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Citation	Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 34(3), 805-815 https://doi.org/10.1038/npp.2008.198
Issue Date	2009-02
Doc URL	http://hdl.handle.net/2115/50392
Type	article (author version)
File Information	Neuropsychopharmacology34-3_805-815.pdf



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**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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Abbreviated Running Title: DEX and Li reciprocally regulate adult neurogenesis in DG

Abstract

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 treatment of them increases it. This effect is thought to be one of therapeutic action 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's proliferation. ADP is possible to be type-2a cell, which corresponds to be the second stage in a model of four differentiation stages in adult hippocampal neural precursor cells. DEX decreased ADP's proliferation, but Li did not have any effect on it. However, Li recovered ADP's proliferation decreased by DEX. The recovery effect of Li was abolished by quercetin (Que), an inhibitor of

β -catenin/ TCF pathway. The intra-nuclear translocation of β -catenin and expression of cyclin D1 are reciprocally regulated by DEX and Li in a similar way of the 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 play a pivotal role in the reciprocal effects between DEX and Li on ADP's proliferation and are new targets of therapeutic agents for stress-related disorders.

Keywords: neurogenesis, stress, hippocampus, dexamethasone, mood stabilizer, Wnt

Introduction

It has been well established that neurogenesis occurs in adult brain of various animal species, including human (Eriksson et al, 1998; Gould et al, 1997; Gage, 2000). Neurogenesis mainly takes place in two discrete regions of adult brain: subventricular zone of lateral ventricles (Alvarez-Buylla and Garcia-Verdugo, 2002) and subgranular zone of the dentate gyrus (DG) in 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 human, elevated levels of glucocorticoids is one of causal events in stress-related disorders (de Kloet et al, 1995; Swaab et al, 2005). In contrast, the administrations of lithium (Li) which is used for treatment of stress-related disorders increase 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, to elucidate how glucocorticoids and lithium regulate neurogenesis might lead to 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).

We here established the culture system of adult rat DG-derived neural precursor cell (ADP) and showed that dexamethasone (DEX), an agonist of glucocorticoid receptor, decreased ADP's proliferation and Li recovered it. In addition, we demonstrated this reciprocal effect between DEX and Li on ADP's proliferation were regulated by GSK-3 β and β -catenin/ TCF pathway.

Materials and Methods

Isolation and culture of ADP. Adult male Sprague-Dawley rats (8 weeks 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 cut with thickness of 1mm using Brain Slicer (Muromachi, Tokyo, Japan) were immersed into dishes containing ice-cold PBS, and the regions containing DG were dissected carefully under dissecting microscope to exclude all regions containing subependymal tissues (Fig.1A).

These tissues were digested with a mixture of Papain, Dispasell and DNaseI as described previously (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), penicillin-streptomycin (Invitrogen) on a non-coating dish and incubated at 37 °C in 5 % CO₂. After overnight incubation, the medium was changed with 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 proliferation medium at 37 °C on laminin (Invitrogen)-ornithin (Sigma) coated dishes and fed with new medium every two or three days by replacing 50 % of the medium. When cell confluency reached 80-90 %, cells were passaged by trypsinization, and the cell density for seeding was approximately 1 x 10⁴ cells/ cm². The cells were monitored with IX-70 optical microscope (Olympus, Tokyo, Japan).

Analysis of ADP. For detecting the expression pattern of markers, 2 x 10⁴ cells/

well were seeded in proliferation medium on laminin-ornithin coated Lab-Tek® 8-chamber slides (Nalge Nunc International, Naperville, IL). After overnight incubation, immunocytochemistry was performed as described below. For testing the proliferation potency, bromodeoxyuridine (BrdU) (Sigma) was added into medium at 10 nM. After 24 hours, immunocytochemistry was performed. For testing the multipotency, cells were treated in differentiation medium (proliferation medium without bFGF) with 10 ng/ ml brain-derived neurotrophic factor (BDNF) (Sigma), 1 μ M 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 the cases of detecting the expression pattern of markers and testing for the multipotency, permeabilization was performed with PBS containing 0.2 % Triton X-100 for 30 min. In the cases of testing for the proliferation potency, permeabilization was performed as follows; Cells were immersed with 50 % formamide in 2 X SSC (0.3 M NaCl, 0.03 M sodium citrate) at 65 °C for 2 hours, incubated in 2 N HCl for 30 min at room temperature and rinsed in 0.1 M borate buffer for 10 min.

Subsequently, 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. 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-gial fibrillary acidic protein (GFAP) (1:2000; Dako, Glostrup, Denmark), rabbit anti-SRY (sex determining region Y)-related HMG (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 hour at room temperature. Secondary antibodies used were: anti mouse Cy3 and anti-rabbit FITC (1:100; Jackson Immuno Reserch, West Grove, PA). Samples were coverslipped with Vectashield containing 4',6-diamino-2-phenylindole (DAPI) (Vector Laboratories). Fluorescent signals were detected using IX-71 fluorescent microscope system (Olympus).

Cell counting with Alamar Blue assay. Alamar Blue assay is a rapid and simple non-radioactive assay to measure the number of cells. Alamar Blue dye is a fluorogenic redox indicator, and is converted from the oxidized form to the reduced form in cells. The reduced form of Alamar Blue dye is highly fluorescent, and 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 surely is 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 in themselves. 1×10^4 cells/ well were seeded in 100 μ l/ well of proliferation medium on laminin-ornithin coated 96-well plates. After overnight incubation, cells were treated with dexamethasone (DEX) (Sigma), lithium chloride (Li) (Wako, Osaka, Japan), SB415286 (Tocris, Ellisville, MO) and/ or quercetin (Que) (Calbiochem, San Diego, CA) at each concentration. After 3 days, 10 μ l/ well of Alamar Blue solution (Invitrogen) were added into medium, and cells were incubated at 37 °C for 3 hours. 50 μ l of medium were dispensed

into plates and the fluorescence intensity of samples were measured by Fluoroscan (Dainippon Sumitomo Pharma, Osaka, Japan) and calculated as described in the manufacture's manual. Statistical analysis was performed by one-way ANOVA and Dunnet post hoc test. Significance was defined as $p < 0.05$. Data are expressed as the means \pm S.E.M.

