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Mood Stabilizing Drugs Expand the Neural Stem Cell Pool in the Adult Brain Through Activation of Notch Signaling

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ABSTRACT

Neural stem cells (NSCs) have attracted considerable attention as a potential source of cells for therapeutic treatment of impaired areas of the central nervous system. However, efficient and clinically feasible strategies for expansion of the endogenous NSC pool are currently unavailable. In this study, we demonstrate that mood stabilizing drugs, which are used to treat patients with bipolar disorder, enhance the self-renewal capability of mouse NSCs in vitro and that this enhancement is achieved at therapeutically relevant concentrations in the cerebrospinal fluid. The pharmacological effects are mediated by the activation of Notch signaling in the NSC. Treatment with mood stabilizers increased an active form of Notch receptor and upregulated its target genes in neural stem/progenitor cells, whereas coculture with γ-secretase inhibitor or the presence of mutation in the *presenilin1* gene blocked the effects of mood stabilizers. In addition, chronic administration of mood stabilizers expanded the NSC pool in the adult brain, which subsequently increased the cell supply to the olfactory bulb. We suggest that treatment with mood stabilizing drugs could be used to facilitate regeneration following insult to the central nervous system. STEM CELLS *2008;26:1758–1767*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Neural stem cells (NSCs) reside in the adult mammalian brain and provide new neurons in neurogenic regions, such as the olfactory bulb and dentate gyrus of the hippocampus [1, 2]. Although the physiological roles of adult neurogenesis are not yet fully understood, they may contribute to olfaction [3, 4] and a certain type of memory [5]. In addition, NSCs in the adult brain may play significant roles in pathological conditions. In the rodent brain, ischemic insult induces the proliferation of neural progenitor cells, progeny of NSC, in the subventricular zone (SVZ) and subgranular layer of the hippocampal dentate gyrus [6–8]. Neural progenitor cells in the SVZ were recruited to the lesion, differentiated to neurons, and contributed to functional recovery [9, 10]. If endogenous NSCs could be efficiently activated to proliferate and differentiate to neurons through the use of currently available pharmacologic agents, it would open novel treatment modalities for the reversal of neurologic dysfunction. However, no drugs are currently known that directly act on the NSC and induce proliferation of neural stem/progenitor cells in the adult brain.

Studies of NSC proliferation have been hampered by the cells' characteristics. That is, adult NSCs in the SVZ divide slowly (estimated cell cycle time ≥15 days) and asymmetrically to self-renew and produce transit-amplifying neural progenitor cells [11]. Therefore, even with extensive labeling with 5-bromo-2′-deoxyuridine (BrdU), only a small portion of NSCs can be labeled, which makes it difficult to determine the absolute number of NSCs in the adult brain. Nevertheless, $BrdU^+$ cells that remain in the SVZ 4 weeks after BrdU injections are considered to be relatively quiescent NSCs because rapidly proliferating neural progenitor cells dilute the label below the threshold of immunofluorescent detection, leave the SVZ, or die [12, 13]. An alternative way to assess NSCs is an in vitro colony-forming neurosphere assay, in which NSCs from the adult SVZ proliferate in serum-free media in the presence of growth factors and form floating colonies called neurospheres [14]. Neurospheres may be derived from subpopulations of transit amplifying neural progenitor cells in addition to bona fide NSCs [15]. Although the nature of epidermal growth factor (EGF)-responsive neurosphere-forming cells in vivo remains to be investigated, the neurosphere assay is useful for determining the effects of drugs on NSC self-renewal.

Lithium, valproic acid (VPA), and carbamazepine (CBZ), collectively called mood stabilizers, are used to treat patients with bipolar disorder. However, the molecular mechanisms underlying their pharmacological actions and the disease itself remain largely unknown. Based largely on in vitro evidence, several molecular models for the therapeutic actions of mood

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stabilizers have been proposed. These include depletion of inositol [16–19], inhibition of glycogen synthase kinase-3β (GSK-3β) [20–23], and modification of MAPK/ERK pathways [24, 25]. In addition, at least one of mood stabilizers (VPA) exhibits activities as a histone deacetylase inhibitor, which may mediate some of mood stabilizing effects [26]. Interestingly, most, if not all, of those studies made their observations at much higher doses than are commonly used to treat bipolar disorder. When an in vitro system is used to identify clinically relevant cellular and molecular targets of mood stabilizers, several caveats are necessary [27]. First, because mood stabilizers are assumed to act in the central nervous system, the effects of the drugs should be observed at doses relevant to therapeutic concentrations found in the cerebrospinal fluid (CSF) or brain. Second, the effects of mood stabilizers are observed only after chronic exposure. Recently, it has been suggested that mood stabilizers enhance neurogenesis, both in vivo [28, 29] and in vitro [29–31]. This model is attractive because it takes several weeks before new neurons mature and are integrated into existing neural networks and this time frame corresponds to that of observed clinical benefits [32]. The precise manner in which mood stabilizing drugs modulate neurogenesis remains to be documented.

MATERIALS AND METHODS

Animals and Drug Administration

CD1 (ICR) mice (Japan SLC, Japan) were kept in the institutional Center for Experimental Animals with free access to food and water. *Presenilin1* mutant mice (C57BL/6 background) were genotyped as described [33]. Because the number of neurosphere-initiating NSCs is relatively stable since 6 weeks of age [34], 8–15 weeks of age mice were used in this study. Eight-week-old adult male mice were given 3 g/L LiCl (Wako Chemicals, Osaka, Japan, http://www.wako-chem.co.jp), supplemented with 0.6% NaCl, or 0.5 g/L VPA (Sigma, St. Louis, http://www.sigmaaldrich.com) by drinking water for 3 weeks. CBZ (Sigma, 1 mg/kg i.p. dissolved in DMSO at 0.5 mg/mL) was given to mice twice daily for 3 weeks. Serum drug concentrations were measured by atomic-absorption spectrometry for lithium or by enzyme immunoassay for VPA and CBZ (Mitsubishi BCL, Japan). All experiments were carried out under the permission of the institutional Animal Research Committee.

Isolation of Forebrain NSCs and the Neurosphere Assay

The protocol used to generate neurospheres in vitro from adult and embryonic brains has been described [13]. Cells from adult SVZ or embryonic medial ganglionic eminence (MGE) were triturated in serum-free media (SFM) [35] and cultured in the presence of 20 ng/mL EGF and 10 ng/mL fibroblast growth factor-2 (FGF-2) together with 2 μg/mL heparin (Sigma) [14]. After 6 days in vitro, the numbers of floating sphere colonies (neurospheres) possessing a diameter $>$0.08 mm were counted. The total number of neurosphere-forming NSCs in the adult brain was calculated from the following: number of neurospheres, volume applied in the culture, and volume in which cells were suspended (usually 700 μL) [34, 36].

