Published in final edited form as:

Hear Res. 2013 October ; 304: 137–144. doi:10.1016/j.heares.2013.07.001.

Lithium Alters the Morphology of Neurites Regenerating from Cultured Adult Spiral Ganglion Neurons

S. M. Shah^{a,b,c,d,1}, C. H. Patel^{a,b,2}, A. S. Feng^{a,b,c}, and R. Kollmar^{a,b,c,e,3,*}

^aDepartment of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

^bBeckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

^cNeuroscience Graduate Program, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

^dMedical Scholars Program, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

^eDepartments of Cell Biology and Otolaryngology, SUNY Downstate Medical Center, Brooklyn, NY 11203, USA

Abstract

The small-molecule drug lithium (as a monovalent ion) promotes neurite regeneration and functional recovery, is easy to administer, and is approved for human use to treat bipolar disorder. Lithium exerts its neuritogenic effect mainly by inhibiting glycogen synthase kinase 3, a constitutively-active serine/threonine kinase that is regulated by neurotrophin and “wingless-related MMTV integration site” (Wnt) signaling. In spiral ganglion neurons of the cochlea, the effects of lithium and the function of glycogen synthase kinase 3 have not been investigated. We, therefore, set out to test whether lithium modulates neuritogenesis from adult spiral ganglion neurons. Primary cultures of dissociated spiral ganglion neurons from adult mice were exposed to lithium at concentrations between 0 and 12.5 mM. The resulting neurite morphology and growth-cone appearance were measured in detail by using immunofluorescence microscopy and image analysis. We found that lithium altered the morphology of regenerating neurites and their growth cones in a differential, concentration-dependent fashion. Low concentrations of 0.5 to 2.5 mM (around the half-maximal inhibitory concentration for glycogen synthase kinase 3 and the recommended therapeutic serum concentration for bipolar disorder) enhanced neurite sprouting and branching. A high concentration of 12.5 mM, in contrast, slowed elongation. As the lithium concentration rose from low to high, the microtubules became increasingly disarranged and the growth cones more arborized. Our results demonstrate that lithium selectively stimulates phases of neuritogenesis that are driven by microtubule reorganization. In contrast, most other drugs that have previously been tested on spiral ganglion neurons are reported to inhibit neurite outgrowth or affect only elongation. Lithium sensitivity is a necessary, but not sufficient condition for the

© 2013 Elsevier B.V. All rights reserved.

*Corresponding author: Departments of Cell Biology and Otolaryngology, SUNY Downstate Medical Center, 450 Clarkson Ave., Box 5, Brooklyn, NY 11203, USA. Tel: x1-718-221-6559; fax: x1-718-270-3732. richard.kollmar@downstate.edu (R. Kollmar).

¹Current address: Department of Medicine, Yale School of Medicine, New Haven, CT 06510, USA.

²Current address: Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD 21210, USA.

³Current address: Departments of Cell Biology and Otolaryngology, SUNY Downstate Medical Center, Brooklyn, NY 11203, USA.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

involvement of glycogen synthase kinase 3. Our results are, therefore, consistent with, but do not prove lithium inhibiting glycogen synthase kinase 3 activity in spiral ganglion neurons. Experiments with additional drugs and molecular-genetic tools will be necessary to test whether glycogen synthase kinase 3 regulates neurite regeneration from spiral ganglion neurons, possibly by integrating neurotrophin and Wnt signals at the growth cone.

Keywords

Cochlea; Glycogen synthase kinase 3; Growth cones; Lithium chloride; Mice; Nerve regeneration

