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Lithium enhances long-term potentiation independently of hippocampal neurogenesis in the rat dentate gyrus

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Abstract

We measured the temporal and spatial profiles of neural precursor cells, hippocampal long-term potentiation (LTP), and signaling molecules in neurogenesis-induced adult rats. Chronic lithium treatment produced a significant 54% and 40% increase in the numbers of bromodeoxyuridine [BrdU(+)] cells after 12 h and 28 days, respectively, after treatment completion in the dentate gyrus (DG). Both LTP obtained from slices perfused with artificial cerebrospinal fluid (ACSF-LTP) and LTP recorded in the presence of bicuculline (bicuculline-LTP) were significantly greater in the lithium group than in the saline controls. Although the number of BrdU(+) cells, approximately 90% of which were double-labeled with a neural marker neuronal nuclear protein, were markedly increased in the granule cell layer (GCL) 28 days after the completion of the 28-day lithium treatment, the magnitude of LTP observed at this time was similar to that observed 12 h after completing the 28-day lithium treatment. However, protein levels of calcium and calmodulin-dependent protein kinase II, p-Elk and TrkB were highly elevated until 28 days after the 28-day lithium treatment. Acute lithium treatment for 2 days also enhanced LTP, which was accompanied by the elevated expression of p-CREB, but not by neurogenesis. Our results suggest that the enhancement of LTP is independent of the increased number of neurons *per se* and it is more closely associated with key molecules, which are probably involved in neurogenesis.

Keywords: dentate gyrus, lithium, long-term potentiation, neurogenesis, rat, synaptic plasticity.

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Lithium is widely used as a mood-stabilizing drug to treat manic-depressive disorders (Manji *et al*. 1999), and although it has been used in the psychiatric domain for many years, lithium targets and actions are still unclear at the biochemical and neurophysiological level. It has been previously demonstrated that lithium affects several signaling cascades in the brain; it increases bcl-2 levels (Kempermann *et al*. 1997; Chen *et al*. 1999; Manji *et al*. 1999), the mRNA levels of G proteins (Li *et al*. 1991), cyclic AMP levels (Masana *et al*. 1992), the phosphorylation of CREB (cAMP-response element binding protein; Ozaki and Chuang 1997), the accumulation of inositol 1,4,5-triphosphate (IP3) in *in vitro* brain slices (Dixon *et al*. 1994), BDNF (brain-derived neurotrophic factor) expression (Fukumoto *et al*. 2001), glutamate release into medium (Dixon *et al*. 1994), and the inhibition of glycogen synthase kinase (GSK3; Grimes and Jope 2001). More recently, it has been demonstrated that chronic lithium increases neurogenesis in the DG of adult rodents (Chen *et al*. 2000). The adult neurogenesis occurs in restricted brain regions, especially in the subventricular zone and the subgranular layer of the hippocampus (Kempermann and Gage 1999; Gould and Gross 2002). Adult neurogenesis is affected by age, exercise

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Abbreviations used: ACSF, artificial cerebrospinal fluid; BrdU, bromodeoxyuridine; CaMKII, calcium and calmodulin-dependent protein kinase II; CREB, cAMP response element binding protein; DAPI, 4′, 6-diamidino-2-phenylindole; DG, dentate gyrus; EPSP, excitatory postsynaptic potential; FITC, fluorescein isothiocyanate; GCL, granule cell layer; GFAP, glial fibrillary acidic protein; MAPK, mitogen-activated protein kinase; MAP2, microtubule associated protein-2; MPP, medial perforant pathway; NeuN, neuronal nuclear protein; NGS, normal goat serum; PBS, phosphate-buffered saline; SGZ, subgranular zone; TrkB, tyrosine kinase receptor B; TS, tetanic stimulation.

(Kempermann *et al*. 2002), antidepressants (Duman *et al*. 2001), stress (McEwen 2001), enriched environment (Nilsson *et al*. 1999; Kempermann *et al*. 2002), and ischemia (Kee *et al*. 2001). Despite a substantial body of evidence that mammalian dentate gyrus (DG) produces a large number of neurons in adult individuals (Kempermann *et al*. 1997; Chen *et al*. 2000), there is still a dearth of knowledge concerning its functionality and physiology. One attractive issue is the functionality of the adult-generated young neurons and the contribution they make to hippocampal plasticity and hippocampal dependent learning. A reduction in the number of newly generated neurons was found to impair hippocampal-dependent trace memories (Shors *et al*. 2001), and a blockade of neuron proliferation by irradiation decreased long-term potentiation (LTP) in the DG (Snyder *et al*. 2001). A locomotor activity such as running increased neurogenesis and LTP (Van Praag *et al*. 1999). However, it remains to be resolved whether hippocampal neurogenesis is causally linked to the synaptic plasticity and/or other factors. We undertook the present study to determine whether lithium-induced hippocampal neurogenesis independently of synaptic plasticity or signaling molecules in the DG of the adult rat hippocampus.

Materials and methods

Animals and treatments

Adult male Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN, USA) were housed in a 12-h light/dark cycle animal facility. Rats were treated intraperitoneally with saline or Li_2CO_3 (4 mEq/kg/day) for 2 days or 28 days as described previously (Chen *et al*. 1999). Saline was provided *ad libitum* to the lithium-treated rats to reduce potential toxicity. After treating with saline or lithium for 14 days, rats received single injections of bromodeoxyuridine (BrdU) at 50 mg/kg of body weight at a concentration of 10 mg/mL, intraperitoneally daily for 14 successive days (van Praag *et al*. 1999). For rats treated with saline or lithium only for 2 days, BrdU was injected daily in a similar way for 2 days. Blood samples were collected retro-orbitally at 12 h and 28 days after completing the lithium and BrdU treatments under pentobarbital anesthesia. Two hippocampi were then rapidly isolated; one was used for LTP and the other was used for immunohistological study or IP3 assay. The IP3 assay was performed using an assay kit, and the manufacturer's protocol (Amersham Pharmacia, Piscataway, NJ, USA). The lithium concentrations in the serum of rats treated for 2 and 28 days were 1.2 ± 0.08 mM and 0.97 ± 0.20 mM, respectively (therapeutic concentration in humans is 0.6–1.2 mM) 12 h after the last injection, and this returned to basal level 28 days after the last injection. No significant differences in the body weights of the control and lithium-treated rats were observed. BrdU (Sigma, St Louis, MO, USA) was dissolved in 0.9% NaCl and filtered in a sterile condition at 0.2 μm.