BrdU Labeling and Immunohistochemistry

Mice were injected with BrdU (Sigma) (65 mg/kg, i.p.) every 3 hours for a total of five injections, and sacrificed 1 hour or 4 weeks after the last injection [13]. Coronal cryosections at 14-μm thickness were immunostained with mouse anti-BrdU antibody 1:1,000 (BD Biosciences, San Diego, http://www.bdbiosciences.com) at 4°C overnight, followed by secondary Alexa594-conjugated goat anti-mouse IgG antibody (Molecular Probes Inc., Eugene, OR, http://www.probes.invitrogen.com). Sections were counter-labeled with the nuclear stain Hoechst 33,342 (1 μg/mL; Sigma), and the total number of BrdU-labeled cells in the SVZ was estimated using the optical dissector method.

Reverse Transcription-PCR

Total RNA isolation, cDNA synthesis, and RT-PCR analysis were carried out as described [13]. cDNA was amplified in a thermal cycler with denaturation at 95°C for 30-second, annealing at 56°C for 40-second and extension at 72°C for 40-second for 32 (*Hes1*, *Hes5*, and *β-actin*) or 35 cycles (the others). Quantitative RT-PCR analyses for *Hes5* and *β-actin* were performed using the LightCycler system (Roche, Tokyo, http://www.roche-diagnostics.jp) as described [34, 37].

Luciferase Reporter Assay

Firefly luciferase reporter plasmids [38] were generously provided by Dr. Kageyama (Kyoto University). Primary neurospheres were triturated into single cells and then transfected with the luciferase reporter plasmid together with synthetic Renilla luciferase reporter plasmid (Promega) as an internal control. Transfection was performed using Lipofectamine 2000 (Invitrogen) for 3 hours at 37°C and the cells were cultured to generate secondary neurospheres with or without drugs. Four days after the transfection, neurospheres were collected and cell extracts were assayed for luciferase activity (Luciferase Assay System, Promega, Madison, WI, http://www.promega.com). The firefly luciferase activities were normalized by Renilla luciferase activities in each sample.

SDS-PAGE and Immunoblotting

Primary neurospheres or tissues from the adult SVZ were homogenized in lysis buffer and subjected to SDS-PAGE and Western blotting. Primary antibodies used are as follows: anit-Hes1 (1:1,000; gift by Dr. Sudo, Toray Corporation) [39], anti-cleaved Notch1 (1:1,000; Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com), anti-Notch1 (1:1,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com), anti-actin (AC-15, 1:20,000; Sigma), anti-phospho-GSK3β (Ser9) (1:1,000; Cell Signaling Technology), and anti-GSK3β (1:5,000; Cell Signaling Technology).

In Vitro γ-Secretase Assay

γ-Secretase was prepared from secondary neurospheres derived from the adult SVZ using a method described in [40]. The membrane fraction that contains γ-secretase was solubilized in 1% CHAPSO. Full-length Notch1 tagged with myc-epitope [41, 42] was expressed in Neuro2a cells and the membrane fraction of the cells were prepared as γ-secretase. The γ-secretase solution and myc-tagged Notch substrate were mixed, and the reaction was stopped by adding Laemmli sample buffer at zero time points or after the incubation at 4°C for 16 hours. The reaction samples were separated by SDS-polyacrylamide gel and blotted with anti-c-Myc antibody (9E10, Biomol, Plymouth Meeting, PA, http://www.biomol.com).

In Situ Hybridization

Digoxigenin (DIG)-labeled single strand riboprobes for *Hes1* or *Hes5* genes were synthesized using DIG RNA labeling mix (Roche). In situ hybridization was performed as described previously [43]. DIG-labeled RNA probes were visualized with alkaline phosphatase-conjugated anti-DIG antibody (1:2000; Roche) and NBT/BCIP reaction, followed by anti-BrdU immunostaining and visualization using the ABC Elite kit (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) and diaminobenzidine as a chromogen.

Statistics

Statistical analysis was performed using one-way ANOVA followed by Scheffe's or Dunnett's post hoc comparison or using an unpaired two-tailed Student's *t*-test if applicable. Level of significance was set at $p < .05$.

Table 1. Therapeutic concentrations of mood stabilizers

	Lithium	VPA	CBZ
Serum levels	0.4–1.0 mEq/L	50–125 μg/μL (0.35–0.87 mM)	4–12 μg/μL (17–51 μM)
CSF/Serum ratio	0.58[59]	0.15[60]	0.21[61]
CSF levels	0.23–0.58 mEq/L	52–130 μM	3.6–10.7 μM