RNA Extraction and quantitative RT-PCR analysis. 1×10^5 cells/ well were seeded in proliferation medium on laminin-ornithin coated 6-well plates. After overnight incubation, cells were treated with each drug at each concentration. After 3 days, total RNA was extracted from cells with RNeasy extraction kit (Qiagen, Hilden, Germany). Total RNA was converted to cDNA with Quantitect Reverse Transcription kit (Qiagen). PCR was performed with SYBR GreenER qPCR SuperMix for ABI PRISM (Invitrogen) in 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 sec 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 *ATCAGATTCCCCACCCTGA* for nestin, *TTTCTCCAACCTCCAGATCC* and *GGTGGCCTTCTGACACAGAT* for GFAP, *GAGGAGAGAAAAAGAAAGGAGAGAA* and *AAGTGCAATTGGGATGAAAAA* for SOX2, *TAAAAGCCCAGGCCCAAT* and *TGACTGCTAGAAGTTCCATTTGC* for doublecortin (DCX), *CTGCTTTGCTCCTGATCTGA* and *TTCATAGGATACCTGCAATCTTTG* for glucocorticoid receptor (GR), *GATAGAGGCCAAATTAATCTTTCAA* and *CCTTCTGCGTCAGCTTAGGT* for mineralcorticoid receptor (MR), *CAACGCACTTTCTTTCCAGAG* and *AGGGCTTCAATCTGTTCTG* 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 post hoc test. Significance was defined as $p < 0.05$. Data are expressed as the means \pm S.E.M.

Western Blotting. For preparation of total proteins, 1×10^5 cells/ well were seeded in proliferation medium on laminin-ornithin coated 6-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

Mammalian Cell Lysis Kit (Sigma). For preparation of nuclear proteins, 4×10^5 cells/ well were seeded in proliferation medium on laminin-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 Nuclear Extract Kit (Active Motif, Carlsbad, CA). Protein concentration was measured by Protein Assay Kit (Pierce, Rockford, IL), and equal amount of proteins (20 μ l/ well) were loaded onto a 10 % SDS gel. The gel was transferred onto a nitrocellulose membrane (GE Healthcare, Milwaukee, WI) and incubated with primary antibodies, which were used at 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 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 Image J (National Institutes of Health, Bethesda, MD). Statistical analyses were performed by one-way ANOVA and Dunnett post hoc test. Significance was defined as $p < 0.05$. Data are expressed as the means \pm S.E.M.

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 (Fig.1A). Cells were dissociated from these tissues as described in materials and methods, and cultured in proliferation medium on laminin-ornithin coated dishes. In this condition, cells grew at monolayer and did not form any neurospheres. Most cells had flat and round, but slightly

elongated shape with short branches, and were phase-dark (Fig.1B). It took approximately 4-5 weeks (equal to 5-6 times passages) to get enough amount of cells for assays. After around 12 times passages, proliferation potency of cells was decreased, and the shape of cells began to change into smaller and more round one or more elongated and branched one. Therefore we used cells for all assays from P5 to P11.

Characterization of ADP

First, we saw 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 (Fig.1C). Next, we performed immunocytochemistry for nestin, GFAP and SOX2. Protein expression of each marker was detected in around 90 % of the cells through different passages (Fig.1D). Moreover, most of the cells were immunolabeled by both nestin and GFAP antibodies at cytoplasm (Fig.1E), and nestin-positive cells were also immunolabeled by SOX2 antibody at nucleus (Fig.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 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, and that of Prox1 was not detected (data not shown). Next, we examined whether the nestin-positive cells are dividing or not. Most of nestin-positive cells were immunolabeled by BrdU antibody (Fig.2A). The ratio of BrdU-positive cells/ DAPI-positive cells was about 60-70% (Fig.2B).

In addition, we examined whether the cells differentiated to neuron or glia with 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 (Fig.2Ca). Some cells treated with retinoic acid/ fetal bovine serum changed like spread, less-rounded

and expressed GFAP (Fig.2Cb). These cells did not express nestin (data not shown), and the shape of them was larger and less round than that of untreated cells (Fig.1E and Fig.2Cb), although the untreated cells also expressed GFAP (Fig.1E). Small number of cells treated with IGF formed many of short dendrites and expressed O4 (Fig.2Cc). These results indicate that the cells dissociated from DG of adult rat brains express three common markers of neural precursor cells and have the proliferation potency and the multipotency. Therefore, the cells were identified as neural precursor cells, and we call them adult dentate gyrus-derived neural precursor cells (ADPs).

DEX and Li have the reciprocal effects on ADP's proliferation

Glucocorticoids can bind to both GR and MR (Sousa and Almeida, 2002). To elucidate the expression pattern of these glucocorticoid receptors, we examined mRNA expression of GR and MR in ADP. RT-PCR showed that mRNA expression of GR was detected, but not that of MR in ADP (Fig.3A). Thus, it is likely to be adequate to use DEX, a specific agonist of GR, for evaluating the effects of glucocorticoids on ADP's proliferation. We investigated the effect of

DEX on ADP's 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 (Fig.3B). The decreased number of the 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 Tuj 1-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's 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's proliferation. Next, we evaluated the effect of Li on ADP's proliferation. We treated cells with Li in 0, 0.1, 0.3, 1, and 3 mM for 3 days both in the absence and the presence of 5 μ M DEX, and performed Alamar Blue assay. Li had no

effect on ADP's proliferation at any concentrations in the absence of DEX (Fig.3C). On the other hand, Li recovered ADP's proliferation decreased by DEX to the control level at 1 and 3 mM in the presence of 5 μ M DEX (Fig.3D). Taken together, these results suggested that DEX and Li had the reciprocal effects on ADP's proliferation.

