

Glucocorticoids and Lithium Reciprocally Regulate the Proliferation of Adult Dentate Gyrus-Derived Neural Precursor Cells Through GSK-3 β and β -Catenin/TCF Pathway

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Adult hippocampal neurogenesis is decreased in rodent models for stress-related disorders at least partly through an elevated level of glucocorticoids. On the other hand, the mood stabilizer lithium (Li) commonly used for their treatment increases it. This effect is thought to be one of the therapeutic actions of Li, but the molecular mechanism has been poorly understood. Here we established the culture system of adult rat dentate gyrus-derived neural precursor cells (ADPs) and examined the effects of dexamethasone (DEX), an agonist of glucocorticoids receptor, and Li on ADP proliferation. It is possible for ADP to be a type 2a cell, which corresponds to the second stage in a model of four differentiation stages in adult hippocampal neural precursor cells. DEX decreased ADP proliferation, but Li did not have any effect on it. However, Li recovered ADP proliferation decreased by DEX. The recovery effect of Li was abolished by quercetin, an inhibitor of β -catenin/TCF pathway. The intranuclear translocation of β -catenin and expression of cyclin D1 are reciprocally regulated by DEX and Li in a way similar to proliferation. In addition, DEX increased the phosphorylation of Tyr²¹⁶, which renders glycogen synthase kinase-3 β (GSK-3 β) active on it. These results suggest that GSK-3 β and β -catenin/TCF pathway might be important in the reciprocal effects between DEX and Li on ADP proliferation and are new targets of therapeutic agents for stress-related disorders. *Neuropsychopharmacology* (2009) **34**, 805–815; doi:10.1038/npp.2008.198; published online 12 November 2008

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INTRODUCTION

It has been well established that neurogenesis occurs in the adult brain of various animal species, including humans (Eriksson *et al*, 1998; Gould *et al*, 1997; Gage, 2000). Neurogenesis takes place mainly in two discrete regions of the adult brain: the subventricular zone of the lateral ventricles (Alvarez-Buylla and Garcia-Verdugo, 2002) and the subgranular zone of the dentate gyrus (DG) in the hippocampus (Kempermann *et al*, 2006). It has been shown that neurogenesis in DG is affected by many factors, including environment, stress, hormones, and drugs. For example, adult neurogenesis in DG is decreased in rodent models for stress-related disorders (Gould *et al*, 1997; Malberg and Duman, 2003; Pham *et al*, 2003; Jayatissa *et al*, 2006). Although it remains unclear how neurogenesis in DG is decreased in these models, some studies have suggested

that glucocorticoids might be involved in the decrease of adult hippocampal neurogenesis (Gould *et al*, 1997; Cameron and McKay, 1999; Kim JB *et al*, 2004). In humans, elevated levels of glucocorticoids constitute one of the causal events in stress-related disorders (de Kloet *et al*, 2005; Swaab *et al*, 2005). In contrast, the administration of lithium (Li), which is used for treatment of stress-related disorders, increases adult hippocampal neurogenesis (Chen *et al*, 2000; Son *et al*, 2003; Kim JS *et al*, 2004). These studies suggest that neurogenesis might be involved in the therapeutic action of Li. Therefore, elucidating how glucocorticoids and Li regulate neurogenesis might lead to a further understanding of the pathophysiology of stress-related disorders and the development of new therapeutic targets. Li is an inhibitor of glycogen synthase kinase-3 β (GSK-3 β ; Klein and Melton, 1996; Stambolic *et al*, 1996), and GSK-3 β is widely known as a key regulator of β -catenin/TCF pathway (Aberle *et al*, 1997; Orford *et al*, 1997).

In this study, we established the culture system of adult rat dentate gyrus-derived neural precursor cells (ADPs) and showed that dexamethasone (DEX), an agonist of glucocorticoid receptor (GR), decreased ADP proliferation and Li recovered it. In addition, we showed that this reciprocal

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effect between DEX and Li on ADP proliferation was regulated by GSK-3 β and the β -catenin/TCF pathway.

MATERIALS AND METHODS

Isolation and Culture of ADP

Adult male Sprague–Dawley rats (8-week old) were deeply anesthetized with sodium pentobarbital and decapitated. The brains were removed and washed with ice-cold phosphate-buffered saline (PBS; pH 7.4). The coronal sections with a thickness of 1 mm were cut using a Brain Slicer (Muromachi, Tokyo, Japan) and immersed into dishes containing ice-cold PBS, and the regions containing DG were dissected carefully under a dissecting microscope to exclude all regions containing subependymal tissues (Figure 1a). These tissues were digested with a mixture of

papain, dispase II and Dnase I as described earlier (Ray and Gage, 2005). The fraction containing ADP was isolated by Percoll (GE Healthcare, Buckinghamshire, UK) gradient centrifugation and seeded in DMEM: F12 (Sigma, St Louis, MO), including 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and penicillin-streptomycin (Invitrogen) on a noncoating dish, and incubated at 37°C in 5% CO₂. After overnight incubation, the medium was changed with a proliferation medium consisting of Neurobasal (Invitrogen), B27 supplement minus vitamin A (Invitrogen), 1 mM L-glutamine (Invitrogen), penicillin-streptomycin, and 20 ng/ml basic fibroblast growth factor (bFGF; Invitrogen). The cultures were maintained with a proliferation medium at 37°C on laminin- (Invitrogen) and ornithin (Sigma)-coated dishes and fed with a new medium every 2 or 3 days by replacing 50% of the medium. When cell confluency reached 80–90%, the cells were passaged by trypsinization