Electrophysiological recordings and LTP induction

Hippocampal slices were prepared using standard methods as described in previously (Snyder *et al*. 2001). Animals were anesthetized with pentobarbital (60 mg/kg) and decapitated. Their brains were removed and placed in cold artificial cerebrospinal fluid (aCSF; 124 mM NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 10 mM glucose, 1.3 mM $MgSO_4$, 2.0 mM $CaCl_2$). One hemisphere was immediately placed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) and processed for histological analysis. The hippocampus was then dissected from the second hemisphere, cut into 400-μm slices with a tissue chopper and placed in an incubation chamber, which was continuously perfused with aCSF for at least 1.5 h. The field potential recordings were obtained in the medial molecular layer of the DG on stimulating the medial perforant pathway (MPP). Baseline recordings were obtained by electrical stimulating the middle region of the molecular layer. Stimulation was performed with insulated bipolar tungsten electrodes of tip diameter *c*. 40 μm. Input–output curves were obtained after 20–30 min of stable recordings. The stimulation intensity that produced one-third of the maximum EPSP (EPSP; 300–400 μA, 100 μs) was chosen as the test pulse and TS. Only those slices that produced field EPSPs of 1 mV or higher in amplitude were accepted for experimentation. The baseline frequency used for the stimulation was 1 per 20 s. During the experiments, the slices were continuously perfused with aCSF bubbled with 95% O_2 – 5% CO_2 at 3 mL/min. The temperature in the perfusion chamber was kept at 30–32°C. LTP was induced using a protocol developed previously (four trains, of 500 ms each, 100 Hz within the train, repeated every 30 s). The stimulation intensity was kept at the baseline intensity at all times.

Immunohistochemistry for BrdU

For histological analysis, rats were killed and processed as follows. Twelve hours after the last BrdU injection, the rats were deeply anesthetized with pentobarbital and perfused via the ascending aorta with saline until the outflow became clear. They were then perfused with 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 20 min. The brains were rapidly removed and immediately frozen and stored at − 70°C. Serial sections (40 μm/section) were cut coronally through the entire anteroposterior extension of the hippocampi. For BrdU immunolabeling, sections were processed using the method of Kempermann *et al*. (1997). For other immunofluorescent labelings, sections were fixed by 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 20 min, washed with PBS, permeabilized in 100% ethyl alcohol for 10 min and then incubated in 10% normal goat serum (NGS), 0.1% Triton X-100 for 30 min at room temperature. Samples were then incubated in 0.1 M phosphate buffer (pH 7.4) containing primary antibody and 0.1% Triton X-100 overnight at 4°C. Subsequently, samples were incubated at room temperature for an additional 3–4 h with secondary antibodies conjugated to fluorescein isothiocyanate, cyanin 3, or cyanin 5. Secondary antibodies (donkey; Jackson ImmunoResearch, West Grove, PA, USA) were used at a final dilution of 1 : 500 in PBS. The samples were then washed with PBS, treated with 10 mg/mL 4′,6-diamidino-2-phenylindole (DAPI; Sigma) for 10 min and coverslipped in Vestashield Æ (Vector Laboratories, Hercules, CA, USA). Primary antibodies generated in mouse (mo), rat (rt), and rabbit (rb) were used at the following concentrations: mo anti-NeuN (1 : 100; Chemicon, Temecula, CA, USA), rt anti-BrdU (1 : 100; Accurate Chemicals, Westbury, NY, USA), rb p-CREB (1 : 50; ABR, Golden, CO, USA), rb p-Elk (1 : 100; Biolabs, Beverly, MA, USA), mo p-MAPK (1 : 100; Cell Signaling Technology, Beverly, MA, USA), rb BDNF (1 : 200; Santa Cruz Biotechnology, Santa Cruz,

CA, USA), mo TrkB (1 : 100; Transduction Laboratories, Lexington, KY, USA), rb Bcl-2 (1 : 100; Santa Cruz Biotechnology), CaMKII (1 : 100; ABR), p-CaMKII (1 : 50; ABR). Fluorescent signals were detected using a confocal laser-scanning microscope (Leica, Wetzlar, Germany), which allowed simultaneous evaluation of up to four separate fluorophores. When it was necessary to observe nuclei in addition to four immunological markers, cells were counterstained with DAPI.

Double-labeling of BrdU and neuron-specific nuclear protein

For the double-labeling of BrdU and neuron-specific nuclear protein (NeuN), sections that had been pre-treated for DNA denaturation were incubated in 0.01 M PBS containing 1% NGS, 0.3% Triton X-100, and a mo anti-NeuN monoclonal antibody and a monoclonal rt anti-BrdU antibody (1 : 50; Accurate Chemicals) for 24 h at 4°C.

The sections were then incubated in PBS containing Cy3-conjugated donkey anti-rat IgG (Jackson Immunoresearch; 1 : 100) and Cy2-conjugated anti-mouse IgG (Jackson ImmunoResearch; 1 : 200) for 1 h at room temperature. After several washes in PBS, the sections were mounted on gelatin-coated slides with Vectashield Æ. Confocal images were produced at a fixed laser power setting with a 40× oil-immersion objective. Separate optical images of BrdU and NeuN immunoreactivity were captured of the same optical view. The captured images were then pseudocolored green or red. A digital overlay was generated and companion images were superimposed. Co-localization was reflected by superimposing green and red pixels, thus appearing yellow. Image analysis was performed by using the standard system operating software provided with the confocal microscope (Version 1.6).

