in three independent cultures (three samples/drug/given concentration, n = 3). A representative image and quantitative analysis normalized to the -tubulin band are shown. Values are means ± standard error expressed as percentage of values of the control cells (no drug treatment in non-B27-deprived cells). The dotted lines in A, B, and C indicate the levels of control cells. CBZ = carbamazepine; Li = lithium; LTG = lamotrigine; VPA = valproate. *p < 0.05 versus B27 deprived only; **p < 0.01 versus B27 deprived only.

Table 2. Summary of the two-way analysis of variance for changes in BDNF, PSD-95, neuroligin 1, -neurexin, and synaptophysin levels under the drug, B27 deprivation, and interaction of drug and B27 deprivation

<table>
<thead>
<tr>
<th></th>
<th>Li</th>
<th>VPA</th>
<th>CBZ</th>
<th>LTG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BDNF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug</td>
<td>8.048</td>
<td>&lt;0.001</td>
<td>14.878</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B27</td>
<td>75.952</td>
<td>&lt;0.001</td>
<td>84.333</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Drug × B27</td>
<td>6.657</td>
<td>&lt;0.001</td>
<td>11.702</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>PSD-95</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug</td>
<td>9.271</td>
<td>&lt;0.001</td>
<td>10.052</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B27</td>
<td>63.530</td>
<td>&lt;0.001</td>
<td>31.971</td>
<td>&lt;0.001</td>
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<tr>
<td>Drug × B27</td>
<td>5.759</td>
<td>&lt;0.001</td>
<td>6.320</td>
<td>0.015</td>
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<tr>
<td><strong>NLG1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug</td>
<td>3.011</td>
<td>0.088</td>
<td>7.484</td>
<td>0.008</td>
</tr>
<tr>
<td>B27</td>
<td>60.674</td>
<td>&lt;0.001</td>
<td>101.114</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Drug × B27</td>
<td>2.444</td>
<td>0.123</td>
<td>4.471</td>
<td>0.039</td>
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<tr>
<td><strong>-neurexin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug</td>
<td>19.820</td>
<td>&lt;0.001</td>
<td>16.160</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B27</td>
<td>62.026</td>
<td>&lt;0.001</td>
<td>61.856</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Drug × B27</td>
<td>6.826</td>
<td>0.011</td>
<td>6.843</td>
<td>0.011</td>
</tr>
<tr>
<td><strong>SYN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug</td>
<td>3.876</td>
<td>0.050</td>
<td>5.478</td>
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</tr>
<tr>
<td>B27</td>
<td>24.362</td>
<td>&lt;0.001</td>
<td>54.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Drug × B27</td>
<td>9.893</td>
<td>0.003</td>
<td>5.842</td>
<td>0.019</td>
</tr>
</tbody>
</table>

All degrees of freedom = 1.

BDNF = brain-derived neurotrophic factor; CBZ = carbamazepine; Li = lithium; LTG = lamotrigine; NLG = neuroligin; PSD = postsynaptic density protein; SYN = synaptophysin; VPA = valproate.
β-neurexin, and SYP expression in hippocampal cells. These data indicate that Li, VPA, and CBZ exert neuroprotective effects against B27 deprivation-induced cytotoxicity via direct upregulation of synaptic protein levels.

Taken together, these results suggest that Li, VPA, CBZ, and LTG exert neurotrophic effects by promoting dendritic outgrowth; however, the mechanisms of action differ. Furthermore, certain mood-stabilizing drugs may exert neuroprotective effects against cytotoxicity by enhancing synaptic protein levels in hippocampal cultures.

The results of the present study suggest that Li, VPA, CBZ, and LTG, at therapeutically relevant concentrations, have neurotrophic or neuroprotective properties that ameliorate impairments in neural plasticity underlying the pathophysiology of mood disorders. However, the mechanism of action differs among the mood-stabilizing drugs.

Acknowledgements

This research was supported by Basic Science Research Program (2011-0005481 to SW, 2012-20003442 to JGL, and 2012R1A1A3008447 to WS) through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (MEST).

Disclosures

The authors of this paper do not have any commercial associations that might pose a conflict of interest in connection with this manuscript.

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Mood stabilizers on neural plasticity


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Supporting Information
Additional Supporting Information may be found in the online version of this article:
Figure S1. Effects of mood-stabilizing drugs on cell viability in hippocampal neurons. Cells were treated with different doses of mood-stabilizing drugs for four days with or without B27. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, a colorimetric assay for assessing cell viability, was performed in two independent cultures (two samples/drug/given concentration, n = 2). (A) Lithium; (B) Valproate; (C) Carbamazepine; (D) Lamotrigine. Values are means ± standard error expressed as percentage of values of the control cells (+B27, no drug treatment). **p < 0.01 versus control.