1. Introduction

Sensorineural hearing loss due to noise, chemicals, inherited mutations, or age-dependent degeneration in the cochlea is the most common neurological disorder in developed countries, especially among older adults (Davis, 1989; Wilson et al., 1999). When sensory hair cells in the organ of Corti or the spiral ganglion neurons (SGNs) that innervate them are damaged, the SGNs tend to first lose their distal neurites, whereas the neuronal cell bodies in Rosenthal's canal and their afferent projections to the brainstem can persist for years (Zimmermann et al., 1995). The remnants of the individual SGNs can be electrically stimulated by a cochlear implant, an auditory prosthesis that has been used for decades with great success to restore hearing (Clark, 2003). The quality of sound perception might be improved, though, by reducing the distances of hundreds of micrometers between implant and neurons. This could be accomplished by modulating signaling pathways that stimulate and guide neurite outgrowth from the spiral ganglion towards the implant's electrodes. Among the pathways tested so far, only those targeted by the neurotrophins have exhibited such a capability in vivo (Gillespie and Shepherd, 2005; Webber and Raz, 2006). In particular, brain-derived neurotrophic factor, neurotrophin-3, and fibroblast growth factors delivered by viral vectors or an implanted osmotic pump can stimulate neurite regeneration in the cochlea (Glueckert et al., 2008; Miller et al., 2007; Shibata et al., 2010; Shibata et al., 2011; Wise et al., 2005).

The small-molecule drug Li^+ is a potential alternative or supplement to neurotrophin therapy. As an inorganic cation, Li^+ is much stabler and easier to administer than bioactive proteins; it can simply be ingested. The human pharmacology and toxicity of Li^+ are well understood (Grandjean and Aubry, 2009b; Grandjean and Aubry, 2009c; McKnight et al., 2012) since Li^+ salts have been used for almost fifty years to treat bipolar disorder (Grandjean and Aubry, 2009a). Lithium carbonate and citrate are approved as safe and effective prescription drugs (Food and Drug Administration, 2012). In several neurodegenerative diseases, Li^+ has neuroprotective effects (Chiu and Chuang, 2010). Most importantly, Li^+ promotes neurite regeneration and functional recovery in animals with spinal-cord lesions (Dill et al., 2008; Yick et al., 2004).

Li^+ is thought to exert its neuritogenic effect mainly by inhibiting glycogen synthase kinase 3 (GSK3), a constitutively-active serine/threonine kinase (Hur and Zhou, 2010). Despite its simplicity, Li^+ is selective for GSK3 and is not a general kinase inhibitor (Klein and Melton, 1996). In SGNs, neither the effects of Li^+ nor the function of GSK3 have been investigated. However, GSK3 mRNA is present at a high level in the adult mouse cochlea (Shah et al., 2009). Furthermore, two intracellular kinase cascades that commonly regulate GSK3 activity also modulate neurotrophin-dependent neuritogenesis in SGNs: the phosphatidylinositol 3-kinase/Akt kinase pathway (Aletsee et al., 2002a; Mullen et al., 2012) and the p38 mitogen activated protein kinase pathway (Mullen et al., 2012). Both Akt and p38 directly phosphorylate GSK3 and thus inhibit its activity (Hur and Zhou, 2010). Finally,

GSK3-dependent neuritogenesis in SGNs could also be regulated via the canonical pathway of the “wingless-related MMTV integration site” (Wnt) proteins (van Amerongen and Nusse, 2009): Wnt-dependent inactivation of GSK3 affects neuritogenesis in a wide range of neurons (Lucas et al., 1998; Purro et al., 2008). In adult SGNs, the Wnt-receptors frizzled 1, 4, 6, 9, and 10 are expressed in a specific and graded fashion (Shah et al., 2009), and Wnt7a (but not Wnt 1, 4, 5a, 6, or 7b) promotes neurite outgrowth from cultured statoacoustic ganglion explants (Fantetti et al., 2011; D. Fekete, personal communication).

To test whether Li^+ modulates neuritogenesis from adult SGNs, we exposed cultures of dissociated cells (Vieira et al., 2007) to different Li^+ concentrations and measured the resulting neurite morphology and growth-cone appearance in detail. Our results reveal that Li^+ at therapeutic concentrations promotes neurite sprouting and branching, but not elongation, and are consistent with a role for GSK3-mediated microtubule reorganization in SGN neurite regeneration.

2. Materials and Methods

2.1 Animals

Mice, strain CBA/CaJ (Jackson Laboratory, Bar Harbor, ME), were maintained in our own colony at the Beckman Institute of the University of Illinois at Urbana-Champaign. All experiments were conducted in accordance with protocols approved by the University of Illinois Institutional Animal Care and Use Committee.

