RESEARCH ARTICLE

Lithium promotes proliferation and suppresses migration of Schwann cells

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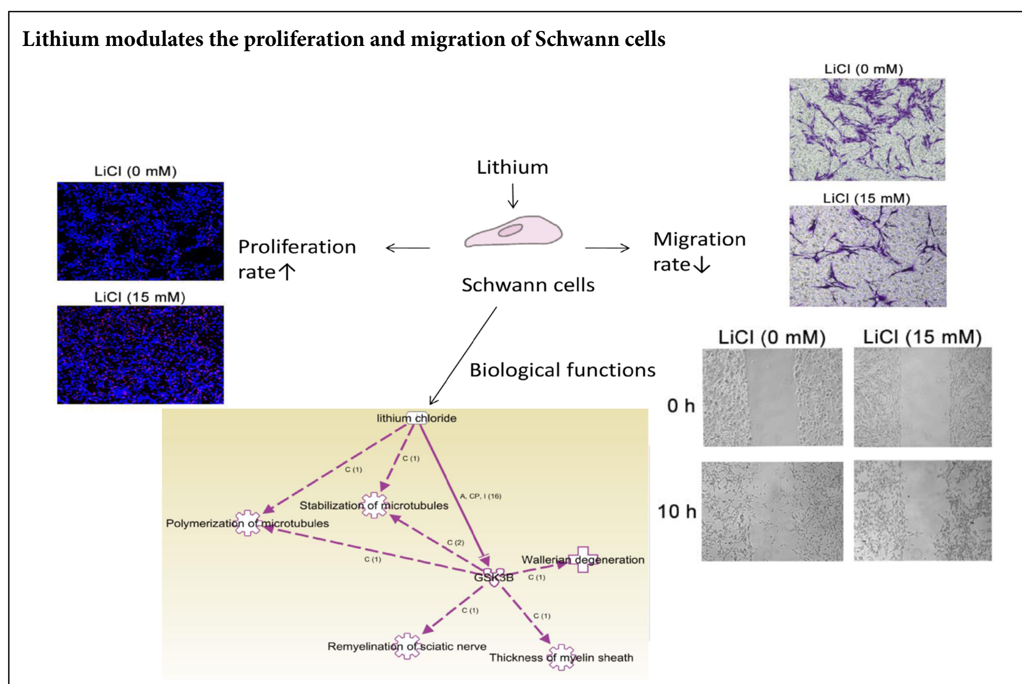
Graphical Abstract

Lithium modulates the proliferation and migration of Schwann cells



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Abstract

Schwann cell proliferation, migration and remyelination of regenerating axons contribute to regeneration after peripheral nervous system injury. Lithium promotes remyelination by Schwann cells and improves peripheral nerve regeneration. However, whether lithium modulates other phenotypes of Schwann cells, especially their proliferation and migration remains elusive. In the current study, primary Schwann cells from rat sciatic nerve stumps were cultured and exposed to 0, 5, 10, 15, or 30 mM lithium chloride (LiCl) for 24 hours. The effects of LiCl on Schwann cell proliferation and migration were examined using the Cell Counting Kit-8, 5-ethynyl-2′-deoxyuridine, Transwell and wound healing assays. Cell Counting Kit-8 and 5-ethynyl-2′-deoxyuridine assays showed that 5, 10, 15, and 30 mM LiCl significantly increased the viability and proliferation rate of Schwann cells. Transwell-based migration assays and wound healing assays showed that 10, 15, and 30 mM LiCl suppressed the migratory ability of Schwann cells. Furthermore, the effects of LiCl on the proliferation and migration phenotypes of Schwann cells were mostly dose-dependent. These data indicate that lithium treatment significantly promotes the proliferation and inhibits the migratory ability of Schwann cells. This conclusion will inform strategies to promote the repair and regeneration of peripheral nerves. All of the animal experiments in this study were ethically approved by the Administration Committee of Experimental Animal Center of Nantong University, China (approval No. 20170320-017) on March 2, 2017.