Involvement of GSK-3 β and β -catenin/ TCF pathway in the reciprocal effects between DEX and Li on ADP's proliferation

Li works as an inhibitor of GSK-3 β through 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's 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's proliferation both in the absence of DEX (Fig.4A) and in the presence of 5 μ M DEX (Fig.4B). Therefore, the direct inhibition of GSK-3 β might be involved in the recovery effect of Li on ADP's 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's proliferation with quercetin (Que), which inhibits β -catenin /TCF pathway through mainly the inhibition of 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's proliferation decreased by DEX in the absence of Li (Fig.4C). On the other hand, Que significantly abolished the recovery effect of Li from ADP's proliferation decreased by DEX at 10 and 30 μ M (Fig.4D). These results indicated that β -catenin/ TCF pathway was involved in the reciprocal effects between DEX and Li on ADP's 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 control G1-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 (Fig.5A) and protein (Fig.5B), and that Li recovered it to the control level. However, Que abolished the recovery effect of Li in both mRNA (Fig.5A) and protein (Fig.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's proliferation.

Nuclear translocation of β -catenin is reciprocally regulated by DEX and Li

β -catenin is translocated from cytosol to 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, 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 (Fig.6A and 6B). Moreover, Que abolished the recovery effect of Li (Fig.6A and 6B). The treatment with Li alone had no effect on the protein level of nuclear β -catenin (data not shown). These results suggested that nuclear translocation of β -catenin might be reciprocally regulated by DEX and Li, as in the cases of ADP's 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 nuclear translocation of it (Aberle et al, 1997; Orford et al, 1997). The activity of GSK-3 β is regulated by two 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 direct inhibition of its catalysed 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 (Fig.7A). DEX remarkably increased the phosphorylation of Tyr²¹⁶, but had no effect on the expression of GSK-3 β and the phosphorylation of Ser⁹ (Fig.7A and 7B). On the other hand, the treatment of Li alone or that of DEX and Li had no effect on all of them (data not shown). Taken together, these results suggested that DEX might decrease ADP's proliferation through increasing the phosphorylation of Tyr²¹⁶ on GSK-3 β and that Li might recover ADP's 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 isolated from dissected DG of adult rat enough carefully to exclude any subependymal tissues, grows at monolayer in the described condition, and can be passaged 10-11times with the same character. ADP expresses nestin, GFAP, SOX2 and BLBP, not DCX and Prox1. In addition, ADP has proliferation potency and multipotency. Some culture systems of neural precursor cell derived from adult rodent

hippocampus have already been reported (Palmer et al, 1997; Palmer et al, 1999; Seaberg et al, 2002; Bull and Bartlett, 2005; Babu et al, 2007). Our culture system is based on them, but is different in some ways as below. Both Seaberg's and Bull's culture systems are derived from mouse DG and the culture condition of them are different from those of ours. Babu's culture system is relatively close to ours, but it is derived from mouse DG and can be passaged much 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 culture system is derived from rat entire hippocampus, but not dissected DG.

Four differentiation stages of precursor cells, including stem and progenitor cells, has 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 first stage (type-1 cells) express nestin, GFAP, SOX2 and BLBP. Cells in second stage (type-2a cells) express nestin, SOX2 and BLBP. The expression of GFAP in this stage is vague. Cells in 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 elongated and blanch shape and is similar to that of type-2a cell with 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 is possible to be type-2a cells. It's 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 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 type-2a 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 embryo (Kim JS et al, 2004) and adult entire hippocampus (Wexler et al, 2008). However, it remains unclear whether Li directly affects

proliferation of neural precursor cells in DG of adult rodents. To elucidate it, we investigated the effect of Li on ADP's proliferation. Although Li had no effect on ADP's proliferation in the absence of DEX, Li recovered ADP's 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, and culture condition. We have no answer 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 further understanding of the character of them, 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's proliferation. These results suggest the involvement of β -catenin/ TCF pathway in the reciprocal effects between

DEX and Li on ADP's proliferation. β -catenin/ TCF pathway is also well known as canonical Wnt pathway. It has been already shown that 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 canonical Wnt pathway regulates the proliferation of DCX positive and elongated cells, which may correspond to type-3 cells, and they 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 and 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's 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-3 β . These results suggest that DEX might negatively regulate β -catenin/ TCF pathway through the phosphorylation of Tyr²¹⁶ on GSK-3 β . Although it has been shown that glucocorticoids negatively regulate cell proliferation through decreasing the phosphorylation of Ser⁹ in osteoblast (Smith et al, 2002), nothing else is known about the effects of glucocorticoids on the phosphorylation states of GSK-3 β . Therefore, our present study is the first report to indicate the effects of glucocorticoids on the phosphorylation of Tyr²¹⁶ on GSK-3 β .

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

exclude the involvement of GSK-3 α . This is the limitation of our present study.

We have shown that DEX and Li reciprocally regulates ADP's 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 further understanding of stress mechanism and the development of new therapeutic targets for psychiatric disorders.

Disclosure/ Conflicts of interest: All of the authors declare they have no conflict of interest.

Acknowledgement: This work was supported in part by Grand-in-aid No.18591269 for Scientific Reserch from the Ministry of Education, Science and Culture, Japan. We thank M. Kihara for her expert technical assistance and K. Suzuki, T. Tanaka, T. Masui and H. Toda for their helpful critique.

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Figure legends

Figure 1. Isolation and characterization of ADP A. Coronal section through dissected regions. The dentate gyrus (DG) of adult rat was dissected as 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 μm . 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 passage 6, 8

and 11. Values are shown as the ratio of each marker positive cells versus DAPI

positive cells. Data are shown as the means \pm S.E.M. E and F.

Immunocytochemistry for nestin, GFAP, and SOX2. E: Nestin (red), GFAP

(green). Scale bar = 120 μm . F: Nestin (red), SOX2 (green). Most cells

co-expressed nestin, GFAP and SOX2. Scale bar = 120 μm .

Figure 2. Tests for the proliferation potency and the multipotency A and B. Cells

showed proliferation potency. Cells were incubated in 10nM BrdU for 24 hour and immunostained with anti-BrdU antibody and anti-nestin antibody. All of BrdU-positive cells were nestin positive. The ratio of BrdU-positive cells was approximately 60-70 %. Scale bar = (A) 30 μ m, (B) 90 μ m, respectively. C. Cells showed multipotency. Cells were treated with (a) 10 ng/ ml BDNF, (b) 1 μ M retinoic acid/ 0.5 % FBS, and (c) 500ng/ ml IGF for 7 days, respectively. Subsequently cells were immunostained with anti-Tuj1 antibody (a) for neuron, anti-GFAP antibody (b) for astrocyte, anti-O4 antibody (c) for oligodendrocyte. Most of BDNF-treated cells were Tuj1-positive (a). Some of cells treated with retinoic acid/ fetal bovine serum were GFAP-positive (b). Small number of IGF-treated cells was O4-positive (c). Each of them showed characteristic shape. Scale bar = (a) 90 μ m, (b) 60 μ m, (c) 20 μ m, respectively.