Cell counting

BrdU(+) cells were counted on a one-in-four sections basis (160 μm apart) using a computer-assisted image analysis system (MetaView Imaging version 3.6, Universal Imaging, Downington, PA, USA). The GCL and SGZ, the latter was defined as a 2-cell body wide zone (approximately 10 μm) along the border of the GCL, were investigated. BrdU(+) cells showing profiles of cell nuclei with a complete nuclear contour were counted in the focal plane in which they appeared largest within the counting box. The GC area was traced by DAPI(+) cells, and the GC reference volume was determined by summing the traced GC areas for each section and multiplying this by the distance between the sections sampled. The number of BrdU(+) cells was then related to the GCL sectional volume and multiplied by the reference volume to produce an estimate of the total number of BrdU(+) cells.

Quantitation in confocal images

To measure the intensity of immunoreactivity in the GCL of the DG, images were acquired with a digital camera (Nikon E800, Tokyo, Japan) and analyzed using an image analysis program (AnalySIS version 3.0, Soft Image Analysis System, Münster, Germany). In each image, a ROI (region of interest), which represented the DG, was determined by freehand drawing, and mean gray values in each ROI were measured. Results represent the ratio of the intensity, which was computed by dividing the mean gray value of a region in an experimental brain by the corresponding value in of a saline control. Generally, four to eight images of each section were averaged to determine a value for a section.

Statistical analysis

Statistical analyses were performed using Statistica (Statsoft, 1996). Statistical analyses for LTP were carried out between pooled slices from individual rats. Data from all slices tested in the same condition from the same rat were averaged to give a single values. Group comparisons for LTP were done using paired *t*-test as one rat from each saline- and lithium-treated group was recorded at the same day under the same conditions. Group comparisons for immunohistochemical results were done using the Student's *t*-test (paired where indicated).

Results

To determine the temporal profile of proliferating cells, lithium was injected twice daily for 28 days (chronic treatment) or 2 days (acute treatment) into 2-month-old rats. Animals were killed either 12 h or 28 days after treatment completion. BrdU labeling of dividing cells was conducted for 14 days starting at 15th day of lithium treatment in the chronic lithium treatment groups or conducted for 2 days in the acute lithium treatment group. Immunohistochemical analysis was performed 12 h and 28 days after treatment completion.

Immunohistochemical analysis revealed that lithium produced a significant 54.2 ± 6.8% increase in BrdU(+) cells compared to the DG saline-injected controls in rats that were killed 12 h after treatment completion (Fig. 1a; $t_{(26)} = 4.65$, $p < 0.001$, paired *t*-test). Even 28 days after treatment completion a significant 40.0 ± 18.1% increase was still observed in BrdU(+) cell numbers (Figs 1b and d; $t_{(16)} = 3.95$, $p < 0.005$). However, lithium treatment for 2 days failed to increase the number of BrdU(+) cells 12 h after treatment completion (Figs 1c and d; $t_{(8)} = 0.85$, $p > 0.8$).

The phenotype of the BrdU(+) cells was examined by double-labeling with BrdU and a neuronal marker, NeuN, 12 h after treatment completion (Fig. 2a). The percentages of cells double-labeled in lithium treated animals and saline control rats were virtually identical 12 h after (Fig. 2b, control rats, 53 ± 5%; lithium-treated rats, 58 ± 4%, $p > 0.4$), 28 days after (control rats, 90 ± 2%; lithium-treated rats, 92 ± 3%, $p > 0.6$) the last injection of lithium and BrdU, and only 4% of the BrdU(+) cells exhibited glial fibrillary acidic protein (GFAP) immunoreactivity after 28 days of lithium treatment (data not shown). These results indicate that even at the early time point (12 h), more than half of the newborn cells exhibited a neuronal phenotype, and that the percentage of cells double-labeled for BrdU and NeuN remained unchanged when investigated 28 days after the last injection of lithium and BrdU despite an overall 54% increase in the number of BrdU(+) cells in lithium-treated rats.

Chronic lithium treatment for 28 days is known to affect neurotransmitter levels (Dixon *et al*. 1994; Moore *et al*. 2000). Therefore, the possibility exists that lithium influences