Key Words: *5-ethynyl-2′-deoxyuridine; Cell Counting Kit-8; cell viability; lithium; migration; peripheral nerve; proliferation; regeneration; Schwann cell; wound healing assay*

Chinese Library Classification No. *R459.9; R363; R364*

Introduction

Lithium is a primary anti-depressant drug and has been used in the treatment of bipolar mood disorder for over 50 years ago. Lithium can also modulate many other nervous system diseases (Kerr et al., 2018; Zhang et al., 2018; Jinhua et al., 2019), including Alzheimer's disease (De Ferrari et al., 2003; Forlenza et al., 2014; Matsunaga et al., 2015), Parkinson's disease (Moors et al., 2017), Huntington's disease (Senatorov et al., 2004; Raja et al., 2015), amyotrophic lateral sclerosis (Fornai et al., 2008; van Eijk et al., 2017), ischemic brain injury (Ren et al., 2003; Shin et al., 2012; Silachev et al., 2015), and spinal cord injury (Wong et al., 2011; Yang et al., 2012; Shah et al., 2013; Fu et al., 2014). Recent studies have demonstrated that lithium can improve nerve regeneration after brachial plexus injury (Su et al., 2014) and sciatic nerve injury (Nouri et al., 2009; Chen et al., 2016), indicating that lithium has the potential to treat peripheral nerve injury.

Peripheral nerve injury is a common clinical problem that may affect a patient's sensation, movement, gland, or organ functions (Gu et al., 2011, 2014). After peripheral nerve injury, Schwann cells, which are the major glial cells of the peripheral nervous system, undergo dedifferentiation, activation, and proliferation (Jessen and Mirsky, 2016; Wong et al., 2017; Qian et al., 2018b; Jessen and Arthur-Farraj, 2019; Jessen and Mirsky, 2019). Proliferated Schwann cells migrate toward the injured nerve stumps, eliminate axon and myelin debris, and form a regenerative tunnel known as the band of Büngner to guide axon regrowth (Namgung, 2014; Clements et al., 2017). Schwann cells then differentiate and myelinate the regenerated axons to facilitate the functional recovery of the injured peripheral nerve (Kidd et al., 2013; Salzer, 2015; Ji et al., 2019).

Investigations of the underlying mechanisms of lithium-induced peripheral nerve regeneration demonstrated that lithium can significantly affect the remyelination process. Lithium treatment increases β-catenin levels in Schwann cells and provokes its nuclear localization and binding to T-cell factor/lymphoid-enhancer factor response elements (Chen et al., 2016). Lithium also increases the promoter activities, transcript levels, and protein levels of the myelin-related genes, peripheral myelin protein 22 and myelin protein zero, elevates the expression of remyelination transcription factors, Oct6 and Sox10, and enhances the thickness of the myelin sheath (Makoukji et al., 2012; Fang et al., 2016). Although the effects of lithium on remyelination by Schwann cells have been well demonstrated, the potential roles of lithium on the modulation of other Schwann cell phenotypes remain unclear.

After peripheral nerve injury, the proliferation and migration of Schwann cells are important for the regeneration and functional recovery of injured nerves and, therefore, many factors that can modulate Schwann cell proliferation and migration have been investigated in our laboratory (Gu et al., 2015; Yi et al., 2016a, b, 2019; Qian et al., 2018a; Wang et al., 2018, 2019; Zhang et al., 2019). Because lithium treatment improves peripheral nerve regeneration, we hypothesize that lithium can modulate Schwann cell proliferation and migration. To test this hypothesis, cultured primary Schwann cells isolated from rat sciatic nerve stumps were treated with different concentrations of lithium chloride (LiCl), and its effects were examined using various assays.

Materials and Methods

Animals

All of the animal experiments in this study were ethically approved by the Administration Committee of Experimental Animal Center of Nantong University, China (approval No. 20170320-017) on March 2, 2017. Neonatal Sprague-Dawley rats aged 1 day were obtained from the Experimental Animal Center of Nantong University, China (animal licenses No. SCXK [Su] 2014-0001 and SYXK [Su] 2012-0031).

Primary Schwann cell culture

Neonatal Sprague-Dawley rats were anesthetized and then killed by cervical dislocation. Sciatic nerve stumps were collected from which primary Schwann cells were isolated. To remove contaminating fibroblasts, isolated cells were treated with an anti-Thy1.1 antibody (Sigma-Aldrich, St. Louis, MO, USA) and rabbit complement (Sigma-Aldrich). Cells were immunostained for a Schwann cell marker using a rabbit anti-S100 antibody (Dako, Carpinteria, CA, USA) and a secondary goat anti-rabbit antibody (Abcam, Cambridge, MA, USA) to determine the purity of the collected Schwann cells. Purified Schwann cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco) in a humidified 5% CO_2 incubator at 37°C. Cultured Schwann cells were passaged less than three times before use.