Figure 3. DEX and Li have the reciprocal effects on ADP's proliferation. A.

Detection of mRNA of GR and MR with RT-PCR. mRNA of GR was detected, but mRNA of MR was not detected. B. DEX decreased ADP's proliferation. AlamarBlue assay was performed 3 days after the treatment. Data are shown as

the means \pm S.E.M of four independent cultures. *p<0.05, compared with control. C. Li had no effect on ADP's proliferation in the absence of DEX. AlamarBlue assay was performed 3 days after the treatment. Data are shown as the means \pm S.E.M of four independent cultures. D. Li recovered DEX-decreased ADP's proliferation. AlamarBlue assay was performed 3 days after the treatment. Data are shown as the means \pm S.E.M of four independent cultures. *p<0.05, compared with control or 0mM.

Figure 4. The involvement of GSK-3 β and β -catenin/ TCF pathway in the reciprocal actions between DEX and Li on ADP's proliferation A. SB415286 had no effect on ADP's proliferation in the absence of DEX. AlamarBlue assay was performed 3 days after the treatment. Data are shown as the means \pm S.E.M of four independent cultures. B. SB415286 recovered DEX-decreased ADP's proliferation. AlamarBlue assay was performed 3 days after the treatment. Data are shown as the means \pm S.E.M of four independent cultures. p<0.05, compared with control or 0 μ M. C. Que had no effect on ADP's proliferation in the absence of Li. AlamarBlue assay was performed 3 days after the treatment.

Data are the means \pm S.E.M of four independent cultures. D. Que abolished the recovery effect of Li on DEX-decreased ADP's proliferation. AlamarBlue assay was performed 3 days after the treatment. Data are shown as the means \pm S.E.M of four independent cultures. * $p < 0.05$, compared with control, 5 μ M DEX or 0 μ M.

Figure 5. Cyclin D1 expression is reciprocally regulated by DEX and Li through 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, 3mM 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 versus GAPDH mRNA. Data are shown as the means \pm S.E.M 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, 3mM Li and/ or 30 μ M Que. After 3 days, nuclear proteins were prepared, 20 μ g of them were loaded into each lane and western blotting was performed. Pictures are shown from

typical experiment that was repeated 3 times. Data were shown as the means \pm S.E.M of three independent cultures. * $p < 0.05$, compared with control, DEX or Li.

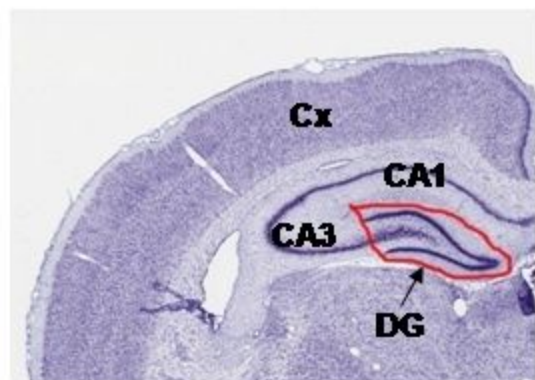
Figure 6. Nuclear translocation of β -catenin is reciprocally regulated by DEX and Li. A and B. DEX decreased nuclear β -catenin, Li recovered it, and Que abolished the recovery effect of Li. Cells were treated with 5 μ M DEX, 3mM Li and/ or 30 μ M Que. After 3 days, nuclear proteins were prepared, 20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

Figure 7. Effects of DEX on two phosphorylated residues: Ser⁹ and Tyr²¹⁶ of GSK-3 β . A and B. DEX increased the phosphorylation of Tyr²¹⁶, not but Ser⁹ of GSK-3 β . Cells were treated with 5 μ M DEX. After 3 days, 20 μ g of cell lysates were loaded into each lane and western blotting were performed. In A, pictures

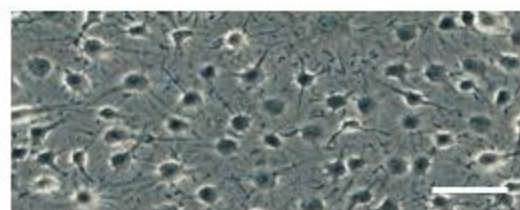
are shown from typical experiment that was repeated 3 times. In B, data were shown as the means \pm S.E.M of three independent cultures. *p<0.05, compared with control.

Figure 1.