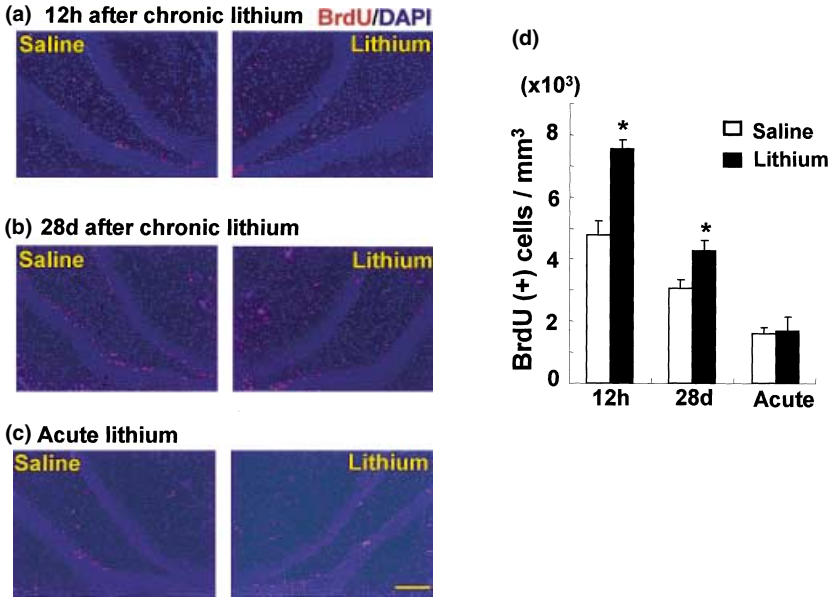

Fig. 1 Effects of acute (2 days) and chronic (28 days) treatment on BrdU immunolabeling in saline- or lithium-treated 2-month-old rats. BrdU(+) cells were observed in the subgranular zone (SGZ) of the DG. Stereological three-dimensional counting revealed that lithium treatment, compared with saline treatment, produced a significant 54.2 ± 6.8% increase (saline 4758 ± 483 cells/mm^3 vs. lithium 7520 ± 310 cells/mm^3, $n = 14$ animals in each group) increase in BrdU(+) cells when analyzed 12 h after treatment completion (a), 40.0 ± 18.1% increase (saline 3048 ± 297 cells/mm^3 vs. lithium 4269 ± 315 cells/mm^3, $n = 9$ animals in each group) 28 days after treatment completion (b). Lithium did not significantly affect the volume of either the right or the left DG and produced a significant increase in the density of BrdU(+) cells in the DG. (c) In lithium-treated rats for 2 days, lithium did not significantly increase the number of BrdU(+) cells vs. saline (saline 1571 ± 235 cells/mm^3 vs. lithium 1670 ± 445 cells/mm^3, $n = 5$ animals in each group). (d) Plot showing the effects of 2 and 28 days lithium treatment on the total number of BrdU(+) cells in the DG. Data are presented as mean ± SEM. Student's *t*-test: $^*p < 0.05$. 12 h, 12 h after chronic lithium; 28d, 28 days after chronic lithium; acute, acute 12 h after acute lithium. Scale bar, 100 μm.

cognition and synaptic plasticity. To determine whether 28 days of lithium treatment affects synaptic plasticity, LTP was studied in rats killed 12 h after treatment completion. For these LTP experiments, the MPP was stimulated. Tetanic stimulation (TS) in the DG produced a small (approximately 10%), but consistent LTP, which lasted at least 40 min (Fig. 3A) in the control group. An identical TS induced a slightly larger LTP in lithium-treated animals, and the difference between the two was significant [controls, 15 slices from 13 rats (109 ± 2%); lithium-treated, 14 slices from 13 rats (128 ± 4%); $t_{(12)} = 4.22$, $p < 0.01$, paired *t*-test; Fig. 3A, left]. It is well known that the excitation of granule cells (GCs) is strongly modulated by GABAergic synapses both *in vitro* and *in vivo*. Therefore, slices were perfused with ACSF in the presence of bicuculline (10 μM), a $GABA_A$-receptor blocker, which supposedly blocks 95% of monosynaptic and 86% of polysynaptic $GABA_A$ current (Liu *et al*. 1998). The addition of bicuculline into the ACSF produced an effective disinhibition of slices and allowed the induction of an additional, larger LTP (40–60%; Fig. 3A, right). Recordings made in the presence of bicuculline (bicuculline-LTP) showed that the EPSP slope was significantly increased 40 min after TS compared with those of the ACSF-LTPs in both the lithium-treated and untreated groups [lithium-treated: ACSF-LTP, 128 ± 4%, bicuculline-LTP, 158 ± 6% (13 slices from 13 rats), $t_{(12)} = 3.81$, $p < 0.002$; saline-treated: ACSF-LTP, 109 ± 2%, bicuculline-LTP, 127 ± 8% (16 slices from 13 rats), $t_{(12)} = 2.17$, $p < 0.05$; paired *t*-test; Fig. 3A]. Wang *et al*. (2000) reported that young neurons in the SGZ of the adult hippocampus were unaffected by $GABA_A$ inhibition (bicuculline-resistant) and displayed robust LTPs. In contrast, mature GCs never produced LTPs without $GABA_A$ inhibition. According to these findings, LTP induction in slices perfused with a standard ACSF ought to be produced by mainly newly produced young neurons, whereas the LTPs obtained in the presence of bicuculline ought to be attributable to both mature and immature neurons. Similarly, a slightly larger increase in ACSF-LTP in lithium-treated animals could be due to young neurons produced by lithium or by other factors activated by lithium. Our results show that ACSF-LTP was increased in lithium-treated animals versus control animals, which raises the possibility that lithium may increase the LTP expression, by inducing the production of young neurons that can resist the GABAergic inhibition of the surrounding interneurons as suggested by Wang *et al*. (2000). In addition, our result showed that bicuculline-LTP was also increased in lithium-treated animals versus saline-treated animals,