Cell viability assay

The viability of Schwann cells was determined by the Cell Counting Kit-8 assay (CCK-8; Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. Briefly, cultured primary Schwann cells were seeded onto a 96-well cell culture plate and treated with 0, 5, 10, 15, or 30 mM LiCl for 24 hours. CCK-8 (10 μL) was then added to the cell culture medium for an additional 4 hours. The optical density (OD) was measured at 450 nm with a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The fold change of cell viability was equal to 100% × (Ae–Ab)/(Ac–Ab); Ae = OD_{450nm} of the experimental group; Ab = OD_{450nm} of the blank control; Ac = OD_{450nm} of the control (0 mM LiCl).

Cell proliferation assay

Schwann cell proliferation was determined using the Cell-Light^{TM} 5-ethynyl-2′-deoxyurdine (EdU) DNA Cell Proliferation Kit (Ribobio, Guangzhou, China). Primary cultured Schwann cells were suspended in complete medium containing DMEM and 10% fetal bovine serum, seeded onto a poly-L-lysine-coated 96-well plate, and treated with 0, 5, 10, 15, or 30 mM LiCl for 24 hours. EdU (45 μL of 100 μM per well) was then added to the cell culture medium for an additional 24 hours. Schwann cells were then fixed in 4% paraformal-

dehyde (Sigma-Aldrich) at room temperature for 30 minutes and cell nuclei were stained with Hoechst 33342. A DMR fluorescence microscope (Leica Microsystems, Bensheim, Germany) was used to take EdU immunofluorescence images. Image fields were randomly selected and the total numbers of cells and EdU-positive cells were counted. The proliferation rate of Schwann cells was calculated by dividing the number of EdU^+ cells by the total number of cells.

Cell migration assay

The migratory ability of Schwann cells was determined using 6.5 mm Transwell chambers with 8 μm pores (Costar, Cambridge, MA, USA). Primary cultured Schwann cells were treated with 0, 5, 10, 15, or 30 mM LiCl for 24 hours, suspended in DMEM, and then seeded in the upper chamber at a density of 300 cells/μL. The bottom chamber was first pre-coated with fibronectin (10 μg/mL) and then 600 μL complete medium containing DMEM and 10% fetal bovine serum was added. Schwann cells were cultured in the Transwell chambers for an additional 24 hours. Cells that migrated to the lower chamber were stained with 0.1% crystal violet for 15 minutes while those left on the upper chamber were wiped away with a cotton swab. Images were captured with a DMR fluorescence microscope (Leica). Migrated cells were dissolved in 33% acetic acid solution and the cell migration rate was determined by measuring the OD of crystal violet from randomly selected images.

Wound healing assay

The migratory ability of cultured Schwann cells was further determined using the wound healing assay. Schwann cells were treated with 0, 5, 10, 15, or 30 mM LiCl for 24 hours. A linear scratch was then made on the Schwann cell monolayer and the cells then cultured for an additional 10 hours. Images were captured with a DMR fluorescence microscope at 0 and 10 hours after wounding. Randomly selected image fields were first processed by Photoshop CC (Adobe, San Jose, CA, USA), and the relative clean area was then calculated using ImageJ software (https://imagej.nih.gov/ij/).

Bioinformatic analysis

LiCl-interacting molecules, diseases, and biological functions were detected using QIAGEN's Ingenuity Pathway Analysis software program (Ingenuity Systems Inc., Redwood City, CA, USA). Relationships were revealed by the Ingenuity Pathways Knowledge Base in the Ingenuity Pathway Analysis software (Ingenuity Systems Inc.; Kramer et al., 2014). Schwann cell behavior-related functions were selected to construct a bioinformatic network.

Statistical analysis

Data were analyzed by Prism 6.0 Software (GraphPad Software, San Diego, CA, USA) and are presented as the mean ± SEM. Statistical analysis was performed using one-way analysis of variance and Dunnett's *post hoc* test. A P-value < 0.05 was considered statistically significant.