2.2 Spiral ganglion neuron cell culture

Adult SGNs were obtained by dissecting the modioli and dissociating the spiral ganglia from mice that were six to eight weeks old. The resulting cell suspensions, which contained also glial and other non-neuronal cells, were plated in chambered glass slides and maintained as primary cultures for 72 hours in defined medium without adding serum or purified growth factors. A detailed step-by-step protocol of our culture method has been published previously (Vieira et al., 2007); here we used the following modifications: Instead of splitting a dissected modiolus longitudinally, it was fragmented with fine forceps to free the entire spiral ganglion; the concentration of bovine serum albumin used to quench the proteases after dissociation was increased from 1 to 10 mg/ml; 1 $\mu\text{g}/\text{ml}$ DNase I (Worthington Biochemical, Lakewood, NJ) was present during tituration; before the last centrifugation, the dissociated cells were passed through a cell strainer with a 40- μm nylon mesh (BD Biosciences, San Jose, CA) to remove clumps; the glutamine concentration in the culture medium was raised from 0.5 to 1 mM; and chambered glass slides (Lab-Tek, cat. no. 178599; Nalge Nunc, Rochester, NY) were used for all cultures. Since the two outermost wells on each side could not be imaged in their entirety, only the twelve wells in the center of each slide were used, permitting the comparison of six treatments with internal duplicates per slide. Each slide was seeded with a pool of dissociated SGNs from twelve mice that was distributed evenly in 150- μl aliquots among the twelve wells, resulting in a typical inoculum of $4 \cdot 10^3$ neurons per well. Lithium chloride (Sigma-Aldrich, St. Louis, MO) at final concentrations between 0.5 and 12.5 mM (up to 40 mM in pilot experiments) was added to the medium at the time of plating; the osmolarity of the medium increased by 1.8 mOsm/l per 1 mM of lithium chloride. Three independent runs were conducted for each culture experiment on different dates, with separate dissections and media preparations.

2.3 Immunofluorescence microscopy

Nuclei and neuronal microtubules were detected by two-color immunofluorescence microscopy as described previously (Shah et al., 2009). The cultures were fixed in the chambered slides with 4% w/v paraformaldehyde in phosphate-buffered saline (PBS; 137

mM NaCl, 2.7 mM KCl, 10 mM NaH₂PO₄, 2 mM KH₂PO₄, pH 7.0 to 7.4) at 4°C for at least one hour, followed by permeabilization with 0.3% v/v Triton X-100 in PBS for 15 min. After blocking for 30 min with PBS containing 0.1% v/v Tween-20 plus 5% v/v normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA), the cells were incubated for 30 min with 2 µg/ml of a mouse monoclonal antibody against the neuronal marker beta 3 class III tubulin (TUBJ1, Covance, Princeton, NJ; Hallworth and Luduena, 2000) in blocking solution, followed by three 5-min washes with PBS containing Tween-20. Next, the cells were incubated for 30 min protected from light with 3 µg/ml Alexa-594-conjugated donkey anti-mouse antibody (Molecular Probes, Eugene, OR) and 0.3 µM of the nuclear stain 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) in blocking solution, followed again by three washes. After mounting with an aqueous medium containing 10% w/v Mowiol 4-88 (Sigma-Aldrich), 5% v/v glycerol, and 2.5% w/v 1,4-diazobicyclo-[2.2.2]-octane in Tris-HCl buffer (pH 8.5), the slides were sealed at the edges with nail polish and stored at 4°C. No tubulin labeling was observed when the TUBJ1 primary antibody was omitted in control experiments (data not shown).

Each well of the chambered glass slide was imaged in its entirety as a series of about 130 tiles with a 10× objective on a motorized compound microscope (Zeiss Axiovert 200M; Carl Zeiss, Thornwood, NY). Each series was stitched together into a single high-resolution image (Fig. 1A) with Axiovision MosaicX software (release 4.7; Carl Zeiss). In separate sessions, randomly-chosen growth cones were imaged with a 63× oil-immersion objective (Fig. 1C). All images were processed identically after acquisition, regardless of treatment. Figures were created by assembling and labeling the digital images with Photoshop and Illustrator software (Adobe Systems, San Jose, CA).