A



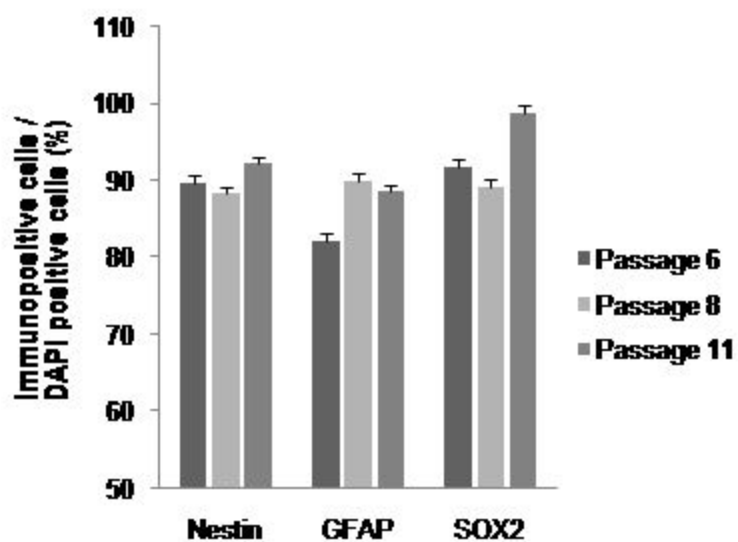
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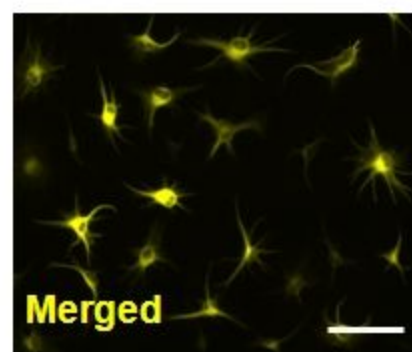
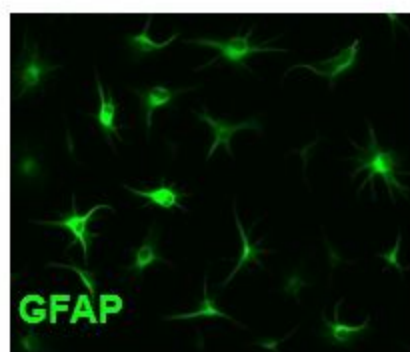
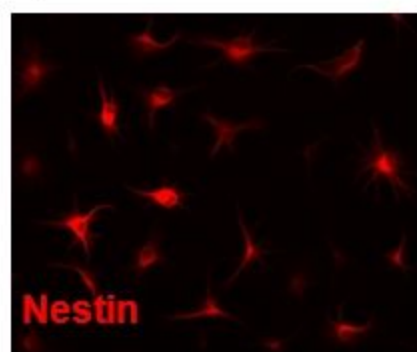
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D



E



F

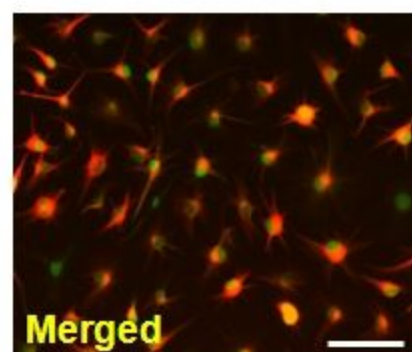
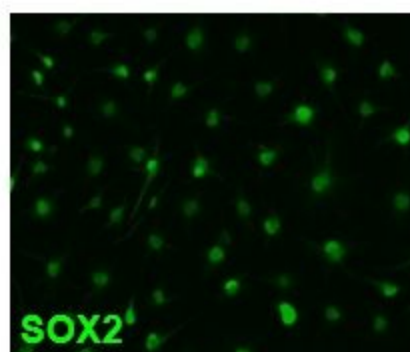
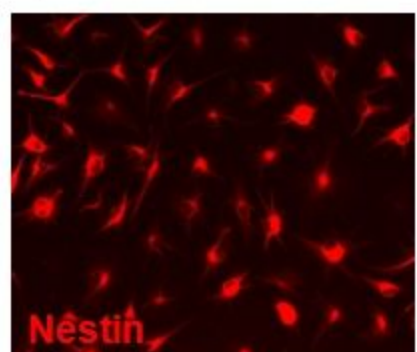
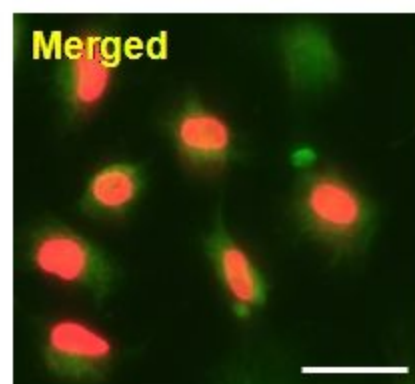
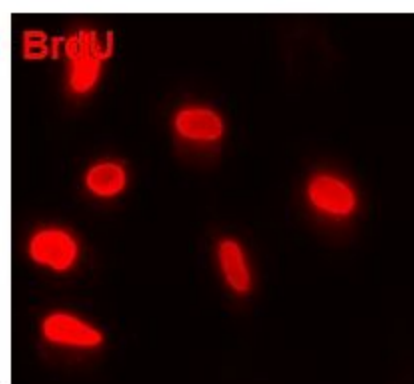
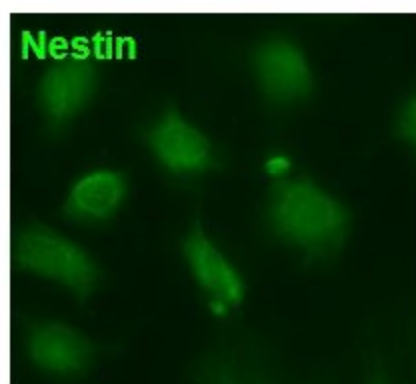
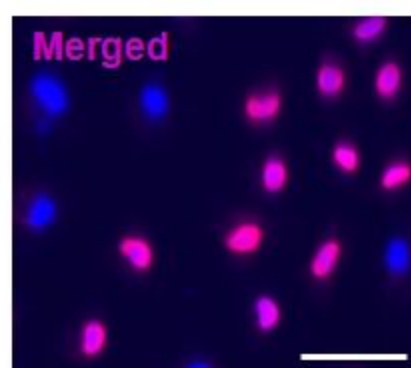
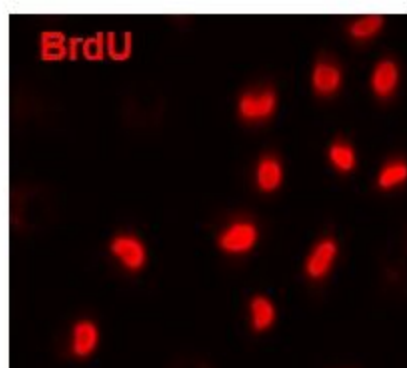
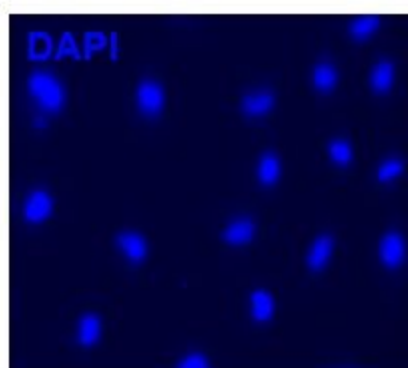


Figure 2.