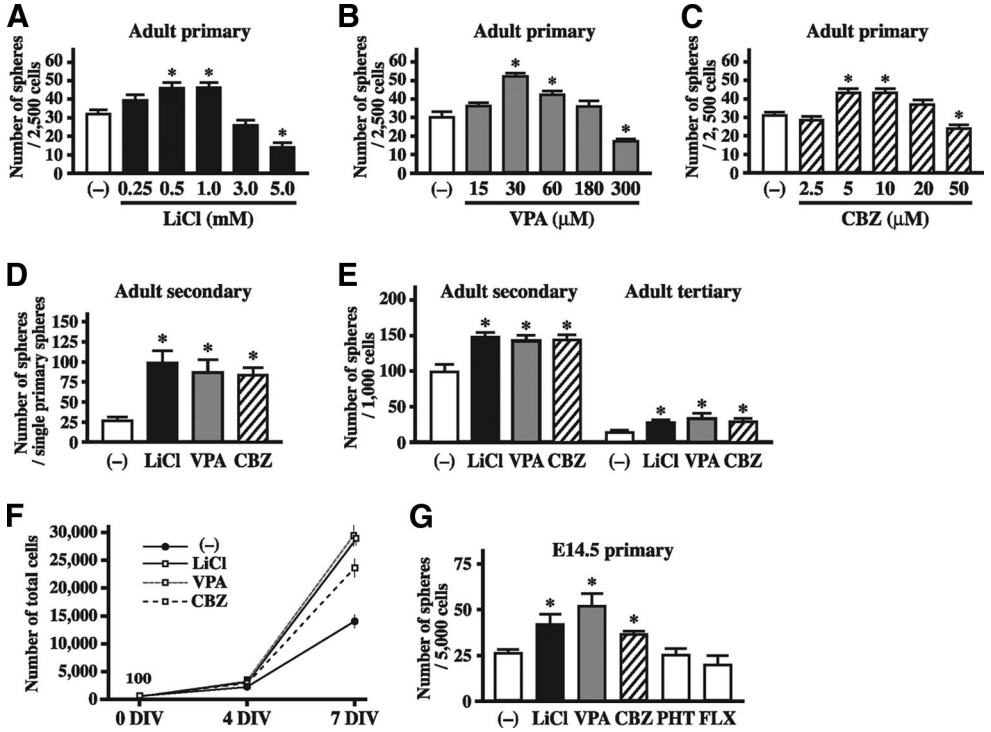

Figure 1. Effects of mood stabilizers on neurosphere formation in vitro. **(A–C):** Cells from the adult SVZ were cultured in the presence of LiCl **(A)**, VPA **(B)**, or CBZ (HBC complex, Sigma) **(C)**. All drugs increased the numbers of primary neurospheres when added at therapeutic concentrations and decreased the numbers at high concentrations as compared to control ($n \geq 6$ for each concentration from at least three independent experiments). **(D–F):** The resultant primary neurospheres were subcloned to generate secondary and tertiary neurospheres in the absence of the drugs. **(D):** Single primary neurospheres were triturated and one tenth of the cells were cultured to form secondary neurospheres. The numbers of resultant secondary neurospheres are expressed as per single primary neurospheres. ($n = 5$ animals, 4–8 neurospheres from each animal). **(E):** Neurospheres were serially passaged in bulk and cultured in the absence of the drugs at a clonal density (two cells/μL). Cells from the primary neurospheres treated with 0.5 mM LiCl, 60 μM VPA or 5 μM CBZ, produced more secondary and tertiary neurospheres than their control counterpart ($n = 5$). **(F):** Cells from the primary neurospheres grown in the presence of 0.5 mM LiCl, 60 μM VPA or 5 μM CBZ were plated at 0.5 cells/μL and cultured in the absence of the drugs. Total cell numbers after 4 or 7 days in vitro were counted ($n = 4$). **(G):** Effects of mood stabilizers at the therapeutic concentrations (0.5 mM LiCl, 60 μM VPA and 5 μM CBZ) were examined using cells from the E14.5 medial ganglionic eminence. Mood stabilizer treatment increased primary neurosphere formation, whereas 10 μM PHT or 1.0 μM FLX showed no effect ($n \geq 4$). Data represent means ± SEM. *, $p < .05$. Abbreviations: CBZ, carbamazepine; FLX, fluoxetine; PHT, phenytoin; VPA, valproic acid.

RESULTS

In Vitro Effects of Mood Stabilizers

To examine the effects of mood stabilizing drugs on primary neurosphere formation, cells from the SVZ of 8-week-old male mice were cultured at low cell density (five cells/μL) in the presence of the drugs. LiCl at 0.5–1.0 mM (within the therapeutically relevant concentrations in the CSF) (Table 1), increased the number of primary neurospheres, whereas LiCl at concentrations greater than 3.0 mM showed toxic effects on primary neurospheres ($F_{(5, 61)} = 24.69$, $p < .001$) (Fig. 1A). Similarly, at doses equivalent to therapeutically relevant CSF concentrations, both VPA ($F_{(5, 57)} = 35.85$, $p < .001$) and CBZ ($F_{(4, 56)} = 11.96$, $p < .001$) increased the number of primary neurospheres from the adult SVZ (Fig. 1B, 1C).

These results could be interpreted in two ways: mood stabilizers could enhance the survival of cultured NSCs, [1] or mood stabilizers could increase the self-renewal capability of neural stem/progenitor cells [2] and thereby increase the number of neurosphere-initiating cells, although these possibilities are not mutually exclusive. To distinguish between those possibilities, we first investigated whether or not mood stabilizers prevent cell death by means of counting dead cells by trypan blue exclusion. The number of dead cells 24 hours after the plating was comparable between drug-treated and non-treated cells (14.71 ± 4.07% for control, 15.29 ± 4.18% for LiCl, 13.47 ± 3.81% for VPA, 13.88 ± 3.60% for CBZ, mean ± SEM, $F_{(3, 64)} = 0.75$, $p = .53$). We then subcloned primary

neurospheres that had been grown in the presence of 0.5 mM LiCl, 60 μM VPA, or 5 μM CBZ and cultured the cells in the absence of the drugs to generate secondary neurospheres. Because primary neurospheres are clonally derived from single NSCs when the adult SVZ cells are cultured at low cell density [44] (see Discussion), the number of secondary neurospheres derived from each primary neurosphere represents the number of symmetric expansive divisions of the original NSCs during the culture. We cultured one-tenth of the cells from each primary neurosphere to keep the cell densities below two cells/μL and found that a greater number of secondary neurospheres were generated from single primary neurospheres treated with mood stabilizers than those without treatment ($F_{(3,\ 16)} = 7.024$, $p = .003$) (Fig. 1D). It is unlikely that these effects are mediated by histone deacetylase inhibitor activities because trichostatin A, another histone deacetylase inhibitor, neither increased the number of primary neurosphere nor enhanced the self-renewal but, inversely, alleviated the primary neurosphere formation at 10 nM or higher concentrations ($F_{(4,\ 11)} = 35.46$, $p < .001$) (supplemental online Fig. 1).

The self-renewal capacity of NSCs was also analyzed through serial bulk passaging of neurospheres, in which drug-treated or control neurospheres were dissociated in bulk and cultured at a constant low cell density (two cells/μL) in the absence of the drugs (Fig. 1E). Bulk passaging demonstrated that NSCs treated with mood stabilizers in the primary culture exhibited enhanced self-renewal, with increased numbers of both secondary ($F_{(3,\ 16)} = 10.36$, $p < .001$) and tertiary neurospheres ($F_{(3,\ 16)} = 5.46$, $p = .009$) as compared to controls (Fig. 1E). The proliferation of cells from drug-treated primary neurospheres was examined by plating cells at a very low density (0.5 cells/μL) and counting cell numbers 4 or 7 days later (Fig. 1F). Cells obtained from primary neurospheres grown in the presence of mood stabilizers proliferated more rapidly than those from control neurospheres ($F_{(3,\ 12)} = 10.45$, $p = .001$) (Fig. 1F). These results suggest that in vitro treatment with therapeutically relevant doses of mood stabilizers enhances the self-renewal of NSCs from the adult brain.

The effect of mood stabilizers on NSCs derived from the MGE ventricular zone of E14.5 embryos was also evident ($F_{(4,\ 64)} = 10.41$, $p < .001$), whereas another anti-convulsant, phenytoin, or anti-depressant, fluoxetine, had no such effect (Fig. 1G), suggesting that the effect was specific to mood stabilizing drugs. Individual neurospheres treated with mood stabilizers produced βIII tubulin$^+$ neurons, GFAP$^+$ astrocytes, and O4-antigen$^+$ oligodendrocytes under differentiation conditions (online supplemental Fig. 2), suggesting that the multipotentiality of NSCs was not altered by the drug treatment.