2.4 Image Analysis

All neurons in a well image were analyzed by using ImageJ software (Schneider et al., 2012). The analyst was blinded to the treatment associated with each image. First, the cell bodies were counted and the number of neurite sprouts from each recorded; a sprout was defined as any beta 3 class III tubulin-containing process that had broken through the concave outline of a neuronal soma (da Silva and Dotti, 2002). The NeuronJ plugin (Meijering et al., 2004) was then used to trace and measure the individual neurites (Fig. 1B) and to classify them as primary (the process that emanates from the cell body and represents the longest path), secondary (branching from a primary neurite), or tertiary (branching from a secondary or tertiary neurite); if necessary, multiple options were traced to find the longest path.

Growth cone images were analyzed by measuring the dimensions of the microtubule-containing central domain (C-domain) with ImageJ (Fig. 1C). Circularity was calculated from the outline as $4 \cdot (\text{area}/\text{perimeter}^2)$. To assess symmetry, the C-domain was bisected by a line that extended the neurite stalk; if the areas of the two parts differed by a factor greater than two, the growth cone was scored as asymmetric.

2.5 Experimental Design and Statistical Analysis

Statistical analysis was performed by using Minitab 15 (Minitab Inc., State College, PA) and R software (R Core Team, 2012). The neuron and neurite data shown in Fig. 2 were analyzed as mixed-effects models, with treatment as a fixed and run as a random factor; interactions were not significant. The growth-cone data in Fig. 4 were analyzed with treatment as a fixed factor only. Analysis of variance was performed using the general linear model, with Poisson regression for counts (neurons per well) and logistic regression for fractions (neurons with n sprouts and circularity). To maximize the homogeneity of variances and the normality of residual distributions, the data were subjected to a logit (for

logistic regression) or logarithmic transformation. Means and bias-corrected, accelerated confidence intervals were obtained by non-parametric bootstrapping of 10^5 replicates. Pairwise comparisons were based on the bootstrapped confidence intervals of the differences between the fitted values for the Li^+ treatments and for the negative control (0 mM Li^+). P values less than 0.05, after correction with the Bonferroni-Holmes procedure for multiple comparisons, were considered significant.

3. Results

3.1 Low $[\text{Li}^+]$ enhances sprouting and branching, high $[\text{Li}^+]$ slows elongation

First, we investigated whether Li^+ affects neurite regeneration from adult SGNs. No neurons survived in pilot experiments at $[\text{Li}^+]$ at 20 mM and above (data not shown); we, therefore, chose to test a range of $[\text{Li}^+]$ from 0.5 to 12.5 mM. To facilitate drug application and neurite analysis, dissociated and axotomized neurons were maintained in serum-free culture. Following three days' exposure in bulk solution, all neurites were visualized by immunofluorescence and traced to measure their lengths (Fig. 1A & B). The morphology was then analyzed in detail for all 935 neurons with 661 primary and 1,356 secondary or tertiary neurites. Neuronal survival was not altered by Li^+ ; the mean was lower at 12.5 mM, but not significantly different from the mean at 0 mM (Fig. 2A; $P = 0.75$). Sprouting, measured as the fractions of neurons with one or more primary neurites, increased significantly at 2.5 mM Li^+ (Fig. 2B; $P = 0.0045$). Similarly, branching, measured as the ratio of the number of secondary and tertiary neurites to the number of primary neurites, increased significantly at 0.5 mM Li^+ (Fig. 2C; $P = 0.0020$). Elongation of the primary neurites at low to intermediate $[\text{Li}^+]$ was indistinguishable from the Li^+ -free control; only at 12.5 mM did we observe a significant reduction of neurite length (Fig. 2D; $P = 0.0015$). The low values of the correlation coefficient (pseudo- $R^2 = 0.16$, Fig. 2D) indicated that the within-group and between-group variabilities were similar and that neurite elongation was possibly influenced by other factors besides drug treatment and experimental run, such as the degree of damage sustained by individual SGNs during dissociation. Similar effects were manifest for secondary- and tertiary-neurite lengths (data not shown). In contrast, the neuritic tree length, measured as the sum of all neurite lengths divided by the number of primary neurites, was significantly increased at 0.5 mM and reduced at 12.5 mM Li^+ (Fig. 2E; $P = 0.0020$ in both cases). These results indicated two different regimes of Li^+ action on neurite morphology—a unimodal stimulation of sprouting and branching at low $[\text{Li}^+]$ versus a slowing of elongation at the highest $[\text{Li}^+]$.