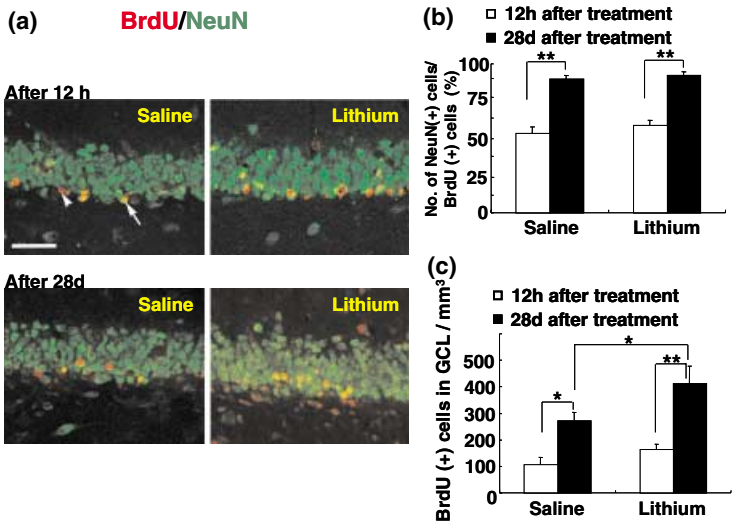

Fig. 2 Phenotype of the BrdU(+) cells by double-labeling studies with BrdU and the neuronal marker, NeuN. (a) Double-labeling studies were performed 12 h and 28 days after completing treatment for 28 days. (b) In the control rats, 53 ± 5% of the BrdU(+) cells were double-stained with NeuN (arrowhead); 12 h after lithium treatment, the percentage of cells double-stained for BrdU and NeuN was virtually the same as the control rats (58 ± 4%). The percentages of cells double-labeled 28 days after treatment completion in control and lithium treated animals were significantly greater compared with 12 h after in each treatment group (control: 12 h after, 53 ± 5%, 28 d after, 90 ± 2%, $p < 0.01$; lithium-treated rats: 12 h after, 58 ± 4%, 28 days after, 92 ± 3%, $p < 0.01$). (c) The number of BrdU(+) cells per unit volume (mm^3) in GCL. The number of BrdU(+) cells only in GCL from lithium-treated animals was significantly increased 28 days after the last injection of lithium and BrdU, compared to that at 12 h after the last injection (12 h after, 163 ± 21/mm^3; 28 days after, 411 ± 66/mm^3, $p < 0.01$). Saline controls (12 h after, $n = 7$ animals), saline controls (28 days after, $n = 7$ animals); lithium-treated (12 h after, $n = 7$ animals), lithium-treated (28 days after, $n = 7$ animals). Fluorescent signals were detected using a confocal laser scanning microscope, and separate optical images of BrdU (red) and NeuN (green) immunoreactivity were captured from the same optical view. *$p < 0.05$, **$p < 0.01$. Arrowhead: BrdU(+) cells; arrow: BrdU(+)/NeuN(+) cell. Scale bar, 50 μm.

suggesting that a growth in the numbers of bicuculline-sensitive young neurons might also be induced by lithium. However, there remain other possibilities, including the possibility that lithium might activate other factors necessary for the establishment of LTP.

It takes about 4 weeks for newly divided cells to migrate over the GCL and function as mature granule cells (Kee *et al.* 2001). The number of BrdU(+) cells only in GCL from lithium-treated animals was found to be remarkably increased 28 days after the last injection of lithium and BrdU, compared to that at 12 h after the last injection ($p < 0.01$), and still greater than that in saline-treated animals at 28 days after the last injection [Fig. 2c; saline-treated (28 days after), 272 ± 31/mm^3; lithium-treated (28 days after), 411 ± 66/mm^3; $p < 0.03$; saline-treated (12 h after), 106 ± 27/mm^3; lithium-treated (12 h after), 163 ± 21/mm^3; $p > 0.05$], and approximately 90% of BrdU-positive cells in GCL co-expressed neuron markers at 28 days after the last injection (Fig. 2b). These results indicate that an additional net increase in NeuN-immunoreactive cells was produced by lithium in the GCL. We next investigated whether this lithium-induced increase in NeuN-positive cells could affect synaptic plasticity by recording LTP 4 weeks after treatment completion. A larger ACSF-LTP was induced in lithium-treated animals than in saline-treated control animals when tested 28 days after the lithium treatment and the difference between the two was significant [Fig. 3B; saline-treated, 12 slices from 11 rats (110 ± 4%); lithium-treated, 15 slices from 11 rats (135 ± 3%); $t_{(10)} = 4.78$, $p < 0.001$, paired *t*-test]. Bicuculline-LTP was also significantly increased compared with the corresponding controls [saline-treated, 11 slices from nine rats (137 ± 2%); lithium-treated, 12 slices 10 rats (159 ± 6%); $t_{(8)} = 2.91$, $p < 0.05$, paired *t*-test]. However, the magnitude of both ACSF-LTP and bicuculline-LTP did not change so much whether it was recorded 12 h or 28 days after completing the lithium treatment [Fig. 3E: (i) ACSF-LTP: 12 h, 128 ± 4%; 28d, 135 ± 3%; $t_{(10)} = 0.47$, $p > 0.7$; (ii) bicuculline-LTP: 12 h, 158 ± 6%; 28d, 159 ± 6%; $t_{(9)} = 0.11$, $p > 0.92$, paired *t*-test], indicating that LTP might not be directly associated with a net increase in the number of mature neurons in the GCL, and that, whatever the effect of lithium might be, it possibly lasts for a long time, certainly 4 weeks after lithium treatment.

In order to verify further that lithium-induced neurogenesis is related to LTP, we administered lithium and BrdU for 2 days in 2-month-old rats. This allowed lithium to produce a non-neurogenic biochemical effects, but not to induce neurogenesis. Indeed, the total number of BrdU(+) cells