Results

Lithium increases Schwann cell viability

To evaluate the role of lithium in the viability of Schwann cells, cultured Schwann cells were treated with 0, 5, 10, 15, or 30 mM LiCl for 24 hours. The impact of lithium on cell viability was determined using a commercial CCK-8 kit. CCK-8 assays showed that viability was significantly increased in Schwann cells treated with 5 mM LiCl compared with control ($P < 0.05$; **Figure 1**). Viability was higher in Schwann cells treated with higher concentrations of LiCl (**Figure 1**). These observations demonstrated that LiCl was not cytotoxic. Instead, LiCl treatment increased the viability of Schwann cells and might promote the proliferation of Schwann cells.

Lithium increases the proliferation of Schwann cells

To determine the effect of LiCl on Schwann cell proliferation, an EdU-based cell proliferation assay was conducted. Cultured Schwann cells were immunostained with Hoechst 33342 (blue), while proliferating Schwann cells were immunostained with EdU (red) (**Figure 2A**). In the 0 mM LiCl group, approximately 12.20% of Schwann cells were proliferating as shown by EdU immunofluorescence (**Figure 2A** and **B**). In contrast, increased cell proliferation was observed in Schwann cells treated with different concentrations of LiCl. Specifically, the ratios of proliferating cells increased to 23.67%, 29.92%, 37.58%, and 41.52% for cells treated with 5, 10, 15, and 30 mM LiCl, respectively ($P < 0.05$; **Figure 2B**). Consistent with the observations from the CCK-8 assay, results from the EdU assay indicated that LiCl increased the proliferation of Schwann cells in a concentration-dependent manner.

Lithium decreases the migratory ability of Schwann cells

To analyze whether lithium treatment impacts the migratory ability of Schwann cells, cultured Schwann cells were treated with different concentrations of LiCl, and their migration rate was evaluated by Transwell assays. After culture for 24 hours, many Schwann cells had migrated toward the bottom chamber in the presence of 0 mM LiCl (**Figure 3A**). However, when Schwann cells were exposed to LiCl, fewer Schwann cells migrated toward the bottom chamber. Compared with Schwann cells treated with 0 mM LiCl, the migration rate was slightly reduced by 5 mM LiCl treatment ($P > 0.05$) and was significantly reduced by treatment with higher concentrations of LiCl (10, 15, and 30 mM, $P < 0.05$; **Figure 3B**).

The migratory ability of Schwann cells was further determined by the wound healing assay. After culture for 10 hours, wound gaps with equal widths were made on confluent Schwann cell monolayers. Cells were then treated with 0, 5, 10, 15, or 30 mM LiCl (**Figure 4A**). Phase contrast microscopy showed that in all groups, the wound gaps were narrowed at 10 hours after scratching, indicating that Schwann cells migrated to the cleaned area and filled the gap. However, the migration of Schwann cells was less robust in Schwann cells treated with 5, 10, 15, or 30 mM LiCl compared with untreated cells (**Figure 4A**). Schwann cells treated with higher concentrations of LiCl had relatively larger

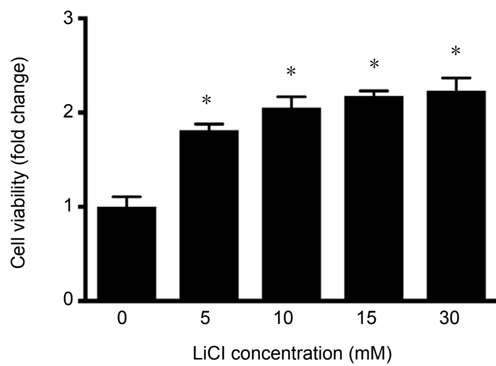

Figure 1 Effect of LiCl on Schwann cell viability.
The viability of Schwann cells treated with 0, 5, 10, 15, or 30 mM LiCl was determined by Cell Counting Kit-8 assays. Histograms show representative results from triplicate experiments. Data are expressed as the mean ± SEM (n = 3; one-way analysis of variance followed by Dunnett's *post hoc* test). *P < 0.05, *vs.* 0 mM (Schwann cells treated with 0 mM LiCl).

