Lithium Promotes Adult Neural Progenitor Differentiation Via GSK3β-Dependent Signaling Pathways

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Abstract: Lithium is one of the standard drugs in the treatment of bipolar disorder, although its molecular modes of action are not well understood. It is a potent inhibitor of the multifunctional enzyme Glycogen Synthase Kinase 3β (GSK3β), which also plays a central role in neurogenesis via the developmental Wnt signaling pathway. In the present study, we analyzed the influence of lithium on GSK3β signaling in adult neural progenitor cells (NPCs) from the rat subventricular zone. Protein expression patterns of NPCs cultured in the presence of 20 mM lithium chloride were compared to those of untreated cells. A proteomic approach based on two-dimensional gel electrophoresis and mass spectrometry showed changes in several GSK3β-related proteins. We demonstrate the inhibition of GSK3β by stabilization and nuclear transfer of its downstream target, β-catenin. Moreover, the phosphorylated (=inhibited) GSK3β protein was strongly enriched in the nuclear fraction of lithium-treated cells. The fraction of cells differentiated into astroglia increased moderately, and the fraction of cells differentiated into neurons increased strongly, as shown by immunostaining. In conclusion, lithium promotes NPC differentiation, mainly to neurons, via GSK3β-related pathways.

Key Words: Adult neural progenitor cell, Lithium, Two-dimensional gel electrophoresis, Rat.

INTRODUCTION

Although lithium is one of the standard drugs used in the treatment of bipolar disorder [1, 2], its molecular modes of action in these disorders are still unknown. Lithium is a potent inhibitor of Glycogen Synthase Kinase 3β (GSK3β), a multifunctional enzyme involved in glycogen metabolism, cell polarity, cytoskeleton formation, cell survival, and transcriptional control [3-5]. In this context, the molecular action of lithium in bipolar disorder is mainly based on GSK3β-dependent signaling [6-10]. Further effects of lithium on cellular signaling are mediated by the inositol phosphate pathway [11-13]. Other cellular signals influenced by mood stabilizers and antidepressants are mediated by the 5-hydroxytryptamine (5HT, serotonin) receptor and Brain-Derived Neurotrophic Factor (BDNF) receptor [14].

In recent years, abnormalities in neural progenitor cell (NPC) survival, migration, and differentiation have been discussed as causes of pathogenesis of bipolar disorder (reviewed in [15]), although no direct evidence for impaired neurogenesis in humans with bipolar disorder exist. In the event that neurogenesis is involved in the pathogenesis of bipolar disorder, new treatment options may arise. One of the central developmental pathways in neurogenesis is Wnt signaling, where GSK3β coordinates cell growth and polarity [16, 17]. Wnt signaling is also active in adult neural stem/progenitor cells of the rat brain [18]. Downstream signaling of GSK3β involves the cytoplasmic stabilization of β-catenin, which is then translocated to the nucleus and interacts with the transcription factor TCF/Lef-1 [3]. Of note, phosphorylation of GSK3β inactivates the enzyme.

In the present study, we analyzed the influence of lithium on GSK3β signaling in NPCs from the adult rat subventricular zone. First, we identified GSK3β-related proteins in a proteomic approach comparing NPCs in the presence of lithium to untreated cells. We then demonstrate lithium-induced inhibition of GSK3β by stabilization and nuclear transfer of its downstream target, β-catenin, and its phosphorylation using phospho-specific antibody staining for the GSK3β protein. Next, we addressed the question how lithium-induced GSK3β inhibition changes differentiation in NPCs.

MATERIAL AND METHODS

Neural Progenitor Cell Cultures

NPCs were isolated from adult rat brains using standard procedures as described previously [19, 20]. Protocols are concordant with the policy on the use of animals, as endorsed by the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the National Institutes of Health, and fulfill the local legal requirements. Subventricular zones of 6 rat brains were dissected, washed in 10 mL ice-cold Dulbecco’s Phosphate Buffered Saline (DPBS) supplemented with 4.5 g/L glucose (DPBS/Glc) and centrifuged for 5 min at 1600 x g at 4 °C. The pellet was mechanically homogenized, resuspended in 20 mL DPBS/Glc, and centrifuged for 5 min at 1600 x g at 4 °C. The pellet was enzymatically digested in 10 mL of 0.01 % (w/v) papain, 0.1 % (w/v) dispase II (neutral protease), 0.01 % (w/v) DNase I, 12.4 mM MgSO4 in Hank’s Balanced Salt Solution (HBSS), triturated by a plastic pipette tip, and incubated at room temperature for 40 min. In three washing steps, the homogenate was centrifuged for 5 min at 1600 x g at 4 °C and the pellet
was resuspended in 10 mL Dulbecco’s Modified Eagle’s Medium (DMEM)-Ham’s F12 medium supplemented with 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mM L-glutamine. Cells were resuspended in 1 mL neurobasal-B27 medium and the cell number was counted. Cells were plated in 2 mL dishes at 200,000 cells in B27-neurobasal medium supplemented with 100 units/mL penicillin, 100 units/mL streptomycin, 20 ng/mL EGF, 20 ng/mL FGF-2, and 2 µg/mL heparin. About 0.8 mL of the medium was replaced weekly, and cells were passage every 10-14 days. The NPCs were cultured for 6-10 weeks in 5% CO2 at 37 °C before use. For inhibition of GSK3β, cell cultures were incubated for 3 days in the presence of 20 mM lithium chloride (Sigma-Aldrich, St. Louis, MO, USA).