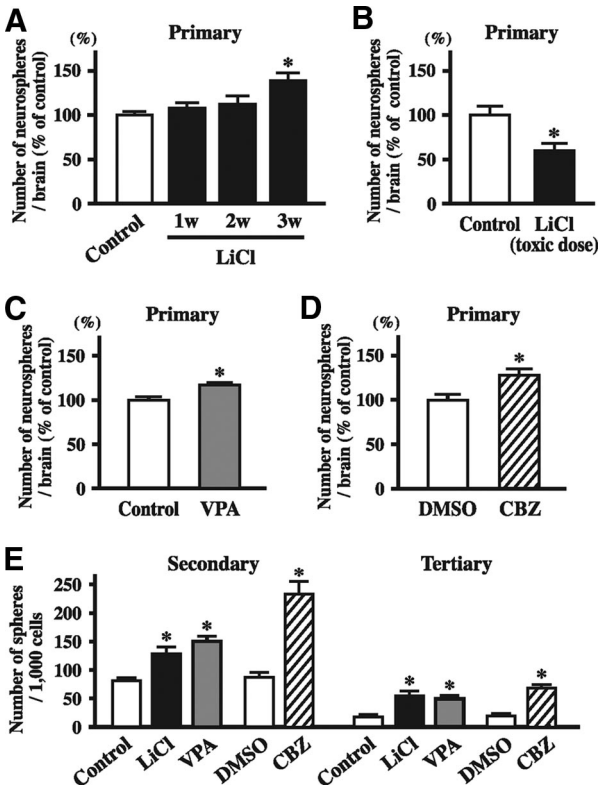

Figure 2. Number of neural stem cells in the adult brain subventricular zone was determined by the neurosphere assay. **(A):** The total number of neural stem cells in the lateral portion of the SVZ was increased in mice treated with LiCl (0.86 $\pm$ 0.23 mEq/l, mean $\pm$ SD) for 3 weeks ($n = 10$) but not for 1 week ($n = 9$) or 2 weeks ($n = 6$) as compared to controls ($n = 21$). **(B):** The total numbers of neural stem cells in the mice, which were treated with higher doses of LiCl and subsequently showed toxic levels of lithium in their sera (4.36–6.59 mEq/l) ($n = 3$), were reduced relative to control ($n = 4$). **(C, D):** Mice were given VPA or CBZ for 3 weeks and were subjected to the neurosphere assay. The total numbers of neural stem cells in the lateral portions of the SVZ were increased in mice treated with VPA ($n = 6$) as compared to controls ($n = 8$) **(C)**, and in mice treated with CBZ ($n = 10$) as compared to DMSO-injected controls ($n = 8$) **(D)**. **(E):** Serial bulk passages of neurospheres were performed at a clonal density (two cells/μL) every 6 days. The primary neurospheres derived from mice given LiCl ($n = 9$, serum Li^+ concentrations at 0.22–1.19 mEq/L) or VPA ($n = 9$) or CBZ ($n = 3$) produced more secondary and tertiary neurospheres than their control counterpart. Data represent means $\pm$ SEM. *, $p < .05$. Abbreviations: CBZ, carbamazepine; DMSO, dimethyl sulfoxide; VPA, valproic acid.

In Vivo Effects of Mood Stabilizers

Next, we used the neurosphere assay to determine whether lithium treatment increased the number of neurosphere-forming NSCs in the SVZ of the adult brain. Because each primary neurosphere is clonally derived from a single NSC when cultured at low cell densities, the total number of NSCs in the SVZ can be estimated by counting primary neurospheres [34, 36, 45]. When LiCl was administered to mice for 3 weeks (3 g/L in drinking water), serum lithium concentrations reached 0.86 $\pm$ 0.23 mEq/L (mean $\pm$ SD), a value close to that observed during therapeutic management of bipolar patients [32]. The total number of neurosphere-forming NSCs in the lateral portion of the SVZ was increased in lithium-treated animals compared to controls, and this increase was statistically significant only after 3-week treatment but not after 1- or 2-week treatment ($F_{(3,\ 42)} = 8.36$, $p < .001$) (Fig. 2A). In contrast, treatment with higher doses of LiCl, which subsequently resulted in higher serum concentrations of 4.36–6.59 mEq/L, reduced the number of neurosphere-forming NSCs in the adult brain ($F_{(1,\ 6)} = 8.12$, $p = .036$) (Fig. 2B).

We asked if a similar stimulatory effect occurred following treatment with VPA or CBZ. Mice given VPA (0.5 g/L in drinking water) or CBZ (1 mg/kg body weight by intraperitoneal injection, twice daily) achieved much lower drug concentrations in their sera (~6.0 μg/mL for VPA and 0.5 μg/mL or lower for CBZ) than observed during treatment of bipolar patients [32]. Nevertheless, administration of VPA and CBZ produced similar increases in the numbers of neurosphere-forming NSCs. Compared to controls, the total number of NSCs in the SVZ was increased in both VPA-treated mice and in CBZ-treated mice ($F_{(1,\ 12)} = 11.10$, $p = .006$ and $F_{(1,\ 16)} = 9.38$, $p = .007$, respectively) (Fig. 2C, 2D). In contrast, the number of NSCs in the SVZ from mice treated with phenytoin (200 mg/L drinking water, 3 weeks), an anticonvulsant lacking mood stabilizing action, was comparable to control (95.1 $\pm$ 7.6% of control, $n = 3$).

To determine the effect of mood stabilizer administration on the self-renewal capacity of NSCs, primary neurospheres were serially passaged in bulk and cells were cultured at low cell density (two cells/μL) in the absence of the drugs. NSCs derived from the SVZ of mice which had been given mood stabilizers and showed serum drug concentrations at or below the therapeutic doses, exhibited enhanced self-renewal. That is, we observed increased numbers of both secondary ($F_{(2, 21)} = 10.36$, $p < .001$ for LiCl and VPA and $t_{(4)} = 5.85$, $p = .004$ for CBZ) and tertiary neurospheres ($F_{(2, 20)} = 9.74$, $p = .001$ for LiCl and VPA and $t_{(4)} = 9.10$, $p < .001$ for CBZ) as compared to appropriate controls (Fig. 2E). Thus, these results suggest that administration of mood stabilizers enhances the self-renewal of NSCs in the adult brain and expands the NSC pool in the SVZ.

At this point, we focused on lithium treatment for further in vivo analyses, because administration of LiCl by drinking water for 3 weeks or longer consistently resulted in serum Li^+ concentrations in the range 0.4–1.2 mEq/L, whereas 3-week treatments with VPA or CBZ produced low and variable drug concentrations. In these trials, the number of NSCs in the SVZ of treated mice was assessed by a different method. After 3 weeks of treatment, mice received 5 BrdU injections every 3 hours and were sacrificed 4 weeks after the last injection. During the 4-week period following the last injection, transit-amplifying neural progenitor cells, having incorporated BrdU, diluted the label by further divisions, differentiated and migrated away from the SVZ, or died [46]. $BrdU^+$ cells that remained in the SVZ for 4 weeks are considered relatively quiescent NSCs. $BrdU^+$ cells in the SVZ were increased in number in the mice given LiCl as compared to control ($t_{(10)} = -3.97$, $p = .003$) (Fig. 3A).