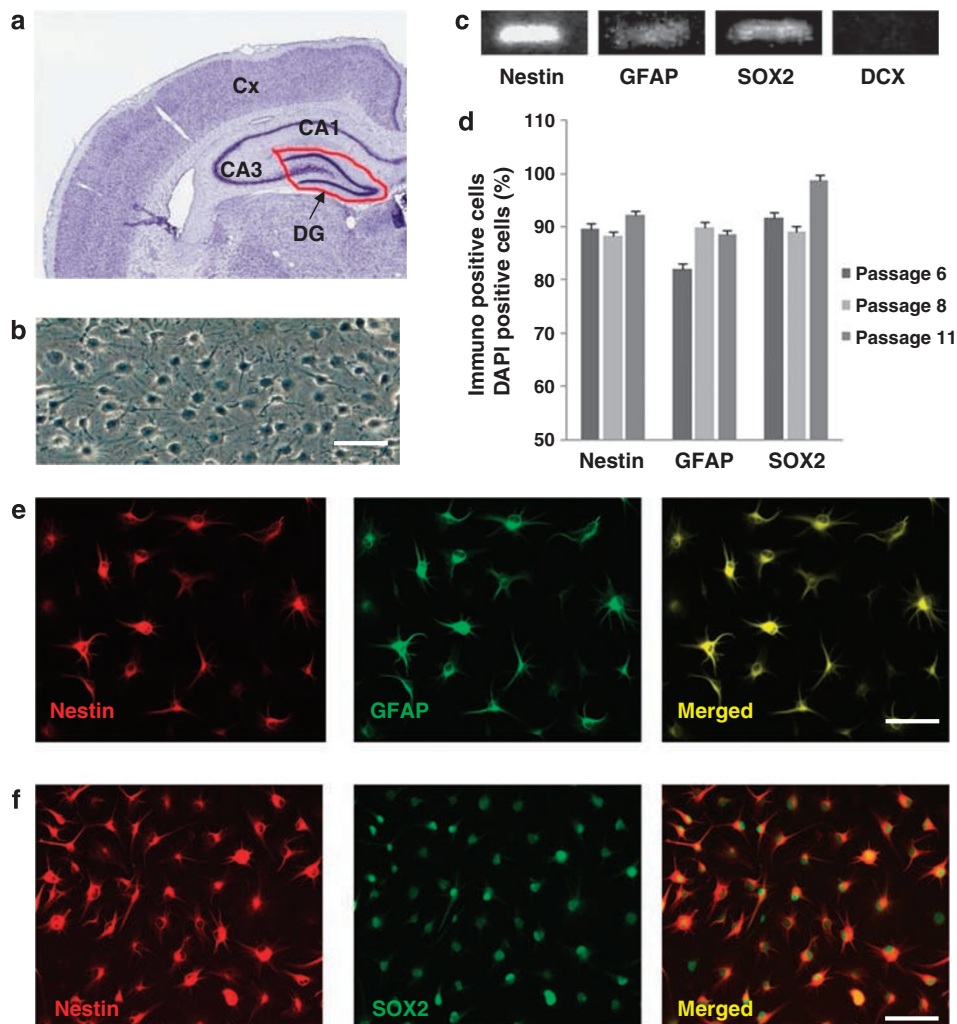


Figure 1 Isolation and characterization of adult rat dentate gyrus-derived neural precursor cells (ADP). (a) Coronal section through dissected regions. The dentate gyrus (DG) of adult rat was dissected as a red line, and cells were cultured as described in 'Materials and Methods'. Dissection was made carefully to exclude all other regions containing subependymal tissues. (b) The shape of cells. Phase-contrast image was shown at 3 days after passage. Cells grew as a monolayer. Scale bar = 90 μ m. (c) Detection of the genes involved in neural precursor cell and immature neuron with RT-PCR. mRNAs of nestin, GFAP, and SOX2 were detected, but DCX was not. (d) The ratio of immunopositive cells with markers for neural precursor cells in passages 6, 8, and 11. Values are shown as the ratio of each marker-positive cell vs 4',6-diamino-2-phenylindole (DAPI)-positive cell. Data are shown as the means \pm SEM. (e, f) Immunocytochemistry for nestin, glial fibrillary acidic protein (GFAP), and SOX2. (e) Nestin (red), GFAP (green). Scale bar = 120 μ m. (f) Nestin (red), SOX2 (green). Most cells coexpressed nestin, GFAP, and SOX2. Scale bar = 120 μ m.

and the cell density for seeding was approximately 1×10^4 cells per cm^2 . The cells were monitored with an IX-70 optical microscope (Olympus, Tokyo, Japan).

Analysis of ADP

For detecting the expression pattern of markers, 2×10^4 cells per well were seeded in a proliferation medium on laminin- and ornithin-coated Lab-Tek II 8-chamber slides (Nalge Nunc International, Naperville, IL). After overnight incubation, immunocytochemistry was performed as described below. For testing proliferation potency, bromodeoxyuridine (BrdU; Sigma) was added into the medium at 10 nM. After 24 h, immunocytochemistry was performed. For testing multipotency, cells were treated in differentiation medium (proliferation medium without bFGF) with 10 ng/ml brain-derived neurotrophic factor (BDNF; Sigma), 1 μM retinoic acid (Invitrogen)/0.5% fetal bovine serum, or 500 ng/ml insulin-like growth factor (IGF; Invitrogen). After 7 days, immunocytochemistry was performed.

Cells were fixed with 4% paraformaldehyde for 15 min. In cases involving detecting the expression pattern of markers and testing for multipotency, permeabilization was performed with PBS containing 0.2% Triton X-100 for 30 min. In the cases of testing for proliferation potency, permeabilization was performed as follows: Cells were immersed with 50% formamide in $2 \times \text{SSC}$ (0.3 M NaCl, 0.03 M sodium citrate) at 65°C for 2 h, incubated in 2 N HCl for 30 min at room temperature, and rinsed in 0.1 M borate buffer for 10 min. Subsequently, the cells were incubated in PBS containing 3% goat serum (Vector Laboratories, Burlingame, CA) for 20 min and then with primary antibodies containing 3% goat serum at 4°C overnight. The primary antibodies used were: mouse anti-nestin (1:2000; BD Biosciences, Franklin Lakes, NJ), rabbit anti-nestin (1:50; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-glial fibrillary acidic protein (GFAP, 1:2000; Dako, Glostrup, Denmark), rabbit anti-SRY (sex-determining region Y)-related high mobility group-box gene 2 (SOX2, 1:2000; Chemicon, Temecula, CA), rabbit anti-brain lipid-binding protein (BLBP, 1:300; kindly gifted by Masahiko Watanabe, Hokkaido University Graduate School of Medicine), rabbit anti-Prox1 (1:2000; Chemicon), mouse anti-Tuj1 (1:5000; Covance, Princeton, NJ), and mouse anti-O4 (1:2000; Chemicon). Cells were incubated in PBS containing secondary antibodies for 1 h at room temperature. The secondary antibodies used were: anti mouse Cy3 and anti-rabbit FITC (1:100; Jackson Immuno Research, West Grove, PA). Samples were coverslipped with Vectashield containing 4',6-diamino-2-phenylindole (DAPI; Vector Laboratories). Fluorescent signals were detected using the IX-71 fluorescent microscope system (Olympus).

Cell Counting with Alamar Blue Assay

Alamar Blue assay is a rapid and simple nonradioactive assay to measure the number of cells. Alamar Blue dye is a fluorogenic redox indicator that is converted from the oxidized form to the reduced form in cells. The reduced form of Alamar Blue dye is highly fluorescent, and the fluorescence in Alamar Blue assay reflects the number of cells (Ahmed *et al*, 1994; Nakayama *et al*, 1997). In addition,

we confirmed that fluorescence in Alamar Blue assay is surely proportional to the simply counted number of ADPs (data not shown). BrdU-based assays were often used for cell counting, but BrdU-positiveness reflects the duplication of DNA, but not the number of cells. Therefore, we used Alamar Blue assay to estimate the effects of drugs on the number of cells. A total of 1×10^4 cells per well were seeded in 100 μl per well of proliferation medium on laminin- and ornithin-coated 96-well plates. After overnight incubation, cells were treated with DEX (Sigma), Li chloride (Wako, Osaka, Japan), SB415286 (Tocris, Ellisville, MO), and/or quercetin (Que; Calbiochem, San Diego, CA) at each concentration. After 3 days, 10 μl per well of Alamar Blue solution (Invitrogen) was added into the medium and cells were incubated at 37°C for 3 h. The medium (50 μl) was dispensed into plates, and the fluorescence intensity of samples was measured by Fluoroscan (Dainippon Sumitomo Pharma, Osaka, Japan) and calculated as described in the manufacturer's manual. Statistical analysis was performed by one-way ANOVA and Dunnet's *post hoc* test. Significance was defined as $p < 0.05$. Data are expressed as the means \pm SEM.