3.2 Raising $[\text{Li}^+]$ results in microtubule disarrangement and growth-cone arborization

Next, we examined the effect of Li^+ on the growth cones of regenerating SGN neurites. A set of 85 randomly-chosen growth cones from the cultures described in the preceding section was imaged at higher magnification, and their microtubule-containing C-domains were measured as shown in Fig. 1C. Inspection of the images revealed that three features changed dramatically with increasing $[\text{Li}^+]$: First, the neurite shafts thickened progressively from 0 to 12.5 mM (Fig. 3A). Second, the arrangement of the microtubules in the C-domains unraveled from a tightly-bundled continuation in the neurite's direction at 0 mM to a loose mesh of looping strands at 12.5 mM (Fig. 3B). Third, the outlines of the C-domains transformed from narrow "paintbrushes" at 0 mM over round "teardrops" at 0.5 mM into increasingly spread and splayed arborizations at 2.5 to 12.5 mM (Fig. 3B).

The changes in growth-cone morphology clearly manifested themselves in plots of C-domain dimensions and shape parameters: Length bottomed out with the "teardrop" shapes at 0.5 mM Li^+ (Fig. 4A; significantly different from 0 mM at $P = 0.025$), whereas width rose steadily with the arborizations at 2.5 mM and above (Fig. 4A; $P = 0.0015$), resulting in both

cases in triple the width-to-length ratio (Fig. 4B; $P = 0.0020$). Area expanded with the progressive loss of microtubule organization (Fig. 4C; $P = 0.020$ for $[\text{Li}^+] = 7.5$ mM). Circularity peaked at 0.5 mM, as the “teardrops” were more compact than either the “paintbrushes” or the arborizations (Fig. 4D; $P = 0.0020$ for 0.5 and 2.5 mM). Lastly, the fraction of growth cones that were asymmetric grew monotonically and approached 100% at 12.5 mM (Fig. 4E; $P = 0.0090$ for $[\text{Li}^+] = 5.0$ mM). These results indicated again a concentration-dependence of Li^+ action—this time a gradual disarrangement of the microtubule cytoskeleton and increasingly arborized growth cones with rising $[\text{Li}^+]$.

4. Discussion

The principal finding of our pharmacological study of cultured adult SGNs was that Li^+ changed the morphology of regenerating neurites and their growth cones in a differential, concentration-dependent fashion. Low $[\text{Li}^+]$ enhanced neurite sprouting and branching, whereas high $[\text{Li}^+]$ slowed elongation; raising $[\text{Li}^+]$ resulted in microtubule disarrangement and growth-cone arborization. In the following we will discuss correlations between Li^+ exposure, the phases of neuritogenesis, and microtubule dynamics; the effects of Li^+ in comparison to other small-molecule drugs; and the likely molecular target of Li^+ in SGNs.

4.1 Stimulation of selected phases of neuritogenesis by Li^+

Exposure of SGNs to Li^+ stimulated some, but not all of the neurite behaviors associated with the different phases of neuritogenesis (Poulain and Sobel, 2010). To begin with, Li^+ did not promote neurite elongation. Its only effect with respect to neurite length was a two-fold reduction at the highest concentration tested (Fig. 2D). The nearly 50% increase in neuritic-tree length at low concentrations (Fig. 2E) resulted from a doubling of the number of secondary and tertiary neurites (Fig. 2C), not from longer individual neurites. Furthermore, the paintbrush-shaped “simple” growth cones that are associated with rapid elongation (Gordon-Weeks, 2000) were observed only in the absence of Li^+ (Fig. 3B, leftmost panel). The failure to lengthen neurites was not due to an inherent growth limit of individual neurites; we have previously shown that adding brain-derived neurotrophic factor or leukemia inhibitory factor to our cultures of adult SGNs promotes elongation (Vieira et al., 2007).