A



B



C

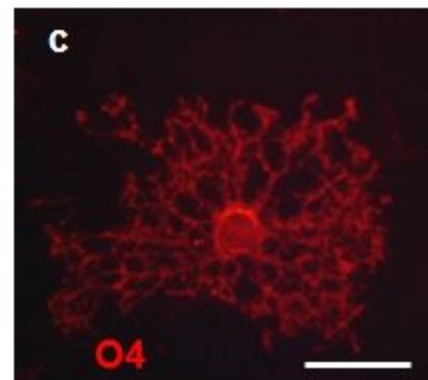
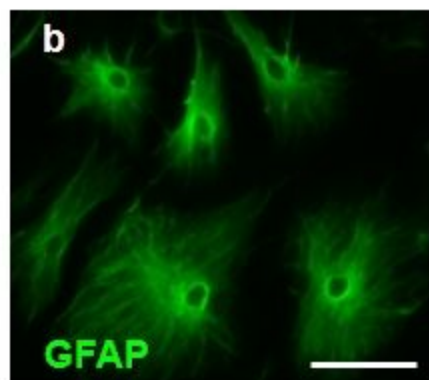
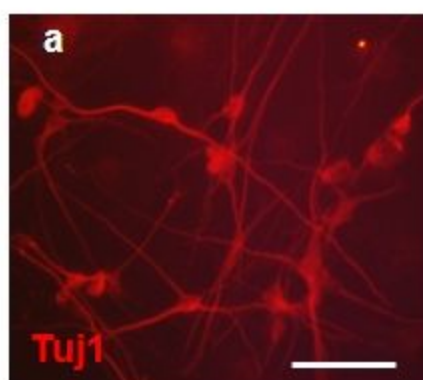
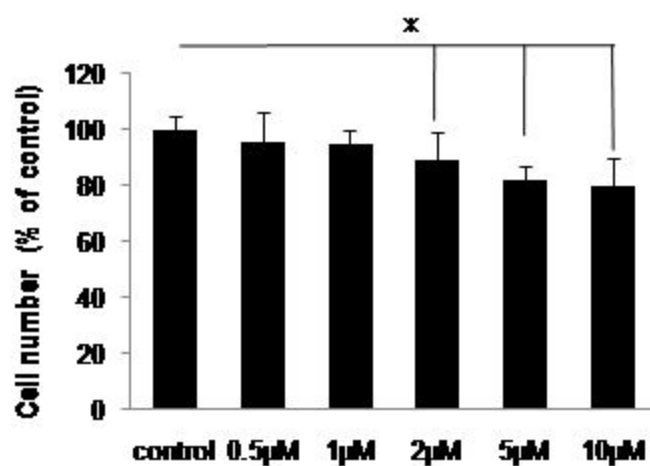


Figure 3.

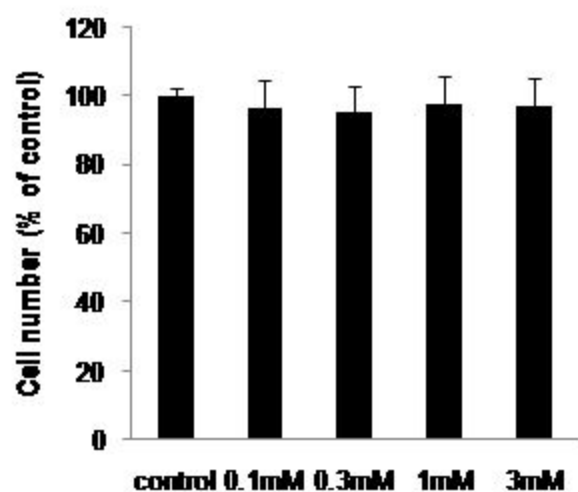
A



B



C



D

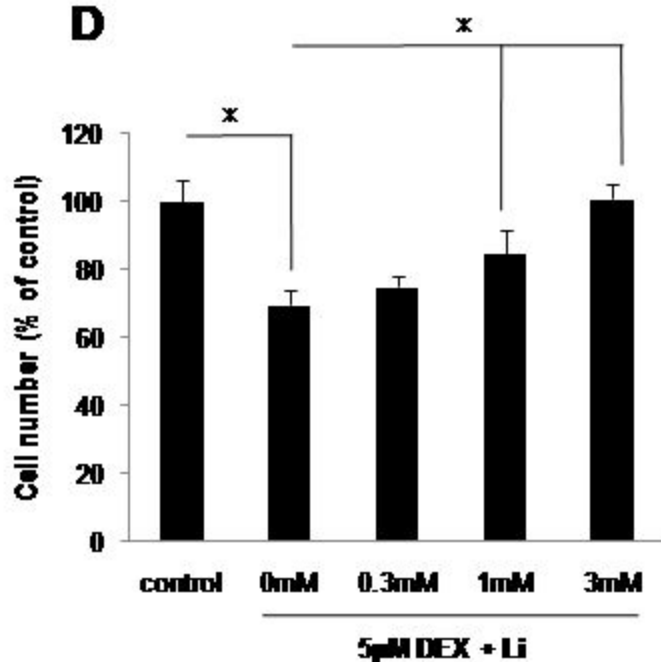


Figure 4.