RNA Extraction and Quantitative RT-PCR Analysis

A total of 1×10^5 cells per well were seeded in a proliferation medium on laminin- and ornithin-coated six-well plates. After overnight incubation, cells were treated with each drug at each concentration. After 3 days, total RNA was extracted from cells with the RNeasy extraction kit (Qiagen, Hilden, Germany). Total RNA was converted to cDNA with the Quantitect Reverse Transcription kit (Qiagen). PCR was performed with the SYBR GreenER qPCR SuperMix for ABI PRISM (Invitrogen) in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster, CA). The conditions of PCR were: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The sequences of forward and reverse primers used were: AGCTGGTCATCAATGGGAAA and ATTTGATGTTAGCGGGATCG for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), CTGAGGCCTCTCTTCTTCCA and ATCAGATTCACCCACCTGA for nestin, TTTCTCCAACCTCCAGATCC and GGTGGCCTTCTGACACAGAT for GFAP, GAGGAGAGAAAAAGAAAGGAGAGAA and AAGTGCAATTGGGATGAAAAA for SOX2, TAAAAGCCAGGCCCAAT and TGACTGCTAGAAGTCCATTTGC for doublecortin (DCX), CTGCTTTGCTCCTGATCTGA and TTCATAGGATACCTGCAATCTTTG for GR, GATAGAGGCCAAATTAATCTTTCAA and CCTTCTGCGTCAGCTTAGGT for mineralocorticoid receptor (MR), CAACGCACCTTCTTTCAGAG and AGGGCTTCAATCTGTTCTCTG for cyclin D1, respectively. GAPDH was used as a control. The results were analyzed by using SDS 2.0 software (Applied Biosystems). Statistical analyses were performed by one-way ANOVA and Dunnet's *post hoc* test. Significance was defined as $p < 0.05$. Data are expressed as the means \pm SEM.

Western Blotting

For preparation of total proteins, 1×10^5 cells per well were seeded in a proliferation medium on laminin- and ornithin-coated six-well plates. After overnight incubation, cells were

treated with each drug at each concentration. After 3 days, lysis of cells and preparation of total proteins were performed with the Mammalian Cell Lysis Kit (Sigma). For preparation of nuclear proteins, 4×10^5 cells per well were seeded in a proliferation medium on laminin- and ornithin-coated 100 mm dishes. After overnight incubation, cells were treated with each drug at each concentration. After 3 days, lysis of cells and preparation of nuclear proteins were performed with the Nuclear Extract Kit (Active Motif, Carlsbad, CA). Protein concentration was measured by the Protein Assay Kit (Pierce, Rockford, IL), and an equal amount of proteins (20 μ l per well) was loaded onto a 10% SDS gel. The gel was transferred onto a nitrocellulose membrane (GE Healthcare, Milwaukee, WI) and incubated with primary antibodies that were used at the following concentrations: mouse monoclonal anti-cyclin D1 antibody (1 : 200; Santa Cruz Biotechnology), mouse monoclonal anti- β -catenin antibody (1 : 200; Santa Cruz Biotechnology), mouse monoclonal anti-GAPDH antibody (1 : 200; Santa Cruz Biotechnology), rabbit polyclonal anti-GSK-3 β antibody (1 : 1000; Cell Signaling, Danvers, MA), rabbit polyclonal anti-pGSK-3 β (Ser⁹) antibody (1 : 1000; Cell Signaling), rabbit polyclonal anti-pGSK-3 β (Tyr²¹⁶) antibody (1 : 1000; Biosource, Camarillo, CA). After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary anti-mouse IgG antibody (1 : 4000; GE Healthcare) or horseradish peroxidase-conjugated secondary anti-rabbit IgG antibody (1 : 4000; GE Healthcare). Protein expression was detected with the Amersham ECL Plus Western Blotting Detection System (GE Healthcare) and Amersham Hyperfilm ECL (GE Healthcare). The pictures were converted to digital files and the intensity of each band was analyzed with ImageJ (National Institutes of Health, Bethesda, MD). Statistical analyses were performed by one-way ANOVA and Dunnett's *post hoc* test. Significance was defined as $p < 0.05$. Data are expressed as the means \pm SEM.

RESULTS

Isolation and Culture of ADP

Tissues from DG of adult Sprague–Dawley rats were dissected carefully to exclude all other regions containing subependymal tissues in which neural stem cells exist (Figure 1a). Cells were dissociated from these tissues as described in Materials and methods, and cultured in a proliferation medium on laminin- and ornithin-coated dishes. In this condition, cells grew at monolayer and did not form any neurospheres. Most cells had a flat and round but slightly elongated shape with short branches, and were phase dark (Figure 1b). It took approximately 4–5 weeks (equal to 5–6 times passages) to get an adequate number of cells for assays. After around 12 times passages, the proliferation potency of cells was decreased, and the shape of cells began to change into smaller and more round ones or more elongated and branched ones. Therefore, we used cells for all assays from P5 to P11.

Characterization of ADP

First, we saw the mRNA expression of three common neural precursor cell markers, such as nestin, GFAP, and SOX2,

and an immature neuron marker, DCX, in the cells with RT–PCR. The mRNA expressions of nestin, GFAP, and SOX2 were detected, but that of DCX was not (Figure 1c). Next, we performed immunocytochemistry for nestin, GFAP, and SOX2. The protein expression of each marker was detected in around 90% of the cells through different passages (Figure 1d). Moreover, most of the cells were immunolabeled by both nestin and GFAP antibodies at cytoplasm (Figure 1e), and nestin-positive cells were also immunolabeled by SOX2 antibody at the nucleus (Figure 1f). These results indicated that the cells dissociated from adult rat DG expressed nestin, GFAP, and SOX2 in both mRNA and protein, and were uniform in the shape and expression pattern through the passages we examined. In addition, we performed immunocytochemistry for BLBP, another neural precursor cell marker, and Prox1, another immature neuron marker. Protein expression of BLBP was detected as in the cases of nestin, GFAP, and SOX2, but that of Prox1 was not detected (data not shown). Next, we examined whether the nestin-positive cells are dividing or not. Most of the nestin-positive cells were immunolabeled by BrdU antibody (Figure 2A). The ratio of BrdU-positive cells/DAPI-positive cells was about 60–70% (Figure 2B).