The slowed elongation at 12.5 mM may have been due in part to Li^+ toxicity resulting from osmotic stress, changes in membrane potential, or other non-specific effects. Survival also began to decline at this concentration (Fig. 2A), although the effect was not statistically significant, and plummeted at 20 mM and above (data not shown). However, while neuronal survival data for Li^+ are rarely reported in the literature, our range of 0.5 to 12.5 mM was at the low end of the 1 to 20 mM LiCl typically used for studies of neuritogenesis (Dill et al., 2008; Hall et al., 2000; Hall et al., 2002; Jiang et al., 2005; Krylova et al., 2000; Krylova et al., 2002; Lucas and Salinas, 1997; Lucas et al., 1998; Owen and Gordon-Weeks, 2003). Finally, from a translational point of view, patients are unlikely to be treated ever with 12.5 mM Li^+ since such a high serum concentration is toxic (Chiu and Chuang, 2010) and reducing neurite elongation is not a desirable therapeutic goal.

In contrast, Li^+ did promote sprouting (Fig. 2B) and branching (Fig. 2C) as well as changes in growth-cone morphology and behavior that are typical for neurite turning and target selection (even though there were no targets present in our cultures): The asymmetric arborizations observed at 2.5 mM Li^+ and above (Fig. 3B, third to sixth panel) and the diminished elongation at 12.5 mM are characteristic for the so-called “complex” growth cones that are stalled in “decision regions” in response to guidance cues (Gordon-Weeks, 2000). Arborization and stalling are also common during the target-selection phase that precedes synaptogenesis, when growth cones encounter target-derived stop and priming cues

(Waites et al., 2005). At the molecular level, the phases of SGN neuritogenesis that were stimulated by Li^+ have in common that they are characterized by local microtubule reorganization instead of long-distance microtubule extension (Poulain and Sobel, 2010).

Our results reveal the initial response of adult SGNs to dissociation and “amputation” *in vitro*: Most of the damaged neurons die, but some survive and execute the initial steps of neuritogenesis (sprouting, elongation, branching) using mechanisms that are responsive to Li^+ . In guinea pigs, low serum Li^+ levels between 0.4 and 0.9 mM have been shown to protect the cochlea of guinea pigs against acoustic overexposure (Horner et al., 1998), whereas chronic Li^+ levels above 3.7 mM cause a reversible hearing loss (Horner et al., 1997); it is not known, however, whether SGNs were among the cellular targets of Li^+ in these studies. It remains to be tested whether Li^+ can promote the complete regeneration of individual SGN neurites and the formation of functional connections that conform to the cochlea’s tonotopic map *in vivo*.