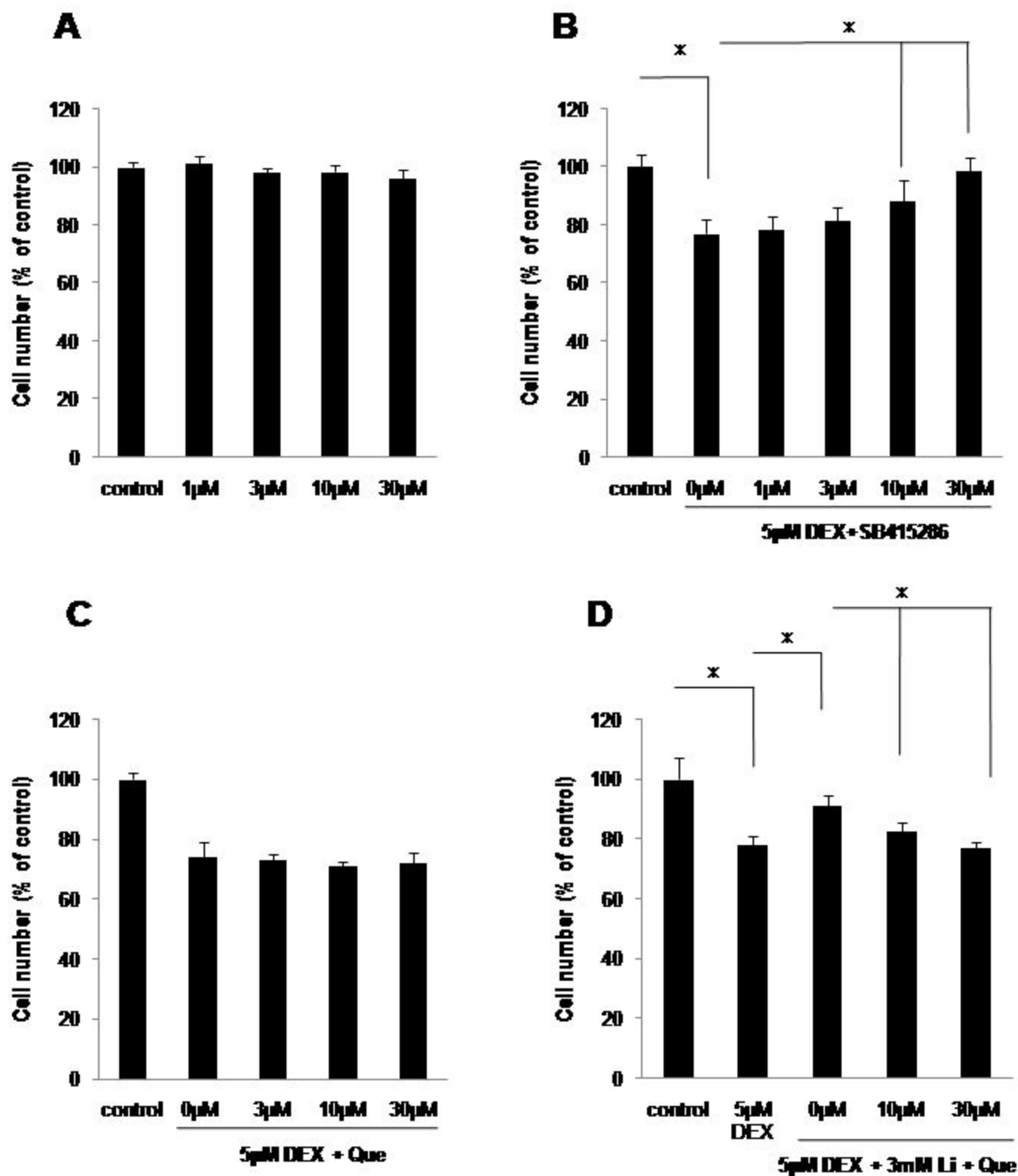
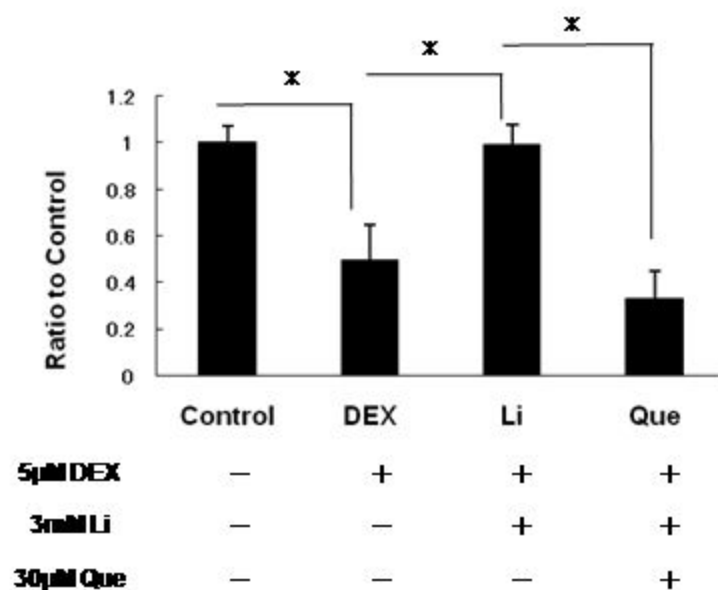


Figure 5.