Next, we asked if the lithium-induced increase of NSCs in the SVZ also increased the number of neural progenitors and subsequent neurogenesis. After 3 weeks of lithium treatment, mice received 5 BrdU injections every 3 hours and were sacrificed 1 hour (total 13 hours, Fig. 3B) or 7 days (Fig. 3C) after the last injection. BrdU labeling over 13 hours that encompasses estimated cell cycle times of neural progenitor cells [12] demonstrated more dividing cells in the SVZ of lithium-treated mice than control ($F_{(1, 15)} = 5.13$, $p = .039$ for SVZ) (Fig. 3B), suggesting that neural progenitor cells were also increased after chronic lithium treatment. As a result of this increase in proliferating cells in the SVZ, more $BrdU^+$ cells survived and migrated to the olfactory bulb ($t_{(8)} = -2.82$, $p = .022$) of LiCl-treated mice than that of control (Fig. 3C). These findings demonstrate that lithium administration expanded the size of the NSC pool and increased neural progenitor proliferation in the SVZ, resulting in increased cell supply to the olfactory bulb in the adult brain.

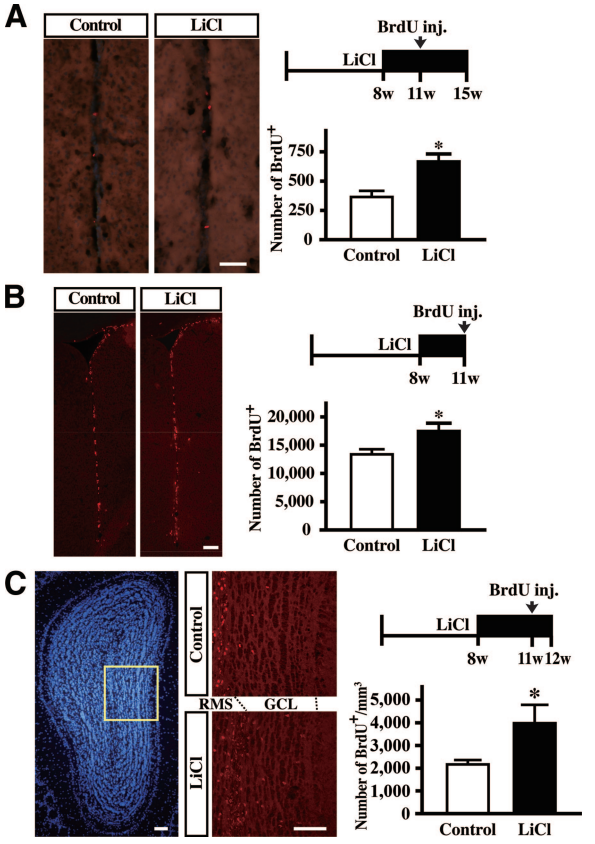

Figure 3. Lithium increases $BrdU^+$ cells in the adult brain. Mice were administered LiCl for 3 weeks, received 5 BrdU injections (65 mg/kg) every 3 hours, and then sacrificed 4 weeks **(A)**, 1 hour **(B)**, or 7 days **(C)** after the last injection. Cryosections were immunostained for BrdU incorporation, which overlapped the nuclear staining with Hoechst 33,342. **(A):** More $BrdU^+$ cells which remained in the SVZ for 4 weeks after the labeling were detected in the mice given LiCl ($n = 6$) than in control ($n = 6$). **(B):** BrdU labeling demonstrated more dividing cells in the SVZ of lithium-treated mice ($n = 10$) than control ($n = 7$). **(C):** $BrdU^+$ cells that migrated to the GCL of the olfactory bulb 7 days after the labeling were counted. Representative higher magnification images for BrdU positivity (red, right panels) show a similar region as indicated by a box in a lower magnification image for Hoechst 33,342 nuclear staining (blue, left panel). More $BrdU^+$ cells were observed in the GCL of lithium-treated mice ($n = 4$) than in that of control mice ($n = 6$). Scale bars = 100 μm. Data represent means ± SEM. *, $p < .05$. Abbreviations: RMS, rostral migratory stream; BrdU, 5-bromo-2′-deoxyuridine; GCL, granular cell layer.

Notch Signaling Mediates the Effects of Mood Stabilizers.

Recent studies suggest that Notch signaling plays a critical role in the maintenance of NSCs [13, 47, 48]. Therefore, we investigated whether mood stabilizers activate Notch signaling in neurospheres. RT-PCR analyses revealed that two downstream genes of the Notch pathway, *Hes1* and *Hes5*, were upregulated in all three-drug-treated neurosphere cultures relative to control (Fig. 4A), which was confirmed by quantitative RT-PCR ($F_{(3, 20)} = 4.94$, $p = .01$) (supplemental Fig. 3A). We next performed a reporter assay using a luciferase gene plasmid, in which the firefly luciferase gene was placed downstream of either the *Hes1* or *Hes5* gene promoter [38]. Treatment with 0.5 mM LiCl, or 60 μM VPA, or 5 μM CBZ enhanced both *Hes1* ($F_{(3, 44)} = 68.34$, $p < .001$) and *Hes5* ($F_{(3, 44)} = 23.48$, $p < .001$) gene promoter activities (Fig. 4B and online supplemental Fig. 3B). Western blotting revealed that all three mood stabilizers increased Hes1 protein in neurosphere cultures ($F_{(3, 8)} = 10.78$, $p = .004$) (Fig. 4C and online supplemental Fig. 3C). Thus, mood stabilizing drugs at therapeutic concentrations upregulate the expression of *Hes1/5* genes, suggesting activation of the Notch signaling pathway.

To obtain direct evidence of Notch signaling activation, we quantified amounts of an active form of Notch1 receptor in neurospheres by Western blotting. Anti-cleaved Notch1 antibody recognizes the Notch intracellular domain, which is a direct measure of the amount of Notch signaling activation. Primary neurospheres exposed to LiCl, VPA, or CBZ contained significantly more cleaved Notch1 than the control neurospheres ($F_{(3, 12)} = 11.63$, $p < .001$) (Fig. 4D and online supplemental Fig. 3D).