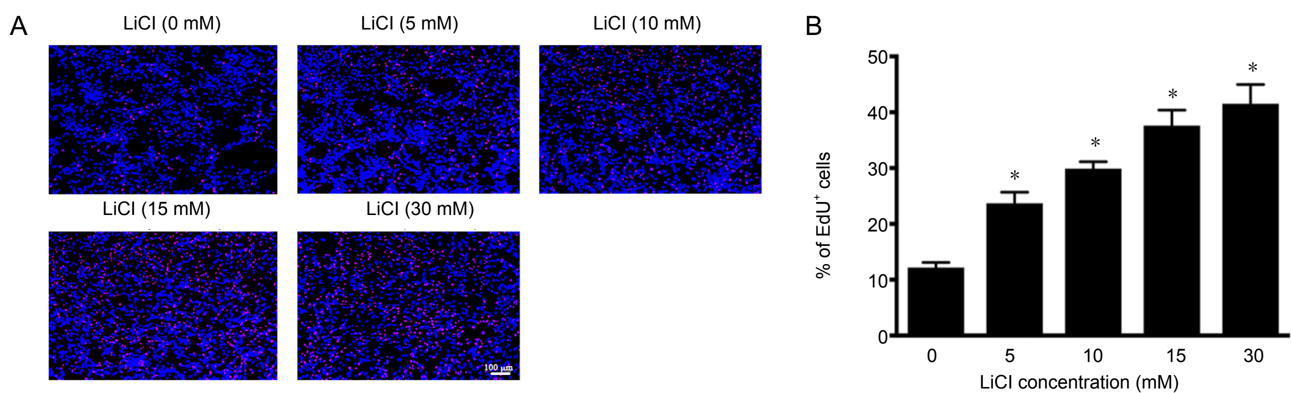

Figure 2 Effect of LiCl on Schwann cell proliferation.
(A) Proliferation of Schwann cells treated with 0, 5, 10, 15, or 30 mM LiCl. Red color indicates 5-ethynyl-2′-deoxyuridine (EdU) staining and blue reflects Hoechst 33342 staining. Scale bar: 100 µm. (B) Cell proliferation rate from quadruplicate experiments. The proliferation rate was calculated by dividing the number of EdU^+ cells by the total number of cells. Data are expressed as the mean ± SEM (n = 4; one-way analysis of variance followed by Dunnett's *post hoc* test). *P < 0.05, *vs.* 0 mM (Schwann cells treated with 0 mM LiCl).

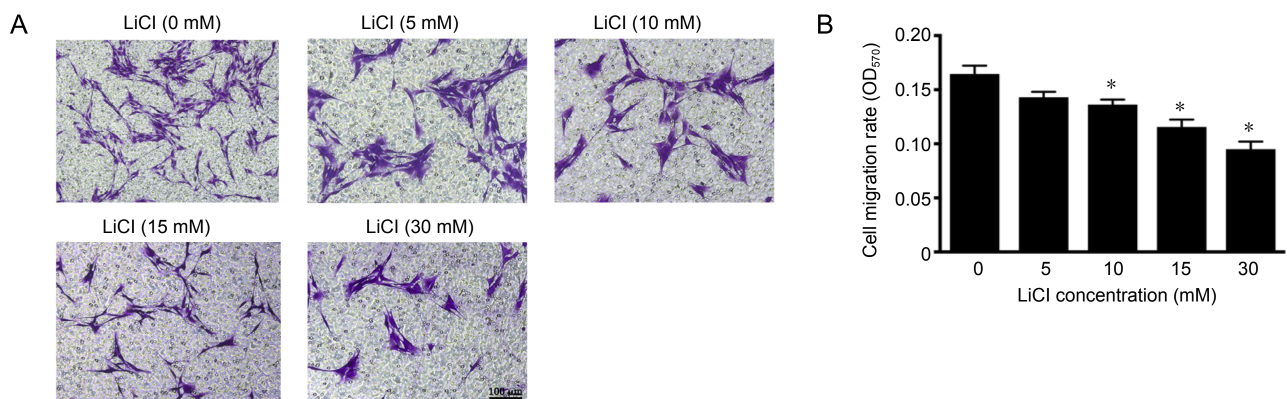

Figure 3 Effect of LiCl on Schwann cell migration through Transwell assay.
(A) Schwann cell migration in the Transwell assay: Schwann cells were treated with 0, 5, 10, 15, or 30 mM LiCl. Cells that migrated to the lower chamber were stained with 0.1% crystal violet, which indicates migrated cells. Scale bar: 100 µm. (B) Cell migration rate from triplicate experiments. Cell migration rate was determined by measuring the optical density (OD) of crystal violet from randomly selected images. Data are expressed as the mean ± SEM (n = 3; one-way analysis of variance followed by Dunnett's *post hoc* test). *P < 0.05, *vs.* 0 mM (Schwann cells treated with 0 mM LiCl).

cleaned areas, indicating that they exhibited relatively slower migration rates compared with control cells (5 mM LiCl, P > 0.05; 10, 15 and 30 mM LiCl, P < 0.05; **Figure 4B**). Together with the outcomes from the transwell-based cell migration assay, these results indicate that LiCl treatment hindered the migration of Schwann cells.