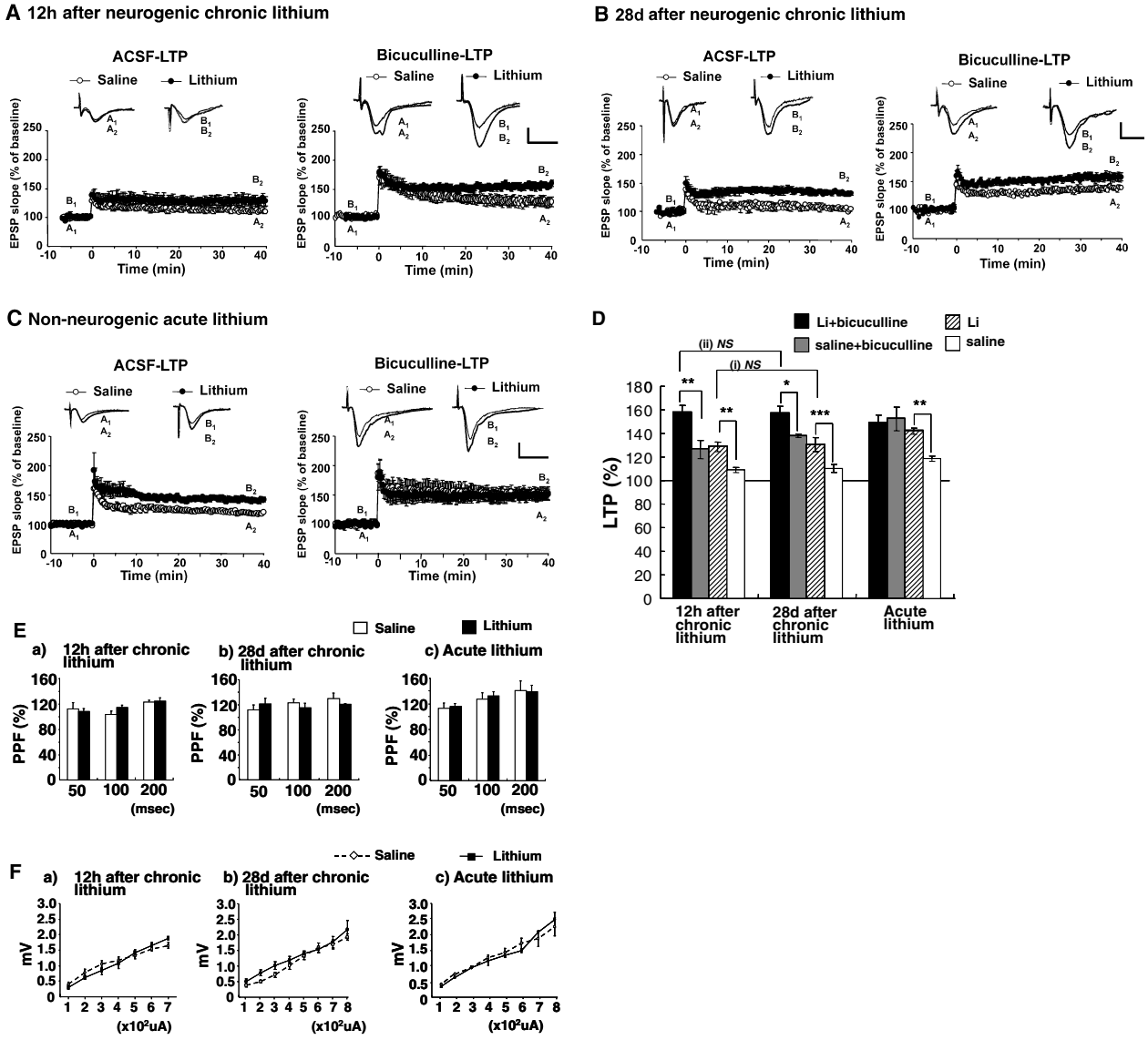

Fig. 3 Time course of LTP in slices from the controls (○) and the lithium groups (●). LTP in the DG of 2-month-old-rats recorded 12 h (A) and 28 days (B) after the lithium-treatment with BrdU on days 14–28, or 12 h after 2 days of lithium treatment (C). (Insets in A–D) Representative examples are shown of evoked responses immediately before (Pre: A1, B1) and 40 min after (Post: A2, B2) LTP induction. Example waveforms represent the averages of 10 responses recorded over a 3-min period. Calibration: 0.5 mV, 10 ms; EPSP, excitatory post-synaptic potential. (D) Plot showing the LTP magnitude. Group comparisons for LTP were done using paired *t*-test as one rat from each saline- and lithium-treated group were recorded at the same day under the same conditions. NS, statistically not significant. a: $n = 13$ rats per each group for ACSF-LTP, $n = 13$ per each group for bicuculline-LTP; b: $n = 11$ per each group for ACSF-LTP, $n = 9$ (control) and $n = 10$ (lithium) for bicuculline-LTP; C: $n = 6$ rats per each group for ACSF-LTP, $n = 5$ per each group for bicuculline-LTP. (E) PPF at 50-, 100-, and 200-ms interpulse intervals. The EPSP slope of the 1st response was calculated relative to the 2nd response. Data traces are superimposed responses to the 1st and 2nd stimuli at different interstimulus intervals. All traces were recorded from the same slice. (F) Input–output curve. No difference was observed between slices from controls and lithium-treated rats ($p > 0.91$).

was much less in these animals compared to that from animals treated for 28 days (Figs 1c and d). Moreover, the number of BrdU(+) cells in these acutely treated animals was not significantly higher than that in the corresponding saline-treated controls (Fig. 1e). However, acute lithium treatment significantly increased ACSF-LTP as compared to acute saline treatment [Fig. 3C, controls, seven slices from six rats (120 ± 3%); lithium-treated, eight slices from six rats (141 ± 4%); $t_{(5)} = 5.58$, $p < 0.002$, paired *t*-test]. In contrast, bicuculline-LTP was not significantly different in acute saline- and lithium-treated rats [Fig. 3C, controls, seven slices from five rats (152 ± 9%); lithium-treated, eight slices

from five rats (149 ± 7%); $t_{(4)} = 0.293$, $p < 0.8$, paired *t*-test]. However, no differences were found between corresponding animal groups in terms of paired pulse facilitation (PPF; Fig. 3E) and the input–output curves of the EPSP amplitudes (Fig. 3F), suggesting no effect of acute or chronic lithium on basal synaptic efficacy or cell excitability (Fig. 3F; acute treatment, $p > 0.81$; 12 h after the last chronic lithium injection, $p > 0.14$; 28 days after the last chronic lithium injection, $p > 0.24$; paired *t*-test). These results demonstrate that acute lithium treatment enhanced ACSF-LTP compared to saline treatment.