If mood stabilizers enhance NSC expansion through Notch signaling, then inhibition of Notch signaling should block this enhancement. Indeed, addition of 1 μM γ-secretase inhibitor,

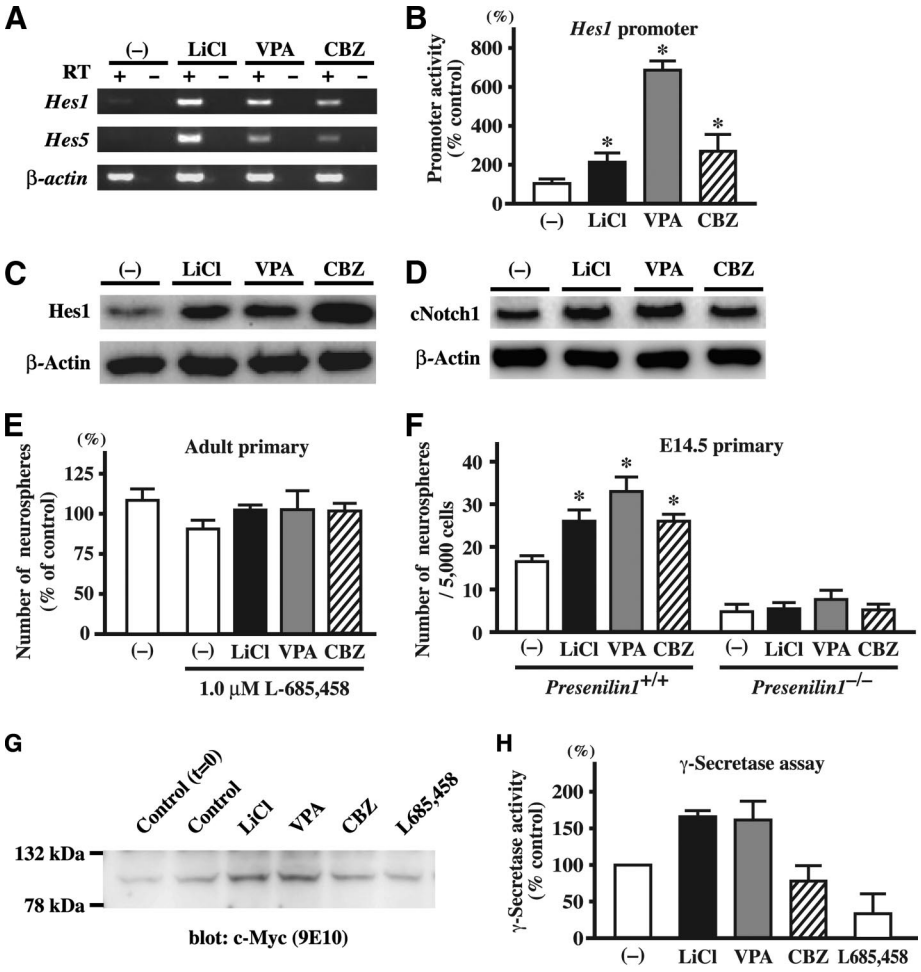

Figure 4. Mood stabilizers activate Notch signaling in neurospheres. **(A, C, D):** Primary neurospheres were grown in the presence of 0.5 mM LiCl, 60 μM VPA, or 5 μM CBZ. **(A):** RNA from the neurospheres was subjected to RT-PCR analysis. RT $\pm$ indicates the presence/absence of reverse transcriptase in the assay. **(B):** Firefly luciferase reporter assay was performed using secondary neurospheres. Treatment with 0.5 mM LiCl, 60 μM VPA, or 5 μM CBZ enhanced *Hes1* gene promoter activities. **(C, D):** Protein extracts from the primary neurospheres were separated on SDS-PAGE, and then immunoblotted with anti-Hes1 **(C)** and anti-cleaved Notch1 **(D)** antibody. **(E):** Primary neurospheres were formed in the presence of therapeutic doses of mood stabilizers together with 1.0 μM γ-secretase inhibitor, L-685,458, which inhibits the activation of Notch signaling. **(F):** The medial ganglionic eminence cells from E14.5 *presenilin1*$^{-/-}$ ($n = 8$) and their wild-type littermates ($n = 8$) were cultured. Mood stabilizers enhanced the formation of primary neurospheres from wild-type cells but showed few effects on those from *presenilin1*$^{-/-}$ cells. **(G, H):** γ-Secretase assay was performed in the presence of 0.5 mM LiCl, 60 μM VPA, 5 μM CBZ, or 10 μM L-685,458. Myc-tagged cleaved Notch1 intracellular domain was immunoblotted with anti-c-myc antibody **(G)**. **(H):** The bands were quantified and relative levels of myc-tagged cleaved Notch1 to control as 100% are shown. All experiments were carried out four or more times. Data represent means $\pm$ SEM. *, $p < .05$. Abbreviations: CBZ, carbamazepine; RT, reverse transcription; VPA, valproic acid.

L-685,458, to cultures abolished the effects of LiCl, VPA, and CBZ on primary neurosphere formation (Fig. 4E). Presenilin1 is a major component of the γ-secretase complex or γ-secretase itself [49] and homozygous mutation for the *presenilin1* gene results in the depletion of NSCs due to the impaired activation of Notch signaling [13]. We examined the effects of mood stabilizers on neurosphere formation from MGE cells of E14.5 *presenilin1*$^{-/-}$ embryos because those embryos die around the time of birth [33]. In contrast to wild-type controls, where mood stabilizers increased the number of primary neurospheres, those drugs exhibited few effects, if any, on primary neurosphere numbers from *presenilin1*$^{-/-}$ NSCs (Fig. 4F). Finally, we examined whether or not mood stabilizers modify γ-secretase activities using γ-secretase source from neurospheres and myc-tagged full-length Notch1 as a substrate. Indeed, γ-secretase activities were enhanced in the presence of therapeutic doses of LiCl and VPA by ~60%, but not in the presence of CBZ (Fig. 4G, 4H). These results suggest that mood stabilizers enhance the self-renewal capability of NSCs by activating Notch signaling and that these effects are mediated by, at least in part, the augmentation of γ-secretase activities.

Both lithium and VPA have been shown either directly or indirectly to inhibit GSK-3β [20–23], a key mediator of Wnt and other signal pathways, and inhibit inositol monophosphatase (IMPase), leading to inositol depletion [16–19]. Therefore, we determined whether or not effects of mood stabilizers on neurospheres were mediated by the inhibition of GSK-3β signaling or inositol depletion. We examined levels of phosphorylated GSK-3β at Ser9, an inactive form of GSK-3β, in the neurospheres by Western blotting following drug treatment. Treatment with therapeutic doses of mood stabilizers did not alter the ratio of phosphorylated to total GSK-3β (Fig. 5A, 5B), whereas high doses of mood stabilizers increased the phosphorylation of GSK-3β ($F_{(3,\ 16)} = 4.86$, $p = .014$) (Fig. 5C, 5D).