A



B

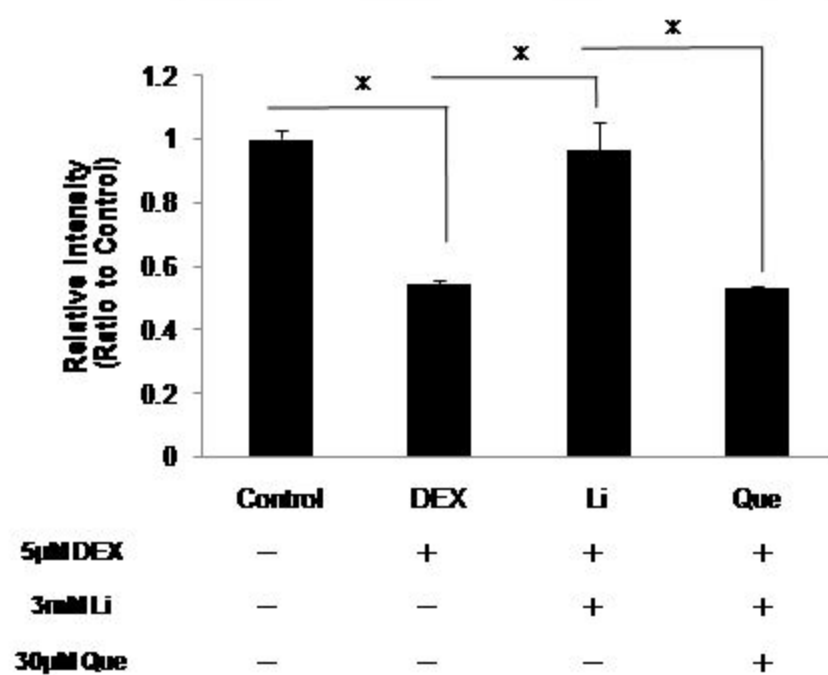
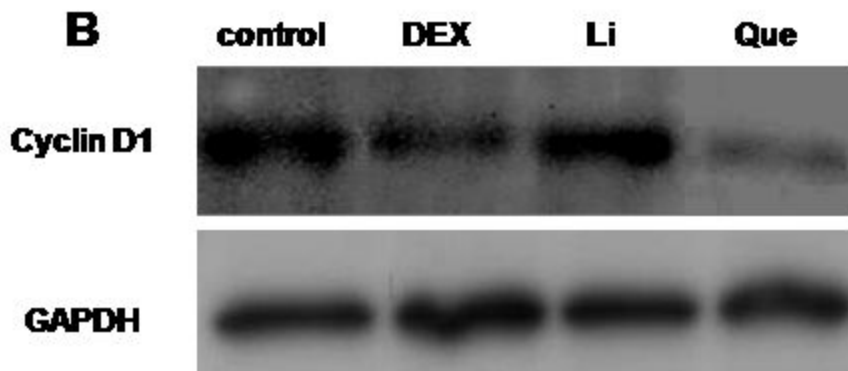
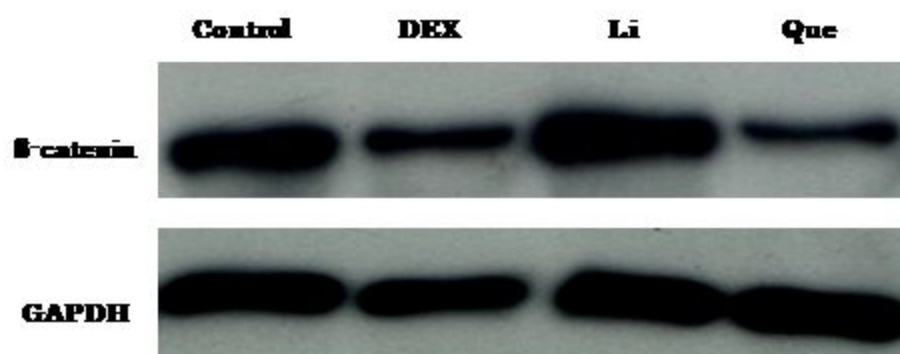


Figure 6.

A



5 μ M DEX	-	+	+	+
3mM Li	-	-	+	+
30 μ M Que	-	-	-	+

B

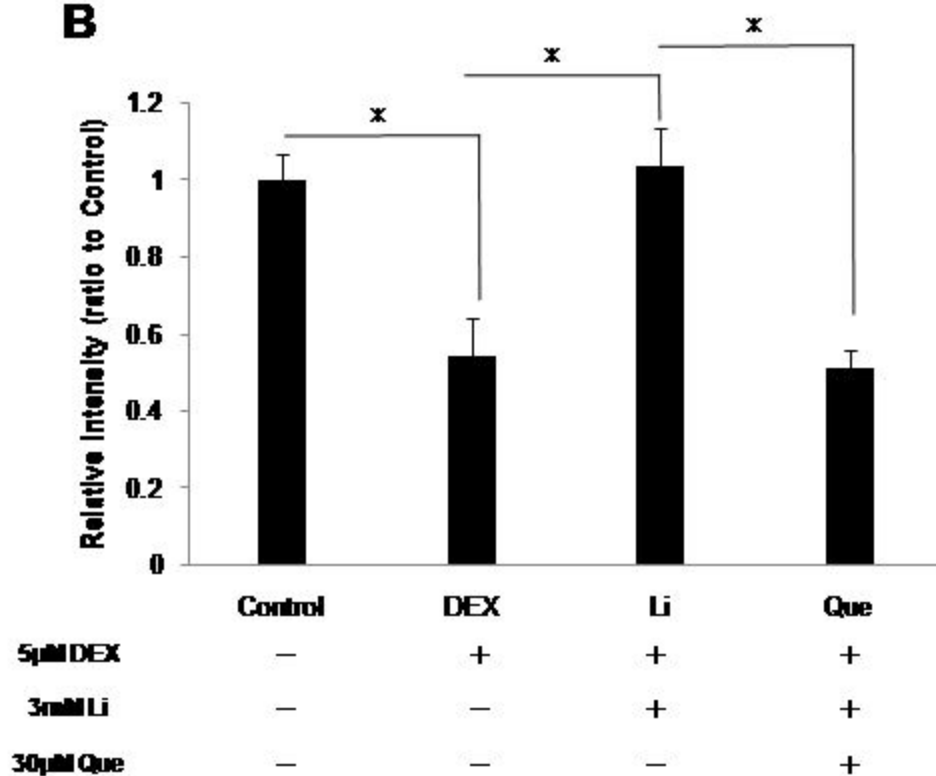
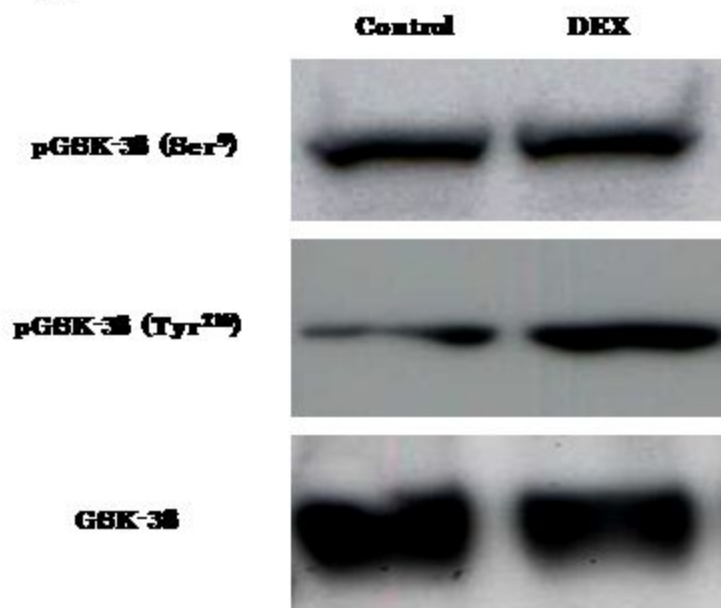


Figure 7.

A



B