Bioinformatic network of lithium-related functions

In addition to functional analyses, we performed Ingenuity Pathway Analysis to investigate lithium-induced functions

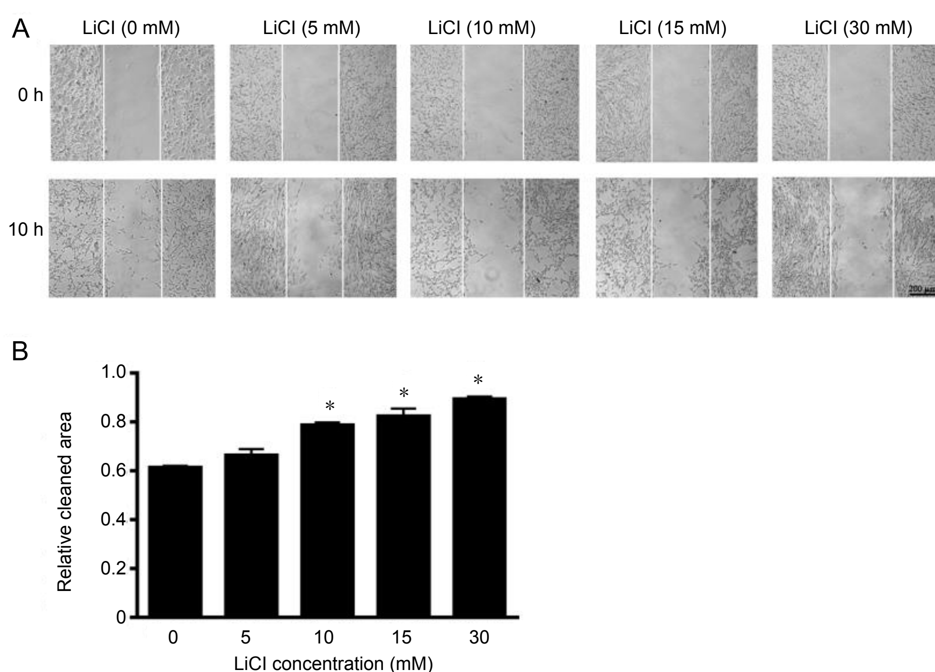

Figure 4 Effect of LiCl on the migratory ability of Schwann cells by the wound healing assay.
(A) Wound healing of Schwann cells treated with 0, 5, 10, 15, or 30 mM LiCl: Vertical white lines mark the wound area at the beginning of the experiment at 0 hour. Scale bar: 200 μm. (B) Histograms show representative results from triplicate experiments. Data are expressed as the mean ± SEM (n = 4; one-way analysis of variance followed by Dunnett's *post hoc* test). *P < 0.05, *vs.* 0 mM (Schwann cells treated with 0 mM LiCl).