Similarly, therapeutic concentrations of these drugs did not change the expression of *IMPase 1/2* genes (Fig. 5E). By

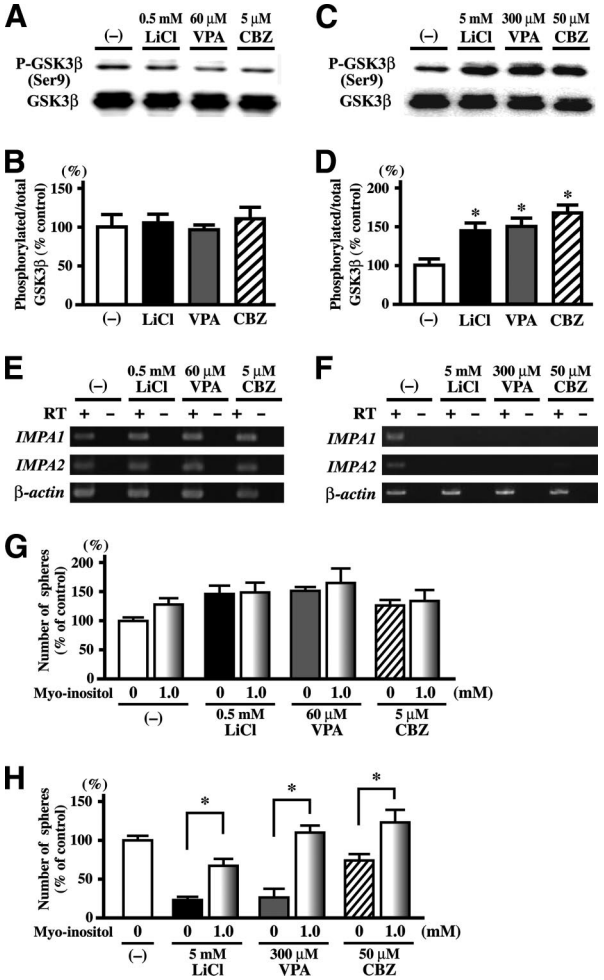

Figure 5. Effects of mood stabilizers on GSK-3β signaling and the inositol pathway. **(A):** Primary neurospheres from the adult SVZ were generated in the presence of 0.5 mM LiCl, 60 μM VPA, or 5 μM CBZ. Protein extracts of the neurospheres were separated on SDS-PAGE and then immunoblotted with anti-GSK-3β or anti-phospho-GSK-3β (Ser9) antibodies. **(B):** The bands were quantified and the ratio of phosphorylated to total GSK-3β was calculated. **(C):** Primary neurospheres were grown and toxic doses of mood stabilizers were added to the culture at 5 days in vitro. After 15-minute treatment, protein extracts of the neurospheres were subject to Western blotting. **(D):** The bands were quantified and the ratio of phosphorylated to total GSK-3β was calculated. **(E, F):** Total RNA was extracted from primary adult neurospheres cultured with mood stabilizers at therapeutic **(E)** or higher concentrations **(F)** and subject to RT-PCR analysis. Mood stabilizers at the therapeutic concentrations showed few effects on the expression levels of *IMPA1/2* in the neurospheres **(E)**, whereas the drugs at the higher concentrations downregulated gene expression **(F)**. RT $\pm$ indicates the presence/absence of reverse transcriptase in the assay. **(G, H):** Effects of inositol rescue were determined by adding 1 mM myo-inositol in cultures of the neurosphere assay. Myo-inositol did not alter the effects of 0.5 mM LiCl, 60 μM VPA, or 5 μM CBZ, which increased the number of primary neurospheres **(G)**, but resumed the toxic effects of 5 mM LiCl, 300 μM VPA or 50 μM CBZ **(H)**. Data represent means $\pm$ SEM. from 3–5 independent experiments. *, $p < .05$. Abbreviations: CBZ, carbamazepine; VPA, valproic acid.

contrast, coculture with higher concentrations of mood stabilizers lowered the expression levels of those genes (Fig. 5F). Addition of myo-inositol to cultured cells rescue the depletion of inositol [18]. Adult SVZ cells were cultured to generate primary neurospheres in the presence of 1.0 mM myo-inositol together with lithium, VPA and CBZ. Myo-inositol itself showed virtually no ability to increase the number of neurospheres and did not reverse the effects of mood stabilizers at therapeutic concentrations (Fig. 5G). By contrast, coculture with 1.0 mM myo-inositol reversed the toxic effects of mood stabilizers, observed in the presence of 5.0 mM LiCl, 300 μM of VPA, and 50 μM of CBZ (Fig. 5H). Together, these results suggest that Notch signaling activation in the neurospheres by therapeutic doses of mood stabilizers is not mediated by the alteration of GSK-3β or inositol signaling.

Notch Signaling Activation In Vivo

Activation of Notch signaling in NSCs by mood stabilizers was evident not only in vitro but also in vivo. In situ hybridization assessment of *Hes1* and *Hes5* gene expression showed that *Hes1/5*-expressing cells were increased in the SVZ of mice treated with LiCl for 3 weeks (Fig. 6A, 6B). The lateral portion of the SVZ was excised and then homogenized in lysis buffer. Comparable amounts of protein were obtained from each mouse (control, 1.79 $\pm$ 0.42 mg (n = 12) vs. LiCl, 1.77 $\pm$ 0.30 mg (n = 12), mean $\pm$ SD), verifying equivalent tissue isolation. Samples of 20 μg protein were separated on SDS-PAGE and immunoblotted with anti-cleaved and anti-total Notch1 antibody (Fig. 6C, 6D). Higher amounts of cleaved Notch1 were detected in the SVZ of the mice treated with lithium for 3 weeks relative to control mice ($F_{(1,\ 18)} = 75.38$, $p < .001$) (Fig. 6E) and an insignificant increase of total Notch1 protein was also observed (Fig. 6F). Increase of cleaved Notch1 protein level was only detected after 3-week, but not 1-week, treatment by lithium (online supplemental Fig. 4A, 4B). These results indicate that lithium treatment results in either increased amounts of activated Notch in individual neural stem/progenitor cells or increased numbers of neural stem/progenitor cells with equivalent levels of activated Notch. In contrast, GSK-3β signaling was not altered by lithium treatment in vivo (online supplemental Fig. 4C, 4D).

DISCUSSION

In the present study, we demonstrated that three different mood stabilizing drugs directly enhanced NSCs' self-renewal capability through activation of Notch signaling. Furthermore, lithium administration expanded the size of the NSC pool in the SVZ of the adult brain, resulting in an increased cell supply to the olfactory bulb.

Several intrinsic and extrinsic factors are known to alter neurogenesis in the adult brain, especially in the dentate gyrus of the hippocampus [11]. However, the growth kinetics of NSCs in the adult brain are largely unknown, at least in part because there are no specific markers currently available that discriminate NSC from transit amplifying neural progenitor cells. In this study, we utilized an in vitro colony-forming neurosphere assay in which NSCs respond to growth factors in serum-free media, proliferate and form clonal neurospheres. The clonality of the neurosphere has recently been challenged as demonstrated by an observation that cells separately prepared from neurospheres constitutively expressing EGFP and neurospheres constitutively expressing β-galactosidase generated a substantial percentage of chimeric neurospheres even when cultured at low cell densities (0.5 cells/μL) [50]. However, in mixing experiments using wild-type and GFP-expressing cells from the adult SVZ, we have never observed primary neurospheres that consisted of both wild-type and GFP$^+$ cells (online supplemental Fig. 5A, 5B). Those results suggest that adhesion and mixing events between two or more neurosphere-initiating NSCs are rare or, at least, negligible when estimating the number of NSCs in the