It has been suggested that the accumulation of IP3 may be the way that lithium works in bipolar affective disorder (Dixon *et al.* 1994), but recently reported that inositol depletion is the therapeutic action of lithium (Williams *et al.* 2002). Therefore, we measured the accumulation of IP3 in acute and chronic lithium-treated rat hippocampi. Neither acute nor chronic lithium treatment had significant effects on the accumulation of IP3, although acute treatment slightly induced the accumulation of IP3 (Table 1). These results suggest that accumulation of IP3 does not play a role in the hippocampal neurogenesis. We next undertook to determine if acute and chronic lithium administration also increase bcl-2, BDNF, trkB, p-CREB, CaMKII (calcium and calmodulin-dependent protein kinase II), p-CaMKII, p-MAPK and p-Elk levels in 2-month-old rats. Chronic lithium-induced increases in the Bcl-2 level were detected in sections obtained 12 h, but not 4 weeks, after the last injection of lithium ($n = 4$ animals, $p < 0.03$; $n = 6$, $p > 0.05$, respectively). Protein levels of BDNF and TrkB were highly significantly increased by lithium-treated rats, which were sacrificed at 12 h and 28 days after chronic lithium treatment, respectively ($n = 5$, $p < 0.002$; $n = 6$, $p < 0.001$, respectively). An increase in p-CREB levels was highly significant 12 h after chronic lithium treatment ($n = 8$, $p < 0.01$), and this remained high until 28 days after the last injection (Fig. 4; $n = 10$, $p < 0.05$). We also examined whether MAPK, and its downstream transcriptional target, the ternary complex factor Elk-1, a key transcriptional-regulator of serum response element (SRE)-driven gene expression (Davis *et al.* 2000), is hyperphosphorylated in lithium-treated rats. We found that the protein levels of p-MAPK and p-Elk were highly and significantly upregulated in chronically treated rats 12 h and 28 days after the last injection, respectively ($n = 7$, $p < 0.01$; $n = 5$, $p < 0.009$, respectively). Therefore, it is likely that MAPK activation is required for LTP-dependent transcriptional regulation and that both CREB and Elk-1 are significantly associated with the MAPK pathway in lithium-treated animals. Notably, the increased immunoreactivity of CaMKII was observed 12 h after the last injection of lithium in lithium-treated animals, compared to the saline controls (Figs 4; $p < 0.03$). Interestingly, CaMKII was further increased 4 weeks after the last injection of lithium (Figs 4; $p < 0.01$) and CaMKII was visible in the perinuclear cytoplasm, as reported previously (Zhang *et al.* 1999). The immunoreactivity of p-CaMKII was not detected probably because of its low expression. In acute lithium-treated rats, the levels of p-CREB, but not p-Elk, BDNF, CaMKII, p-MAPK or Bcl-2, were significantly elevated versus the acute saline-treated controls (Fig. 4; p-CREB: controls, 100.0 ± 6.2% vs. lithium-treated, 152.5 ± 8.2%, $t_{(4)} = 5.07$,

Table 1 Effects of chronic and acute lithium treatment on the accumulation of IP3 (pmole/mg)

	Chronic lithium		Acute lithium
Group	12 h after treatment	28 days after treatment	12 h after treatment
Saline	2.69 ± 0.22	2.35 ± 0.21	2.52 ± 0.24
Li_2CO_3	2.93 ± 0.29	2.44 ± 0.18	3.27 ± 0.19
p-value	> 0.2	> 0.4	> 0.05 (= 0.052)

Animals were treated as indicated in Materials and methods. The IP3 assay was performed using an assay kit TRK1000, and the manufacturer's protocol (Amersham Pharmacia Biotech). Data are obtained from four rats in each treatment. Values are means ± SEM.

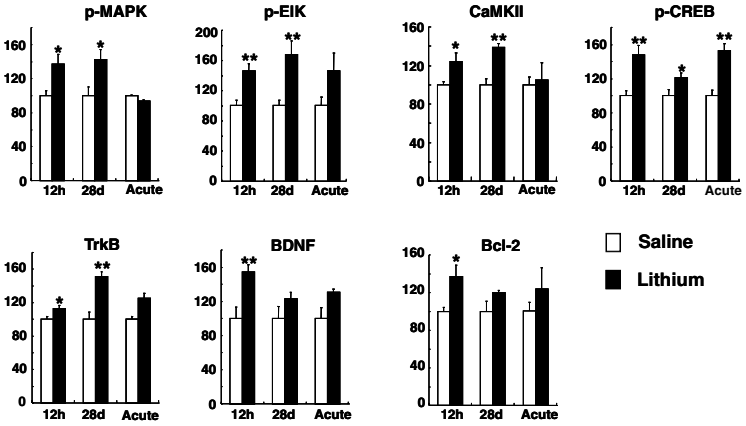

Fig. 4 Effects of acute (2 days) and chronic (28 days) lithium treatment on protein levels in the DG. Rats were treated with lithium, and immunohistochemistry was performed as described in Materials and methods. Results are expressed as percentage of saline-treated controls and represent means ± SEM of four or five rats. 12h: 12 h after chronic lithium treatment; 28d: 28 days after chronic lithium treatment; Acute: 12 h after acute lithium treatment. $^{*}p < 0.05$. $^{**}p < 0.01$.

$p < 0.01$). Our immunohistochemical results indicate that the lithium-induced activation of intracellular signaling pathway contributes to the lithium-induced enhancement of LTP.

Discussion

In the present study, we have tried to determine the contribution made by lithium treatment to hippocampal neurogenesis and LTP. The establishment of an experimental regimen capable of achieving this is potentially complicated as neurogenesis-affecting factors, including drugs such as antidepressants and NMDA receptor antagonists, and psychophysical conditions, such as those induced by stress, voluntary movement, and ischemia, have significant influence on synaptic plasticity. We chose lithium for this purpose and investigated the effects of acute and chronic lithium administration on DG LTP.