In addition, we examined whether the cells differentiated to neuron or glia with the appropriate medium. The cells were treated with 10 ng/ml BDNF, 1 μ M retinoic acid/0.5% fetal bovine serum, or 500 ng/ml IGF for 7 days. Cells were immunostained by anti-Tuj1 antibody for neuron, anti-GFAP antibody for astrocyte, or anti-O4 antibody for oligodendrocyte. Most cells treated with BDNF elongated axon-like branches and expressed Tuj1 (Figure 2C, a). Some cells treated with retinoic acid/fetal bovine serum changed like spread, less-rounded, and expressed GFAP (Figure 2C, b). These cells did not express nestin (data not shown), and their shape was larger and less round than that of untreated cells (Figures 1e and 2C, b), although the untreated cells also expressed GFAP (Figure 1e). A small number of cells treated with IGF formed many of the short dendrites and expressed O4 (Figure 2C, c). These results indicate that the cells dissociated from DG of adult rat brains express three common markers of neural precursor cells and have both proliferation potency and multipotency. Therefore, the cells were identified as neural precursor cells, and we call them ADPs.

DEX and Li have Reciprocal Effects on ADP Proliferation

Glucocorticoids can bind to both GR and MR (Sousa and Almeida, 2002). To elucidate the expression pattern of these GRs, we examined the mRNA expression of GR and MR in ADP. RT–PCR showed that the mRNA expression of GR was detected, but not that of MR in ADP (Figure 3a). Thus, it is likely to be adequate to use DEX, a specific agonist of GR, for evaluating the effects of glucocorticoids on ADP proliferation. We investigated the effect of DEX on ADP proliferation with Alamar Blue assay. The number of cells was not significantly different between control and 0.5, 1, 2, 5, 10 μ M DEX treatment for 1 day or 2 days (data not shown). However, it was significantly decreased with 2, 5, 10 μ M DEX treatment for 3 days (Figure 3b). The decreased

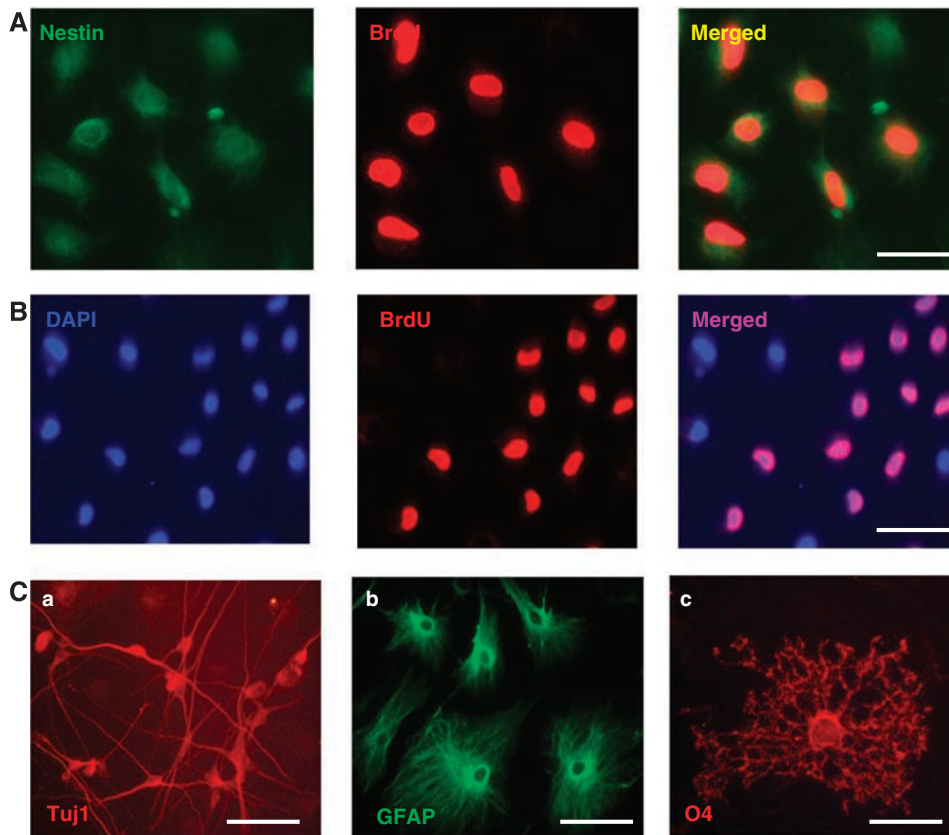


Figure 2 Tests for proliferation potency and multipotency. (A, B) Cells showed proliferation potency. Cells were incubated in 10 nM bromodeoxyuridine (BrdU) for 24 h and immunostained with anti-BrdU antibody and anti-nestin antibody. All of the BrdU-positive cells were nestin positive. The ratio of BrdU-positive cells was approximately 60–70%. Scale bar = 30 μ m (A), 90 μ m (B), respectively. (C) Cells showed multipotency. Cells were treated with (a) 10 ng/ml BDNF, (b) 1 μ M retinoic acid/0.5% fetal bovine serum (FBS), and (c) 500 ng/ml insulin-like growth factor (IGF) for 7 days, respectively. Subsequently, cells were immunostained with anti-Tuj1 antibody (a) for neuron, anti-glial fibrillary acidic protein (anti-GFAP) antibody (b) for astrocyte, anti-O4 antibody (c) for oligodendrocyte. Most of the brain-derived neurotrophic factor (BDNF)-treated cells were Tuj1-positive (a). Some of the cells treated with retinoic acid/FBS were GFAP-positive (b). The small number of IGF-treated cells was O4-positive (c). Each of them showed characteristic shape. Scale bar = 90 μ m (a), 60 μ m (b), 20 μ m (c), respectively.

number of cells has three possibilities: decreased proliferation, accelerated differentiation, and apoptosis. To test the possibility of accelerated differentiation, we performed immunostaining with Tuj1, nestin, GFAP, and O4 antibodies, and there were no Tuj1-positive cells, nestin-negative and GFAP-positive cells, and O4-positive cells (data not shown). To test the possibility of accelerated apoptosis, we performed dUTP nick-end labeling (TUNEL) assay, and any TUNEL-positive cells were not seen (data not shown). In addition, we confirmed that DEX decreased ADP proliferation with BrdU immunocytochemistry (data not shown). Therefore, DEX had no effect on both differentiation and apoptosis in ADP, and we concluded that DEX decreases ADP proliferation. Next, we evaluated the effect of Li on ADP proliferation. We treated cells with Li in 0, 0.1, 0.3, 1, and 3 mM for 3 days, both in the absence and presence of 5 μ M DEX, and performed Alamar Blue assay. Li had no effect on ADP proliferation at any concentrations in the absence of DEX (Figure 3c). On the other hand, Li recovered ADP proliferation decreased by DEX to the control level at 1 and 3 mM in the presence of 5 μ M DEX (Figure 3d). Taken together, these results suggested that DEX and Li had reciprocal effects on ADP proliferation.