**Cell Viability Assay**

Cell viability was measured using a colorimetric assay based on the reduction of tetrazolium salts by cellular dehydrogenases [21, 22] (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, U.S.A.) according to the manufacturer’s protocols.

**Two-Dimensional Gel Electrophoresis (2DE)**

2DE was performed using standard protocols as previously described [19, 20]. Cells were harvested and protein extraction for 2-DE was performed for 60 min at room temperature in a lysis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) Triton X-100, 100 mM DTT, 0.05% (v/v) IGP buffer pH 3-10 (Amersham Biosciences, Uppsala, Sweden), and 0.156% (w/v) Complete protease inhibitor tablets (Roche, Mannheim, Germany). Four cell culture extracts were prepared as biological replicates. Sample protein amounts were determined by the Bradford method. A total of 250 µg (5-10 µL) of the protein solution were suspended in rehydration solution consisting of 6 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) IGP buffer pH 3-10, and a few grains of bromophenol blue to give a final volume of 350 µL. The samples were applied to pH 3-10 nonlinear gradient IEF gel strips for isoelectric focussing using the IPGphor apparatus (Amersham Biosciences, Uppsala, Sweden). The IEF gel strips were rehydrated for 12 hours at 30 V to remove high salt concentrations and to improve protein entry into the gel. Then 200 V, 500 V and 1000 V were applied for 1 hour each. Voltage was increased to 8000 V within 30 min into the gel. Then 200 V, 500 V and 1000 V were applied for 100 minutes to move high salt concentrations and to improve protein entry. IEF gel strips were rehydrated for 12 hours at 30 V to reapparatus (Amersham Biosciences, Uppsala, Sweden). The IEF gel strips for isoelectric focusing using the IPGphor apparatus were incubated for 3 days in the presence of 20 mM lithium chloride (Sigma-Aldrich, St. Louis, MO, USA).

**Gel Image Analysis and Mass Spectrometry**

Gels were scanned and images were analyzed using the Phoretix 2D Expression software (Nonlinear Dynamics, Newcastle-upon-Tyne, UK). Image analysis was performed as described [24]. Normalized spot volumes defined as integral of spot area multiplied by optical densities were compared to normalized means ± standard deviations from 4 biological replicate gels of each group by Student’s t-test for unpaired data [25]. Spots of interest were excised and digested by trypsin for mass spectrometry (Centre for Molecular Medicine, ZMMK, University of Cologne, Germany). Mass spectra were obtained by MALDI-TOF-MS peptide mass fingerprinting and analyzed by searching the NCBI nonredundant protein database with Mascot [26] (http://www.matrixscience.com).

**Preparation of Cytoplasmic and Nuclear Protein Fractions**

Cells were harvested and centrifuged at 1,000 x g for 5 min. Most of the supernatant was discarded and in the rest of the supernatant cells were resuspended and centrifuged again at 1000 x g for 5 min. The supernatant was discarded and pellets weighed. Pellets were resuspended in lysis buffer consisting of 15% sucrose, 10 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, and Complete protease inhibitor (Roche, Mannheim, Germany) in a ratio of 1:3, mixed well, and centrifuged at 600 x g for 15 min. The nuclear pellet and supernatant containing cytoplasmic fraction were frozen separately at -20 °C. Nuclear pellets were washed twice with dH2O in order to remove EDTA which inhibits DNase I. Pellets were incubated with 10 µl of DNase I (0.1 mg/ml) in 1xDNase I buffer (10 X buffer consisting of 1 M Tris Cl (pH 7.5), 25 mM MgCl2, 5 mM CaCl2 in DEPC-H2O) for 20 min at 37 °C. The incubated pellets were triturated through 20G syringes for several times in 50 µl dH2O. The protein concentration was determined [27] and the samples were frozen at -20 °C.