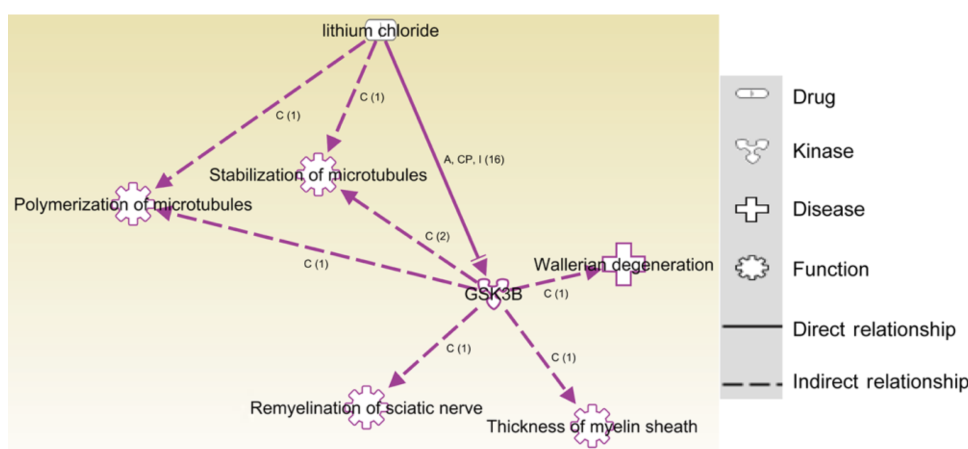

Figure 5 Bioinformatic network of LiCl-related functions.
LiCl-related molecules, diseases and functions are revealed and displayed. Symbol legends are indicated to the right side. A indicates activation; CP indicates chemical-protein interactions; I indicates inhibition; and C indicates causation. Numbers in the brackets indicate the numbers of Ingenuity Pathways Knowledge Base identified relationships.

in the injured nerve stumps during peripheral nerve regeneration (**Figure 5**). The most highly related molecule to LiCl was glycogen synthase kinase 3β (GSK3B). LiCl can significantly decrease the activation of GSK3B in a cell-free system (Haertel-Wiesmann et al., 2000) as well as in various cell types, such as mouse renal glomerulus cells (Xu et al., 2015), mouse vascular smooth muscle A7r5 cells (Deng et al., 2008), mouse interstitial cells from the renal medulla (Rao et al., 2005), and rat osteoblastic osteosarcoma UMR 106-01 cells (Tyson et al., 2002). Inhibition of GSK3B by LiCl can contribute to increased polymerization and stabilization of microtubules (Xu et al., 2015). Moreover, inhibition of GSK3B might decrease Wallerian degeneration (Wakatsuki et al., 2011) and increase myelin sheath thickness (Makoukji et al., 2012). These findings indicate potentially significant roles of LiCl in peripheral nerve regeneration.

Discussion

Emerging evidence shows that lithium is beneficial for Schwann cell remyelination and peripheral nerve regeneration. However, few studies have reported on the impact of lithium on other Schwann cell phenotypes. This study investigated the effects of LiCl treatment on Schwann cell viability, proliferation and migration. Our results show that LiCl significantly increased viability and proliferation of Schwann cells, while inhibiting their migration *in vitro*. The molecular mechanisms underlying these complex effects of lithium on Schwann cells remain largely unknown and further studies are required to address this issue.

In our study, results from CCK8 and EdU assays indicated that lithium could enhance the viability and proliferation of cultured Schwann cells and that this promoting effect was dose dependent. Consistent with our observations, Yoshino and DeVries (1987) showed that lithium could enhance the mitogenic activity of cultured Schwann cells, while Makoukji et al. (2012) found that incubation of MSC80, a Schwann cell line, and primary Schwann cells with LiCl induced significantly elevated mRNA and protein levels of myelin protein zero and peripheral myelin protein 22. However, Pinero et al. (2017) found that lithium inhibited the proliferation and

differentiation of cultured Schwann cells. Their results contradict the findings of ourselves and others. These paradoxical results might be because Pinero et al. treated Schwann cells with LiCl together with cAMP stimulators or cAMP analogs, while in other studies, Schwann cells were treated with LiCl alone. It is possible that LiCl interacts with cAMP. As far as we know, there has been no report on the direct effect of lithium on Schwann cell migration. Our results from both Transwell migration assays and wound healing assays indicate that lithium hinders the migration of Schwann cells.

Lithium treatment suppresses tau protein levels in the rat brain and in primary cultured neurons isolated from rat cortices (Lei et al., 2017). We previously demonstrated that tau protein exists not only in the brain but also in the peripheral nervous system (Yi et al., 2019). Reducing the levels of tau protein in Schwann cells by transfecting siRNA against microtubule-associated protein tau (Mapt), the gene encoding tau, can increase the proliferation of Schwann cells and decrease the migration of Schwann cells (Yi et al., 2019). These outcomes provide a possible clue to the mechanisms underlying the lithium-induced Schwann cell phenotype switch and indicate that in Schwann cells, lithium treatment might suppress tau levels, leading to increased proliferation and decreased migratory ability.

Taken together, this study reveals important effects of lithium on Schwann cell proliferation and migration. Insights from our study will help our understanding of how lithium affects Schwann cell phenotype and peripheral nerve regeneration.

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