Involvement of GSK-3 β and β -Catenin/TCF Pathway in the Reciprocal Effects Between DEX and Li on ADP Proliferation

Li works as an inhibitor of GSK-3 β through the direct inhibition of its catalyzed reaction (Klein and Melton, 1996; Stambolic *et al*, 1996; Davies *et al*, 2000). However, Li also works as an inhibitor of inositol monophosphatase (Atack *et al*, 1995). To confirm that the effect of Li on ADP proliferation is mediated by the direct inhibition of GSK-3 β , we examined the effects of SB415286, a specific inhibitor of GSK-3 β (Coghlan *et al*, 2000). We found that SB415286 mimicked the effects of Li on ADP proliferation both in the absence of DEX (Figure 4a) and in the presence of 5 μ M DEX (Figure 4b). Therefore, the direct inhibition of GSK-3 β might be involved in the recovery effect of Li on ADP proliferation.

GSK-3 β is widely known as a key regulator of β -catenin/TCF pathway (Aberle *et al*, 1997; Orford *et al*, 1997). Therefore, we investigated the involvement of β -catenin/TCF pathway in the reciprocal effects between DEX and Li on ADP proliferation with Que, which inhibits β -catenin/TCF pathway through mainly the inhibition of

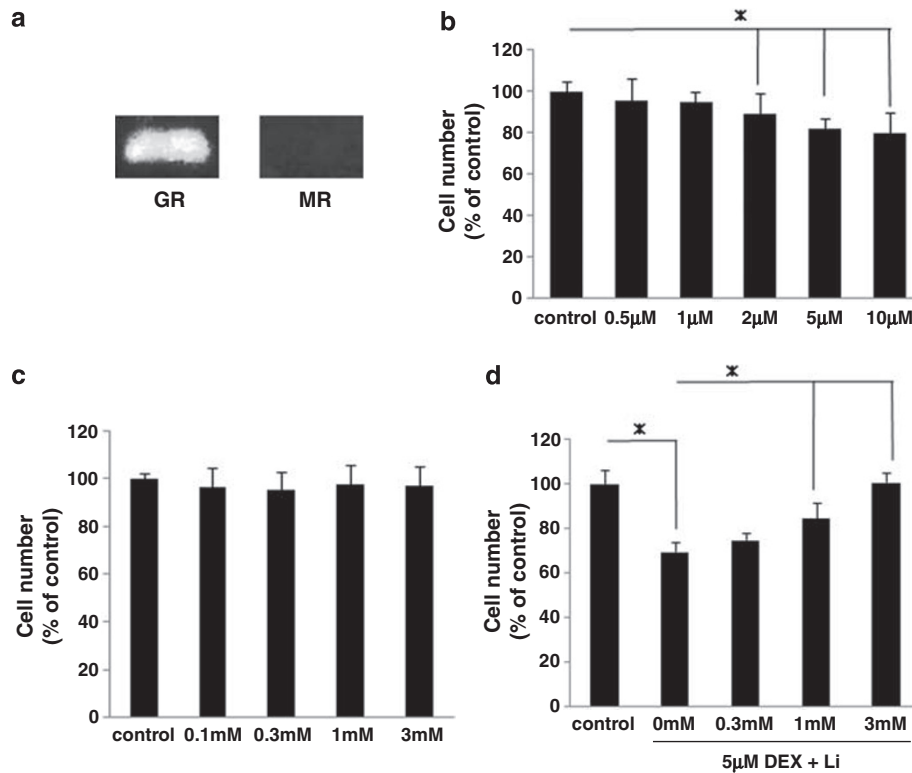


Figure 3 Dexamethasone (DEX) and lithium (Li) have reciprocal effects on adult rat dentate gyrus-derived neural precursor cell (ADP) proliferation. (a) Detection of mRNA of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) with RT-PCR. mRNA of GR was detected, but mRNA of MR was not detected. (b) DEX decreased ADP proliferation. Alamar Blue assay was performed 3 days after the treatment. Data are shown as the means \pm SEM of four independent cultures. * $p < 0.05$, compared with control. (c) Li had no effect on ADP proliferation in the absence of DEX. Alamar Blue assay was performed 3 days after the treatment. Data are shown as the means \pm SEM of four independent cultures. (d) Li recovered DEX-decreased ADP proliferation. Alamar Blue assay was performed 3 days after the treatment. Data are shown as the means \pm SEM of four independent cultures. * $p < 0.05$, compared with control or 0 mM.

nuclear translocation of β -catenin (Park *et al*, 2005). Cells were treated with DEX, Li, and Que for 3 days, and Alamar Blue assay was performed. Que had no effect on ADP proliferation decreased by DEX in the absence of Li (Figure 4c). On the other hand, Que significantly abolished the recovery effect of Li from ADP proliferation decreased by DEX at 10 and 30 μ M (Figure 4d). These results indicated that β -catenin/TCF pathway was involved in the reciprocal effects between DEX and Li on ADP proliferation.

Cyclin D1 Expression is Reciprocally Regulated by DEX and Li Through β -Catenin/TCF Pathway

Cyclin D1 is a common cell-cycle regulator that controls G_1 -S transition through controlling cyclin-dependent kinases, and its expression is regulated by β -catenin/TCF pathway (Tetsu and McCormick, 1999; Shtutman *et al*, 1999). We investigated the effect of DEX, Li, and Que for cyclin D1 expression in ADP. Cells were treated with 5 μ M DEX, 3 mM Li, and/or 30 μ M Que for 3 days, and quantitative RT-PCR and western blotting were performed. Quantitative analysis showed that DEX significantly decreased cyclin D1 expression in both mRNA (Figure 5a) and protein (Figure 5b) and that Li recovered it to the control level. However, Que abolished the recovery effect of Li in both mRNA (Figure 5a) and protein (Figure 5b). Li had no effect on cyclin D1 expression in ADPs in the absence of DEX (data not shown). Taken together, these results indicated that the

expression level of cyclin D1 might be reciprocally regulated by DEX and Li through β -catenin/TCF pathway as in the case with ADP proliferation.

Nuclear Translocation of β -Catenin is Reciprocally Regulated by DEX and Li

β -Catenin is translocated from the cytosol to the nucleus and works as a transcription factor after binding to TCF (Salic *et al*, 2000). We measured the effects of DEX, Li, and Que on nuclear β -catenin in ADP. Cells were treated with 5 μ M DEX, 3 mM Li, and/or 30 μ M Que for 3 days. After that, the nuclear proteins were extracted and western blotting was performed. The protein level of nuclear β -catenin was decreased with DEX and Li recovered it to the control level (Figure 6a and b). Moreover, Que abolished the recovery effect of Li (Figure 6a and b). The treatment with Li alone had no effect on the protein level of nuclear β -catenin (data not shown). These results suggested that the nuclear translocation of β -catenin might be reciprocally regulated by DEX and Li, as in the cases of ADP proliferation and cyclin D1 expression.