**Western Blotting**

Protein extracts of the cytoplasmic and nuclear fractions were suspended in Laemmli sample buffer containing 0.5 M Tris, 10% SDS, 10% glycerol, 0.05% bromophenol blue, and 5% 2-mercaptoethanol as described earlier [28], and denatured at 65 °C for 15 min. Proteins were separated in 12.5 % polyacrylamide gels and transferred to 0.2 µm nitrocellulose membranes by electroblotting (300 mA for 30 min). Membranes were blocked with blocking buffer (1X TBS, 0.1% Tween-20, 5% (v/v) nonfat dry milk) for 1 hour at room temperature and stained with the specific antibody for GSK3β (1:1,000; Cell Signaling Technologies, Danvers, MA, USA) and phosphoGSK3βSer9 (1:1,000; Cell Signaling Technologies, Danvers, MA, USA), or β-catenin (1:2,000; Chemicon, Temecula, CA, USA), respectively, and the secondary antibody HRP-IgG goat-anti-rabbit (1:10,000; Pierce, Rockford, IL, USA). Chemiluminescence was visualized by mixing 0.45 mM p-coumaric acid, 12.5 mM lumiAl [5-amino-2,3-dihydro-1,4-phthalazinedione] in 100 mM Tris, pH 8.5, with 0.018% H2O2 in 100 mM Tris, pH 8.5 and exposure on X-ray films (MRDM; Eastman Kodak, Rochester, NY, USA) for 30-60 s. The bands were quantified using the Phoretix 2D Expression software (Nonlinear Dynamics, Newcastle-upon-Tyne, UK).
NPCs were grown adhesively on poly-L-lysine-coated cover slips for 7 days in presence of LiCl or under control conditions. The cells were fixed for 20 min in 4% paraformaldehyde in PBS, pH 7.4, with 0.2% Tween-20 (PBST). Slides were blocked 1 h at room temperature in Seablock (Pierce, Rockford, IL, USA) and incubated with the respective primary antibody overnight at 4 °C in a humidified chamber using primary antibodies against GFAP (1:1,000; BD Biosciences, Heidelberg, Germany), and Tubulin-ßIII (clone TuJ-1: 1:1,000; Abcam, Cambridge, UK). The next day, slides were washed 3 times for 5 min in PBST and incubated with an appropriate fluorescent secondary antibody for 1 h at room temperature. The secondary antibody was anti-ms Cy2- or Cy3-conjugated IgG (1:200, Jackson ImmunoResearch, West Grove, PA, USA). After washing 3 times for 5 min each in TBST, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, 1:5,000, Molecular Probes, Eugene, OR, USA) for 1 min. Slides mounted in Mowiol-containing fluoprotective media [29] and stored at 4 °C in the dark until fluorescence microscopy was performed.

**Image Analysis and Cytometry**

Images were recorded using a digital camera (DC500, Leica, Bensheim, Germany) on a fluorescent microscope (DM-R HC, Leica Microsystems, Bensheim, Germany; BX50, Olympus, Hamburg, Germany). For image cytometry, images were analyzed using the TissueQuest software (TissueGnostics, Vienna, Austria) as described in detail [30]. Briefly, in the DAPI channel, nuclei were detected by dissection algorithms. Then immunopositive cells were detected by a non-annular signal growth algorithm around the nuclei. The signals for nestin, GFAP, and TuJ-1 were plotted against each other with the help of a non-annular signal growth algorithm around the nuclei. The signals for nestin, GFAP, and TuJ-1 were plotted against each other with the help of a non-annular signal growth algorithm around the nuclei. The signals for nestin, GFAP, and TuJ-1 were plotted against each other with the help of a non-annular signal growth algorithm around the nuclei.

**RESULTS**

**Lithium Decreases Cell Viability**

Comparing viable cells under different doses of LiCl in the cell culture media to untreated control NPCs, we found decreased numbers of NPCs in high doses of LiCl (Fig. 1). The ED50 was about 20 mM, thus we chose this concentration for further experiments.