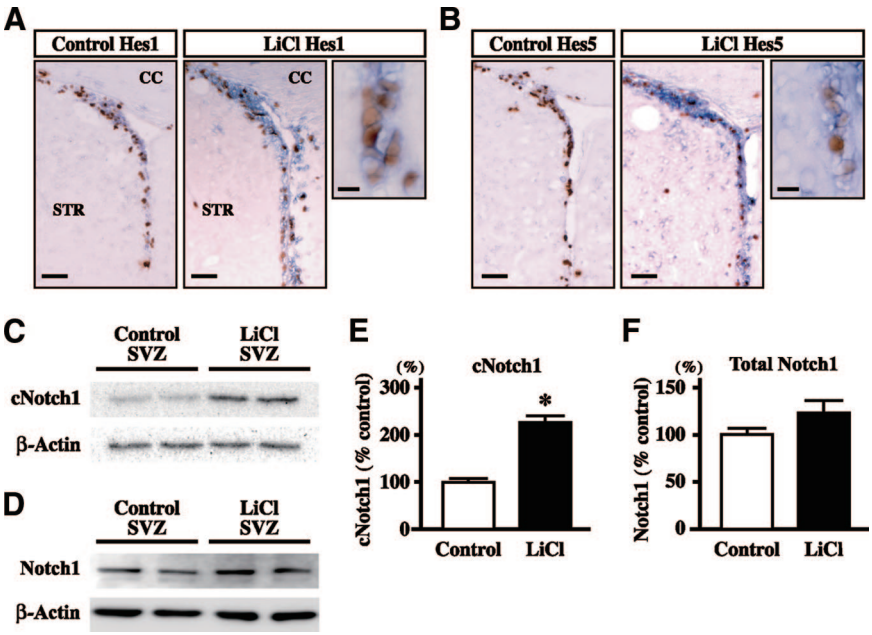

Figure 6. Lithium administration activates Notch signaling in the SVZ of the adult brain. **(A, B):** Mice were given LiCl for 3 weeks and received 5 BrdU injections. Cryosections were analyzed by in situ hybridization for *Hes1* **(A)** and *Hes5* **(B)** gene expression (purple) and double labeled for BrdU incorporation (brown). Lithium treatment for 3 weeks increased the number of *Hes1/5*-expressing cells in and close to the SVZ, where $BrdU^+$ neural stem/progenitor cells resided. Scale bar = 50 μm (lower magnification) or 10 μm (higher magnification). **(C–F):** The SVZ tissue was subjected to Western blotting. Treatment with lithium for 3 weeks resulted in a significant increase of cleaved Notch1 ($n = 8$) **(C, D)** and an insignificant increase of total Notch1 protein ($n = 4$) **(E, F)**. Data represent means ± SEM. *, $p < .05$. Abbreviations: CC, corpus callosum; STR, striatum.

SVZ. The in vivo BrdU labeling analyses support our conclusion that chronic lithium treatment expands the size of the NSC pool in the SVZ. The discrepancy between the data from Singec et al. and our data may be due to the fact that cells derived from neurospheres that keep proliferating in vitro [50] are highly motile and adhere to each other more easily than primary cells from the SVZ. Alternatively, non-adhesive culture plates used in the study by Singec et al. [50] may substantially enhance the cell movement and fusion events during the neurosphere formation (online supplemental Fig. 5C, 5D).

Notch signaling plays critical roles in the development of the central nervous system, as revealed by studies of mice deficient for Notch pathway molecules [51]. Attenuation of Notch signaling leads to the reduction of neural stem/progenitor cells and precocious neuronal differentiation in developing brains, suggesting that activation of Notch signaling keeps the NSC undifferentiated and expands the NSC pool by promoting symmetrical and self-renewing divisions. In addition, NSCs are decreased by 40% in the brains of adult *presenilin1* heterozygous mutant mice, and conversely, introduction of an active form of Notch1 receptor by retrovirus infection increases the number of NSCs in the adult brain [13]. Thus, the modification of Notch signaling activities alters the size of the NSC pool postnatally. Our present results suggest that treatment with mood stabilizers at therapeutic concentrations activates Notch signaling in neural stem/progenitor cells in the adult brain. Activation of Notch signaling results in the expansion of the NSC pool and subsequently increased production of neural progenitor cells in the SVZ and the olfactory bulb. Interestingly, Notch activation is reported in human neuroblastoma cells after treatment with VPA in vitro [52], although this effect was only observed at very high doses (1–2 mM). It remains to be investigated whether sensitivity to the drug differs between NSCs in the adult brain and cancerous cells or whether neuroblastoma cells lose their sensitivity after transformation and extended culture in vitro.

Consistent with other findings [28, 29], our data indicate that all three mood stabilizers increase the number of NSCs and that they therefore enhance neurogenesis in vivo. Increased neurogenesis in the olfactory bulb after chronic treatment with mood stabilizers may simply reflect the increase of NSCs, or alternatively, mood stabilizers may have direct effects on neurons to facilitate their survival or maturation [53]. A multitude of studies suggest that mood stabilizing drugs inhibit GSK-3β activities in neurons [20–23] and that this event may alter several different signaling cascades. One of the main effects of GSK-3β inhibition is accumulation of β-catenin, which has antiapoptotic effects and may enhance the survival of neurons [54, 55]. Mood stabilizers also inhibit IMPase and cause inositol depletion [16–19], which subsequently inhibits the collapse of sensory neuron growth cones and may facilitate axonogenesis in vitro [18]. Although we could detect few effects on GSK-3β or inositol signaling activities in neural stem/progenitor cells, it remains formally possible that the drugs have less apparent but biologically significant effects on these signaling in postmitotic neurons. Our data do not exclude a possibility that mood stabilizers modify other signal pathways than Notch signaling in NSCs as well as non-stem cells in the adult brain.

It is currently unknown whether the effects of mood stabilizers on the NSC and neurogenesis in the adult brain are related to the pharmacological actions of the drugs to ease symptoms of bipolar disorder. It is well documented that neurogenesis is suppressed in animal models of stress or depression [56, 57]. Importantly, hippocampal neurogenesis is necessary for antidepressant drugs to improve behavioral deficits in stressed mice [58]. Therefore, it would be reasonable to hypothesize that impaired neurogenesis is causally related to the depressive state and that mood stabilizers attenuate the symptoms of at least the depressive phase of bipolar disorder by enhancing neurogenesis in the hippocampus. It is currently difficult to explain how mood stabilizers prevent bipolar disorder patients from proceeding to the manic phase, because the molecular and cellular mechanisms underlying the pathogenesis of mania are largely unknown and animal models that fully reproduce the symptoms of mania are not available. Nevertheless, our working hypothesis that the pharmacological action of mood stabilizers is mediated by the time-consuming expansion of NSC pool in the adult brain, which is achieved by Notch signaling activation, is attractive because it may explain why clinical benefits of the drugs are observed only after chronic treatment [32]. It is tempting to investigate the modification of neuronal network following the expansion of NSC pool and enhanced neurogenesis by mood stabilizer treatment.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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