Effects of DEX on the Phosphorylation States of GSK-3 β

Activated GSK-3 β phosphorylates β -catenin and inhibits its nuclear translocation (Aberle *et al*, 1997; Orford *et al*, 1997). The activity of GSK-3 β is regulated by two

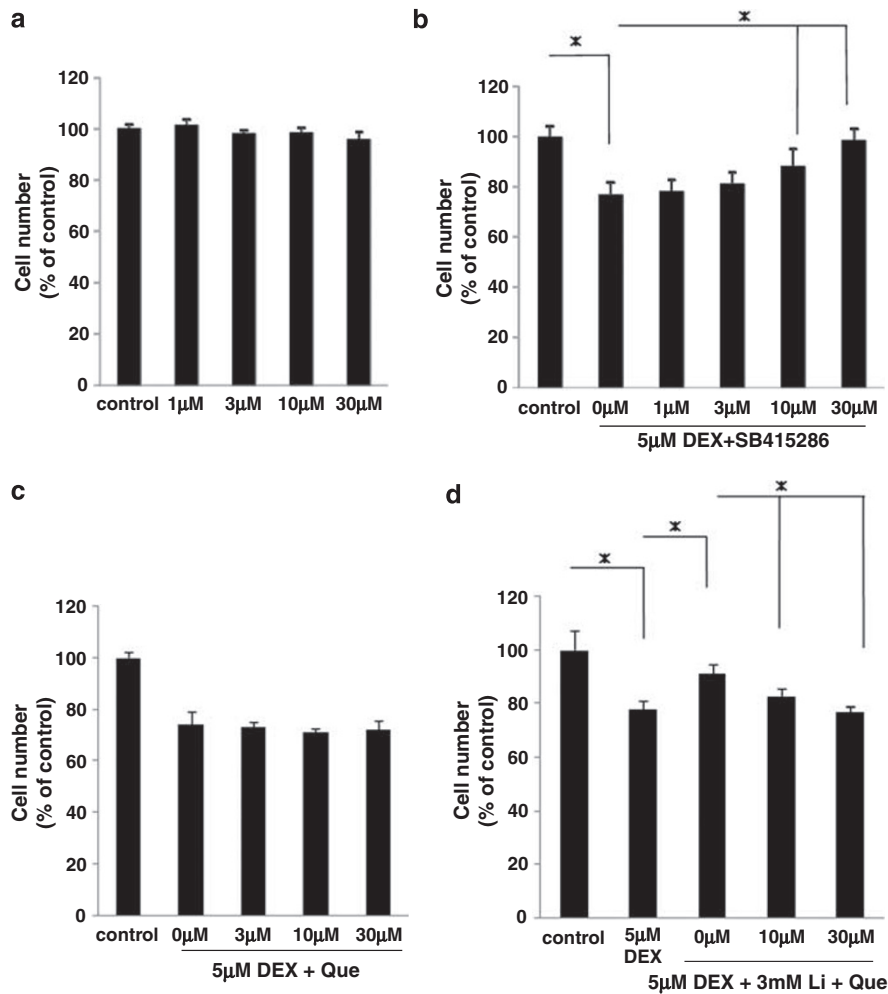


Figure 4 The involvement of glycogen synthase kinase-3 β (GSK-3 β) and β -catenin/TCF pathway in the reciprocal actions between dexamethasone (DEX) and lithium (Li) on adult rat dentate gyrus-derived neural precursor cell (ADP) proliferation. (a) SB415286 had no effect on ADP proliferation in the absence of DEX. Alamar Blue assay was performed 3 days after the treatment. Data are shown as the means \pm SEM of four independent cultures. (b) SB415286 recovered DEX-decreased ADP proliferation. Alamar Blue assay was performed 3 days after the treatment. Data are shown as the means \pm SEM of four independent cultures. $p < 0.05$, compared with control or 0 μ M. (c) Quercetin (Que) had no effect on ADP proliferation in the absence of Li. Alamar Blue assay was performed 3 days after the treatment. Data are the means \pm SEM of four independent cultures. (d) Que abolished the recovery effect of Li on DEX-decreased ADP proliferation. Alamar Blue assay was performed 3 days after the treatment. Data are shown as the means \pm SEM of four independent cultures. * $p < 0.05$, compared with control, 5 μ M DEX or 0 μ M.

phosphorylated residues; Ser⁹ to render it inactive (Cross *et al*, 1995) and Tyr²¹⁶ to render it active (Hughes *et al*, 1993). Moreover, Li works as an inhibitor of GSK-3 β through the direct inhibition of its catalyzed reaction (Klein and Melton, 1996; Stambolic *et al*, 1996). Therefore, we investigated whether DEX and Li affect the expression of GSK-3 β and the phosphorylation of these residues. Cells were treated with DEX and/or Li for 3 days, and western blotting was performed. Ser⁹ is well phosphorylated, but Tyr²¹⁶ is little in the control condition (Figure 7a). DEX increased the phosphorylation of Tyr²¹⁶ remarkably, but it had no effect on the expression of GSK-3 β and the phosphorylation of Ser⁹ (Figure 7a and b). Conversely, the treatment of Li alone or those of DEX and Li had no effect on all of them (data not shown). Taken together, these results suggested that DEX might decrease ADP proliferation by increasing the phosphorylation of Tyr²¹⁶ on GSK-3 β and that Li might recover ADP proliferation, decreased by DEX through the direct inhibition of its

catalyzed reaction, not changing the phosphorylation of Tyr²¹⁶ on it.

DISCUSSION

Here we have established the culture system of ADP. ADP is carefully isolated from the dissected DG of adult rats to exclude any subependymal tissues, grows at monolayers in the described condition, and can be passaged 10–11 times with the same character. ADP expresses nestin, GFAP, SOX2, and BLBP, but not DCX and Prox1. In addition, ADP has proliferation potency and multipotency. Some culture systems of neural precursor cells derived from the adult rodent hippocampus have already been reported (Palmer *et al*, 1997, 1999; Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005; Babu *et al*, 2007). Our culture system is based on them, but is different in some ways as shown below. Both Seaberg's and Bull's culture systems are derived from mouse