We demonstrate for the first time that chronic lithium administration at therapeutic doses robustly increases hippocampal LTP *in vitro* in rats immediately after and 4 weeks after lithium withdrawal. In addition, we show that a blockade of inhibitory transmission with bicuculline increases the ability of the DG to express LTP both in saline- and lithium-treated rats. However, this does not necessarily mean that inhibitory transmission was increased in the lithium-treated rats, as the input–output curves were similar for control and lithium-treated animals (Fig. 3G) and ACSF-LTP in lithium-treated rats was also greater than that in control rats. These results, combined with results previously published (Liu *et al*. 1998; van Praag *et al*. 1999 Snyder *et al*. 2001), suggest a contribution of newly created neurons, induced by the lithium treatment on the increased LTP. It was previously reported that an increase in neurogenesis by exercise increases hippocampal LTP (van Praag *et al*. 1999), and it was suggested that running might indirectly affect neurogenesis and synaptic plasticity via trophic factors and angiogenesis. In relation to this point, it is noteworthy that physical activity potentiates the expression of the BDNF transcript in the rat hippocampus (Russo-Neustadt *et al*. 2000). In addition, it was reported that the level of growth factor (bFGF) itself regulates hippocampal granule neuron production (Cheng *et al*. 2002). A blockade of the proliferation of progenitor cells in GCs was found to block the formation of trace memories (Shors *et al*. 2001) and LTP (Snyder *et al*. 2001). Although none of the previous studies demonstrated that newly generated neurons are functionally active in local circuits and contribute to LTP establishment or to the formation of trace memories, it is possible that the newly created neurons establish new functional circuits, and sequentially lead to the upregulation of key molecules necessary for LTP. In fact, our immunohistochemical results indicate that the expression levels of Bcl-2 and BDNF were upregulated only when blood lithium level remained at therapeutic levels after chronic injection. In contrast, p-Elk, CaMKII, and TrkB were highly upregulated even 4 weeks after lithium withdrawal. These results, together with those of bicuculline-LTP, indicate that the increased LTP observed in lithium-treated rats might be contributed to the increased generation of bicuculline-resistant new cells, and in part, to the upregulation of BDNF, p-MAPK, and p-CREB 12 h after chronic lithium.

However, our results indicate that the magnitude of the LTP obtained from acute and chronic lithium-treated rats is inconsistent with neurogenesis. Both ACSF-LTP and bicuculline-LTP obtained from rats 4 weeks after lithium treatment were not significantly greater than those of rats killed 12 h after chronic lithium treatment, although new neurons were greatly increased in the GCL, as illustrated in Fig. 2. These results suggest that the increased migration and maturation of newborn cells in the GCL might not contribute directly to the magnitude of the LTP. Rather that the magnitude of the LTP is consistent with the immunohistological results, in which the upregulations of CaMKII, p-Elk, and TrkB were even greater in lithium-treated rats killed at 4 weeks than in rats killed 12 h after chronic lithium treatment. Our results obtained from acute lithium-treated rats showed that the LTP was enhanced in the absence of neurogenesis. This further supports the notion that the lithium-induced increase in LTP is not directly related to the increased number of newborn cells. Our data show that the estimated 5000–7000 additional new neurons produced per unit volume (mm^3) of the DG in lithium-treated animals. Thus, the number of new neurons is small compared with the total number of approximately 1 million per unit volume already present in the rat. Thus key molecules, probably associated with neurogenesis, and not the increased number of neurons *per se*, are likely to be the important factors underlying the increased LTP.

The mechanism by which lithium modulates LTP remains to be determined. First, it is important to determine which signals are directly related to neurogenesis. In this regard, it is noteworthy that both the presence (Bender *et al*. 2001) and the absence (Nakagawa *et al*. 2002) of the co-expression of p-CREB and BrdU were observed in newly generated young neurons. Ozaki and Chuang (1997) demonstrated that chronic lithium treatment for 28 days increases the transcription mediated by CRE binding in the rat brain, and further, it has been shown that CRE is a key element in the transcription of BDNF mRNA in the rat brain (Shieh and Ghosh 1999). It is also demonstrated that the release of neurotrophins from mature GCs can induce the phosphorylation of CREB in immature GCs (Bender *et al*. 2001). Otherwise, lithium may directly inhibit GSK3 activation, which results in the CREB activation, as previously suggested (Grimes and Jope 2001). Our observations showing increases in the expressions of p-CREB, BDNF, and Bcl-2 by lithium, indicate that CREB, p-CREB, and Bcl-2 are closely linked in a signaling cascade in the

hippocampus of lithium-treated rats. It is found that therapeutic lithium level used in the present study does not significantly increase the accumulation of IP3, especially in the chronic lithium-treated rats. In fact, lithium had significant effects on the accumulation of IP3 when it was added at high concentration as high as 10–25 mM *in vitro* (Dixon *et al*. 1994) and 10 mEq/kg *in vivo* (Whitworth *et al*. 1990). However, acute lithium treatment at therapeutic level might contribute to the LTP enhancement, independently of neurogenesis, by physiologically relevant accumulation of IP3, as shown in Table 1.

Interestingly, chronic lithium treatment accelerated the production of BrdU(+) cells, but did not change the relative number of neurons (Fig. 2b). It is essential that we distinguish whether the continued increase in the number of BrdU(+) cells is due to enhanced proliferation, survival, or both. Given the chronic time frame of the present studies in which BrdU labeling was conducted 12 h after 14 daily BrdU injections, it is likely that the increased survival of BrdU(+) cells also play a role in the effect of lithium. This is consistent with our data, which shows that both the number of BrdU(+) cells and the expression levels of survival factor Bcl-2 are most greatly enhanced at 12 h after the chronic lithium treatment. We used co-labeling with BrdU and NeuN to estimate the degree of cell differentiation towards a neuronal phenotype. Four weeks after the last injection of lithium, although the number of surviving cells had remarkably declined, the proportion of the cells doubly labeled with BrdU and NeuN remained constant in the lithium group like in the control group. Thus, though lithium appears to accelerate cell production it does not enhance cell differentiation or maturation into neurons.

In conclusion, the novel finding of this study is that the enhanced LTP of the DG of the neurogenesis-increased rat hippocampus is independent of the presence of the newly created neurons, and that increased neurogenesis in GCL *per se* cannot be considered conclusive evidence of increased LTP without considering the functionality of the new neurons and changes in the chemical environment of the hippocampus.

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