**Lithium Alters the Expression of GSK3ß-Related Proteins**

In the present study, we used a proteomic approach to search for changes in the NPC protein expression pattern induced by lithium. Two-dimensional gels of whole cell lysates of NPCs from the subventricular zone of adult rats incubated in the presence of LiCl were compared to those of untreated control NPCs. In the gels of the experimental group exposed to LiCl, we found a total of 285 ± 123 (N=4 biological replicates) protein spots (ranging from 170 to 435), compared to 546 ± 167 (N=4 biological replicates) in the control group (ranging from 238 to 858). We found the highest differential expression values for protein pathways involved in cytoskeletal reorganization and cellular energy metabolism (data not shown). Moreover, we found changes in the expression of proteins related to GSK3ß, either as direct interaction partners or protein targets modified by GSK3ß, or as parts of the downstream signaling (Fig. 2, Table 1). Among the identified proteins, Ruvb-like protein-1 (Ruvbl1) is a DNA helicase which binds β-catenin, a downstream target of GSK3ß which regulates the transcriptional activity through the binding of the transcription factor TCF/LeF-1 [31]. We identified two proteasome subunits, Psal and Psal6, which are involved in the degradation of cytoplasmic proteins, among them β-catenin. The Microtubule-associated protein RP/EB 1 (Mare1) binds the C-terminal domain of the Adenomatosis Polyposis Coli (APC) protein and forms a complex with axin and GSK3ß. This part of the Wnt signaling pathway is also involved in microtubule interaction, regulated by Rho GDP-dissociation inhibitor protein 1 (Gdir), which is a negative regulator of Rho signaling and microtubule association.
GSK3β content by Western blot analysis. We compared cellular extracts from NPCs incubated in the presence of 20 mM LiCl to untreated control cells. When probed with anti-GSK3β antibodies, the cytoplasmic fraction showed a 1.5-fold increase in the LiCl-treated cultures (P<0.0003; N=3) (Fig. 4A). In contrast, the nuclear concentration of GSK3β remained unchanged under exposure to LiCl. Next, we used a phospho-GSK3β-specific antibody to detect inactivated GSK3β protein (pGSK3β) (Fig. 4B). We did not find pGSK3β in the cytoplasm of control NPCs, but a pGSK3β band occurred in the cytoplasm of LiCl-treated NPCs. In contrast, nuclear staining for pGSK3β was detectable, and increased about 3-fold in the LiCl-treated cells (P<0.0238; N=3).

**Inhibition of GSK3β Stimulates Neural Differentiation**

To evaluate the effects of lithium on cell differentiation, we compared the expression of cell differentiation markers after the application of 20 mM LiCl and compared the results to untreated NPCs (N=5 biological replicates). We used GFAP to detect astrocytes, and tubulin-βIII (TuJ1) to detect neurons. Whereas in the control NPCs only few GFAP-positive cells can be seen (Fig. 5A), lithium increased the number of GFAP-positive cells. With regard to TuJ1 expression, no TuJ1-positive cells can be seen in untreated controls (Fig. 5C), whereas lithium strongly induced TuJ1-expression (Fig. 5D). Using the cytometry analysis software TissueQuest, we created FACS-like scattergrams of DAPI fluorescence intensity vs. GFAP or TuJ1 fluorescence intensities, respectively. We found an increased number of GFAP-positive cells in the presence of LiCl as compared to untreated controls (1.5-fold; Fig. 5E, F). With regard to TuJ1 staining, no positive cells are found in the untreated cells, whereas nearly all cells in the LiCl-treated cells became TuJ1-positive (Fig. 5G, H). These data indicate that lithium is a neural differentiation stimulus, increasing the number of neurons and astrocytes, with a stronger effect on neurogenesis.
Lithium Promotes Neurogenesis

Lithium and GSK3β-Related Signaling

Lithium has long been used in clinical neurology and psychiatry as mood stabilizer in bipolar disorder, which affect up to 5% of the population at least once during life [1, 11]. The main advantage of lithium in comparison to other antipsychotic drugs used in the therapy of depression.

DISCUSSION

Functional regulation of GSK3β activity by LiCl.

Comparing cytoplasmic and nuclear protein extracts from NPCs in the presence of LiCl to untreated cells, we found 1.5-fold increased cytoplasmic levels of GSK3β in the presence of LiCl, but no changes in the nuclear concentrations. (B) Probing with a pGSK3β-specific antibody, we found absent cytoplasmic pGSK3β levels in the untreated NPCs, and pGSK3β occurring in the cytoplasm of LiCl-treated NPCs. There was a 3-fold increase in nuclear levels of pGSK3β under LiCl treatment.

Influence of lithium on neuronal and astrocytic marker expression in NPCs.