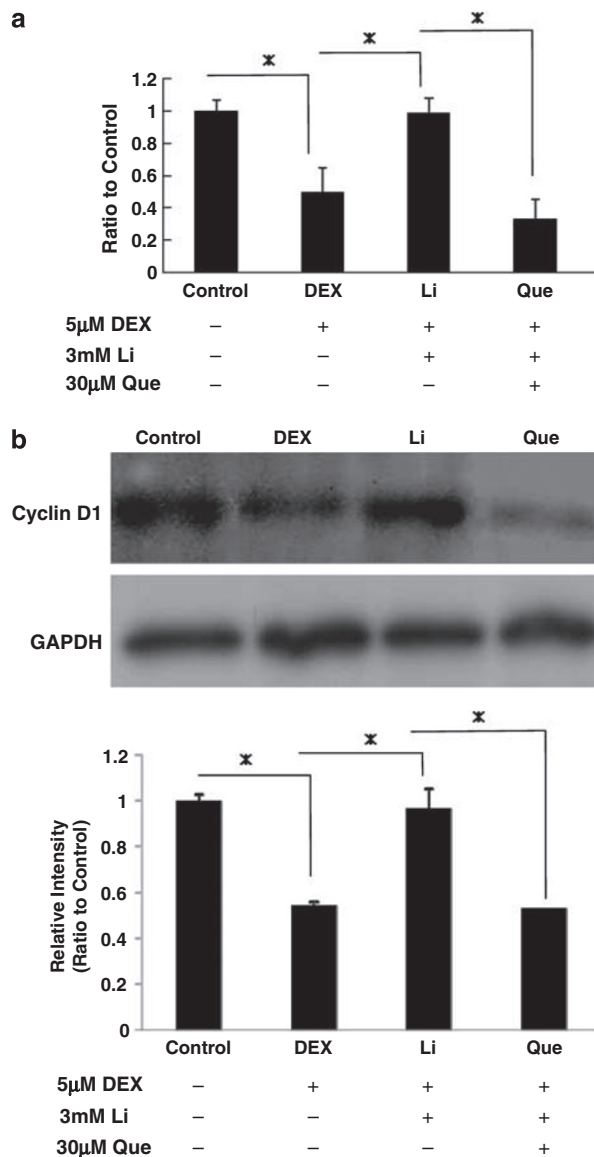


Figure 5 Cyclin D1 expression is reciprocally regulated by dexamethasone (DEX) and lithium (Li) through the β -catenin/TCF pathway. (a) DEX decreased mRNA expression of cyclin D1, Li recovered it, and Que abolished the recovery effect of Li. Cells were treated with 5 μ M DEX, 3 mM Li, and/or 30 μ M Que. After 3 days, RNA isolation and quantitative RT-PCR were performed. Values are shown as the ratio of cyclin D1 mRNA vs glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Data are shown as the means \pm SEM of three independent samples. * p < 0.05, compared with control, DEX, or Li. (b) DEX decreased protein expression of cyclin D1, Li recovered it, and Que abolished the recovery effect of Li. Cells were treated with 5 μ M DEX, 3 mM Li, and/or 30 μ M Que. After 3 days, nuclear proteins were prepared, 20 μ g of them was loaded into each lane, and western blotting was performed. Pictures are shown from a typical experiment that was repeated three times. Data are shown as the means \pm SEM of three independent cultures. * p < 0.05, compared with control, DEX, or Li.

DG, but their culture conditions differ from ours. Babu's culture system is relatively close to ours, but it is derived from mouse DG and can be passaged many more times than ours. In addition, the properties of adult mouse-derived neural precursor cells may be different from those of adult rat-derived ones (Ray and Gage, 2005). Meanwhile, Palmer's

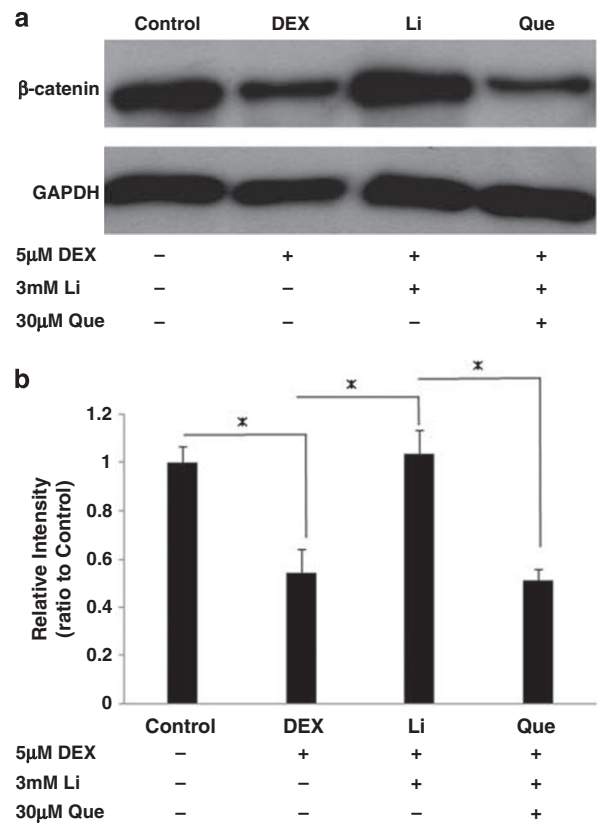


Figure 6 Nuclear translocation of β -catenin is reciprocally regulated by dexamethasone (DEX) and lithium (Li). (a, b) DEX decreased nuclear β -catenin, Li recovered it, and Que abolished the recovery effect of Li. Cells were treated with 5 μ M DEX, 3 mM Li, and/or 30 μ M Que. After 3 days, nuclear proteins were prepared, 20 μ g of them was loaded into each lane, and western blotting was performed. In (a), pictures are shown from a typical experiment that was repeated three times. In (b), data are shown as the means \pm SEM of three independent cultures. * p < 0.05, compared with control, DEX, or Li.

culture system is derived from rat entire hippocampus, but not dissected DG.

Four differentiation stages of precursor cells, including stem and progenitor cells, have been proposed in adult rodent hippocampus *in vivo* (Seri et al, 2001; Fukuda et al, 2003; Kempermann et al, 2004; Steiner et al, 2006): Cells in the first stage (type-1 cells) express nestin, GFAP, SOX2, and BLBP. Cells in the second stage (type-2a cells) express nestin, SOX2, and BLBP. The expression of GFAP in this stage is vague. Cells in the third stage (type-2b cells) express nestin, DCX, and Prox1, but not GFAP and SOX2 (Kempermann, 2006; Gage et al, 2008). Therefore, the expression pattern of markers on ADPs corresponds to type 1 cell or type 2a cell *in vivo*. The shape of ADP is different from that of type 1 cell, with an elongated and blanced shape, and is similar to that of type 2a cell with a flat and round shape (Kempermann et al, 2004). The proliferation potency of ADP is limited; that of type 1 cell is probably unlimited, and that of type 2a cell is limited (Kempermann et al, 2004). Taken together, ADP could possible be type2a cells. It is poorly understood which stage of neural precursor cells contributes to the reactivity of drugs to neurogenesis. However, a recent study indicated that type 2a-like cells might be a target of fluoxetine, which is an

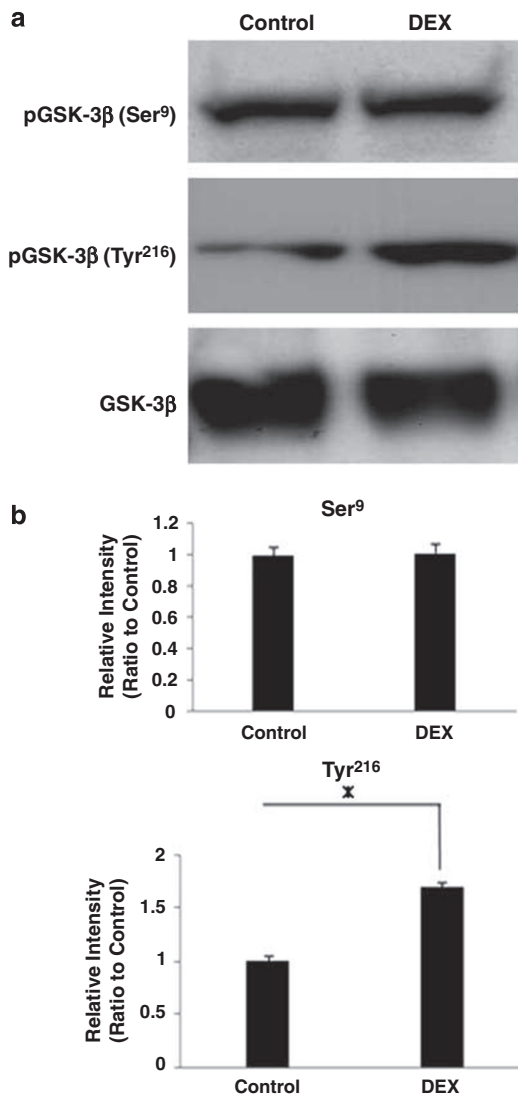


Figure 7 Effects of dexamethasone (DEX) on two phosphorylated residues: Ser⁹ and Tyr²¹⁶ of glycogen synthase kinase-3β (GSK-3β). (a, b) DEX increased the phosphorylation of Tyr²¹⁶, not but Ser⁹ of GSK-3β. Cells were treated with 5 μM DEX. After 3 days, 20 μg of cell lysates were loaded into each lane and western blotting was performed. In (a), pictures are shown from a typical experiment that was repeated three times. In (b), data are shown as the means ± SEM of three independent cultures. **p* < 0.05, compared with control.

antidepressant and can increase adult neurogenesis in DG (Encinas *et al*, 2006). Therefore, it might be beneficial to examine the reactivity of type 2a cells to various drugs and ADPs could be a good model for type2a cells.

Some studies have shown that Li increases adult neurogenesis in DG of rodents *in vivo* (Chen *et al*, 2000; Son *et al*, 2003; Kim JS *et al*, 2004). In addition, it has been shown that Li directly increases neural precursor cells derived from the embryo (Kim JS *et al*, 2004) and adult entire hippocampus (Wexler *et al*, 2008). However, it remains unclear whether Li directly affects the proliferation of neural precursor cells in DG of adult rodents. To elucidate it, we investigated the effect of Li on ADP proliferation. Although Li had no effect on ADP proliferation in the absence of DEX, Li recovered the ADP proliferation decreased by DEX to the control level.

Interestingly, these findings are different from Wexler's results in spite of the closeness of the source of cells. The discrepancy might be due to the difference of the source and character of cells as well as culture condition. We have no answer regarding which culture condition and reactivity to Li is closer to those of *in vivo* neural precursor cells in adult DG, and further investigation to answer this question might lead to a further understanding of their character, including the reactivity to various drugs.

It has been shown that the activation of β-catenin/TCF pathway leads to increasing cyclin D1 expression in tumor-derived cell lines (Tetsu and McCormick, 1999; Shtutman *et al*, 1999). In this study, we found that both cyclin D1 expression and the protein level of nuclear β-catenin are reciprocally regulated by DEX and Li as well as ADP proliferation. These results suggest the involvement of β-catenin/TCF pathway in the reciprocal effects between DEX and Li on ADP proliferation. β-Catenin/TCF pathway is also well known as the canonical Wnt pathway. It has already been shown that the canonical Wnt pathway regulates the proliferation of embryo-derived neural precursor cells *in vitro* (Hirsch *et al*, 2007) and adult hippocampal neurogenesis *in vivo* (Lie *et al*, 2005). However, Lie *et al* showed that the canonical Wnt pathway regulates the proliferation of DCX-positive and elongated cells, which may correspond to type 3 cells and are in the late differentiation stages of neural precursor cells (Kempermann, 2006; Gage *et al*, 2008). Therefore, our present study is the first report to indicate the involvement of β-catenin/TCF pathway in the proliferation of hippocampal neural precursor cells in the early differentiation stages.

Moreover, we first clarified the involvement of glucocorticoids in β-catenin/TCF pathway in adult DG-derived neural precursor cells. There is the possibility that GR directly represses the transcription of cyclin D1 because GR is a transcription factor that can promote or repress the transcription of various genes through direct binding to their promoters (Schoneveld *et al*, 2004). Our results do not exclude this possibility, and this direct mechanism could regulate ADP proliferation in cooperation with β-catenin/TCF pathway.

We found that DEX significantly increases the phosphorylation of Tyr²¹⁶ and has no effect on the phosphorylation of Ser⁹ on GSK-3β. These results suggest that DEX might negatively regulate β-catenin/TCF pathway through the phosphorylation of Tyr²¹⁶ on GSK-3β. Although it has been shown that glucocorticoids negatively regulate cell proliferation through decreasing the phosphorylation of Ser⁹ in osteoblasts (Smith *et al*, 2002), nothing else is known about the effects of glucocorticoids on the phosphorylation states of GSK-3β. Therefore, our present study is the first report to indicate the effects of glucocorticoids on the phosphorylation of Tyr²¹⁶ on GSK-3β.

GSK-3 is well known to be encoded by two different genes, GSK-3β and GSK-3α (Frame and Cohen, 2001; Grimes and Jope, 2001). As most studies of the function of GSK-3 activity in neurogenesis and cell proliferation have focused on the more abundant GSK-3β, our present study also focused on GSK-3β. However, some studies have highlighted a role for GSK-3α in neuronal disease (Phiel *et al*, 2003). In addition, both Li and SB415286 can inhibit not only GSK-3β but also GSK-3α. Therefore, the limitation of

our present study is that it does not exclude the involvement of GSK-3 α .

We have shown that DEX and Li reciprocally regulate ADP proliferation through GSK-3 β and β -catenin/TCF pathway; DEX activates GSK-3 β through the phosphorylation of Tyr²¹⁶; GSK-3 β activated by DEX inhibits β -catenin/TCF pathway, and Li recovers it through inhibiting GSK-3 β activated by DEX. However, it remains unclear how DEX increases the phosphorylation of Tyr²¹⁶ on GSK-3 β . To elucidate it might lead to a further understanding of stress mechanism and the development of new therapeutic targets for psychiatric disorders.

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DISCLOSURE/CONFLICTS OF INTEREST

All the authors declare that they have no conflict of interest.

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