(A) Control NPCs showed only few GFAP-positive cells. (B) Lithium increased the number of GFAP-positive cells about 1.5-fold. (C) No TuJ1-positive cells can be seen in untreated controls. (D) Lithium strongly induced TuJ1-expression in the NPCs (Scale bar, 10 μm). (E)-(H) Immunocytochemistry results using the TissueQuest analysis software. In the scattergrams, the DAPI fluorescence intensity is depicted on the x-axis, and the GFAP or TuJ1 fluorescence intensity on the y-axis. Detection thresholds are indicated by black lines. Single cell fluorescent signals results in single dots. (E, F) The FACS-like scattergram shows an increase in the cell number above the detection threshold in the presence of LiCl as compared to untreated controls. (G, H) With regard to TuJ1 staining, no positive cells are found in the untreated cells, whereas nearly all cells in the LiCl-treated cells became TuJ1-positive. These data show that the number of astrocytes and neurons increase under stimulation with lithium.
seems to be its reduction in suicidality [1]. A major molecular mechanism of lithium action is the inhibition of GSK3β, thus stabilizing β-catenin and mimicking Wnt signaling [34]. During development, Wnt signaling regulates neural precursor proliferation and apoptosis, stem cell self-renewal, lineage decision, as well as axon guidance and outgrowth [35, 36]. In the adult brain, Wnt signaling has been reported as an active signaling pathway in adult hippocampal neural stem/progenitor cells [18]. Moreover, lithium promotes adult hippocampal neural precursor proliferation [37]. In the present study, we found differentially expressed proteins related to GSK3β signaling in NPCs cultures from the adult rat subventricular zone, when treated with lithium. This indicates that lithium is also an important inhibitor of GSK3β in NPCs and activates its downstream signaling.

Increase in Nuclear Transfer of β-Catenin by Inhibition of GSK3β

The cellular target of GSK3β conveying the transcriptional activation in the nucleus is β-catenin, a protein binding the transcription factors TCF/Lef-1 and C/EBP [38]. In the present study, we found increased concentration of β-catenin in the nuclear compartment during lithium treatment, thus confirming the inhibition of GSK3β and activation of its downstream signaling via the β-catenin pathway in NPCs.

In β-catenin knock-out mice, the central nervous system is significantly smaller as well as the number of neuronal precursor cells, whereas in transgenic mice expressing constitutively activated β-catenin, the brain volume is enlarged [39, 40]. Thus β-catenin seems to regulate the balance between neural precursor cell proliferation and differentiation. In the present study, we observed increased nuclear β-catenin levels associated with the activation of β-catenin/TCF/Lef-1-specific gene transcription on the molecular level and increased neurogenesis on the morphological level.

Lithium Treatment Promotes Neuronal Differentiation

The effect of lithium on neural differentiation remains controversial, since increased neurogenesis has been found in P8 rat cerebellar granule cells and E17 rat cortical neurons [41], rat embryonic stem cells and striatal primordial stem cells [42], and the human teratocarcinoma cell line Ntera2/D1 [43]. On the other hand, inhibition of neurogenesis by lithium has been found in mouse embryonic stem cells [44] and mouse teratocarcinoma cells [45].

In the present study, we tested the influence of lithium on differentiation of NPCs by immunostaining for marker proteins of the neuronal and glial lineages and subsequent cytometry. We found that lithium induced the appearance of significant numbers of both neurons and astrocytes, indicating that lithium promotes neurogenesis via transcriptional activation of β-catenin-dependent target genes. Other studies have also found increased numbers of neurons in adult rat neural stem cells in vitro [46] or in vivo [47]. In aged rats, this increase in differentiated neural cells seems to be lost [48].

The increased neurogenesis in the present study as well as in the literature has been attributed to decreased rates of apoptosis and neuroprotective effects of lithium [49]. Lithium strongly up-regulates the expression of anti-apoptotic proteins such as bcl-2 [50, 51] or by inhibition of pro-apoptotic proteins such as caspase-3 [52, 53].

CONCLUSIONS

The findings of the present study indicate that GSK3β signaling plays an essential role in regulating differentiation and proliferation of adult NPCs. Lithium treatment results in the inhibition of GSK3β and transcriptional activation of distinct target genes of via β-catenin and TCF/Lef-1 nuclear transfer. Lithium also produced larger numbers of neurons and astrocytes differentiated from NPCs.

LIST OF ABBREVIATIONS

GFAP = Gial Fibrillary Acidic Protein
GSK3β = Glycogen Synthase Kinase 3β
MALDI-TOF-MS = Matrix Assisted Laser Desorption/Ionisation-Time of Flight mass spectrometry
NPC = Neural Progenitor Cell
pGSK3β = phospho-Glycogen Synthase Kinase 3β
TCF/Lef-1 = T Cell Factor/Lymphoid enhancer factor(Lef)

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Lithium Promotes Neurogenesis

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11