4.2 Comparison of Li^+ effects to those of other small-molecule drugs

Comparing the effects of Li^+ on SGN neurites to those of other small-molecule drugs is complicated by the fact that previous studies have generally focused on a smaller number of morphological parameters and that experimental differences (e.g., growth-factor supplements; spiral-ganglion explants vs. dissociated SGNs) have sometimes led to different outcomes. Most drugs tested on SGNs have been reported only to inhibit neurite outgrowth, variously defined as either the number or the length of neurites and often without distinguishing between primary and higher-order neurites: GF109203X, an inhibitor of protein kinase C (Lallemend et al., 2005); wortmannin, an inhibitor of phosphatidylinositol 3-kinase (Aletsee et al., 2002a; Mullen et al., 2012); Akt inhibitor II of Akt protein kinases (Mullen et al., 2012); U0126, an inhibitor of mitogen activated protein kinase kinase 1 (Aletsee et al., 2001; Aletsee et al., 2002b); AS601245, CEP-11004, and SP600125, inhibitors of c-jun amino-terminal kinase signaling (Atkinson et al., 2011; Bodmer et al., 2002); SB203580, an inhibitor of p38 mitogen-activated protein kinase (Mullen et al., 2012; but see Aletsee et al., 2001; Aletsee et al., 2002b); and FTI-277, an inhibitor of ras farnesyl transferase (Aletsee et al., 2001; Aletsee et al., 2002b; Mullen et al., 2012). Promotion of neurite elongation has been reported only for 8-cpt-cAMP, an inhibitor of cyclic-nucleotide-activated kinases, and forskolin, an activator of protein kinase A (Xu et al., 2012); phorbol 12-myristate 13-acetate, an activator of protein kinase C (Lallemend et al., 2005); KT5720, an inhibitor of protein kinase A, and U0126 (Mullen et al., 2012); and H-1152, an inhibitor of rho-associated kinases (Lie et al., 2010). The profile most similar to that of Li^+ has been reported for *Clostridium difficile* toxin B, an inhibitor of rho GTPases—it inhibits elongation, but promotes sprouting (Brors et al., 2003; Mullen et al., 2012). Unlike Li^+ , however, toxin B is not approved for human use; in fact, together with toxin A, it underlies the severe gastrointestinal inflammation caused by *C. difficile* (Voth and Ballard, 2005).

4.3 GSK3 as the likely target of Li^+ in adult SGNs

Our results are consistent with (but do not prove) Li^+ inhibition of GSK3 activity in the cultured SGNs. First, the changes in neurite morphology and growth-cone appearance qualitatively resemble the response of other neuron types to Li^+ exposure and GSK3 inhibition. For example, in cultures of hippocampal neurons, a permanently-active GSK3B mutant reduces axon sprouting, whereas GSK3 inhibition by Li^+ , other drugs, or RNA interference promotes it (Jiang et al., 2005). Inhibiting GSK3 in pontine or dorsal-root-ganglion neurons with Li^+ results in enhanced branching and enlarged growth cones with unbundled and looping microtubules (Hall et al., 2002). Inhibiting GSK3 in sensory neurons with Li^+ or SB 216763, an ATP competitor, slows axonal elongation and enlarges growth cones (Owen and Gordon-Weeks, 2003).

Second, our dose-response curves (Figs. 2 and 4) quantitatively match the model for GSK3 regulation of neurite morphogenesis formulated by Kim and coworkers (Kim et al., 2006): GSK3 directly targets microtubule-associated proteins in the neurite, such as adenomatosis polyposis coli (APC), collapsin response mediator protein-2 (CRMP2), and microtubule-associated protein 1B (MAP1B) (Kim et al., 2006). Fully active GSK3 phosphorylates all three proteins, inactivating APC and CRMP2 but activating MAP1B, which results in maximal elongation. Partial inhibition of GSK3 prevents phosphorylation of APC and CRMP2 only; as a result, sprouting and branching are promoted, while the elongation rate is maintained. Complete inhibition of GSK3 prevents phosphorylation of MAP1B, too, arresting microtubule dynamics and bringing neuritogenesis to a standstill. We observed promotion of sprouting and branching at low $[Li^+]$ (Fig. 2B & C), near the half-maximal inhibitory concentration (IC_{50}) for GSK3 of 1 to 2 mM (Phiel and Klein, 2001); no promotion of elongation, but a reduction at high $[Li^+]$, where GSK3 is expected to be almost completely inhibited (Fig. 2D); and conspicuous changes of microtubule behavior in the growth cone throughout (Figs. 3 and 4).

The other potential targets of Li^+ in SGNs are the phosphomonoesterases (myo)-1(or 4)-monophosphatase and inositol polyphosphate 1-phosphatase (Pasquali et al., 2010). However, while their IC_{50} 's for Li^+ are similar to those of GSK3—0.8 and 0.3 mM, respectively—there is no published evidence for a role of these enzymes in neuritogenesis.

4.4 Conclusion

Our results demonstrate that Li^+ in SGNs selectively promotes those phases of neuritogenesis that are associated with microtubule reorganization, such as sprouting and branching. As a next step, one could assess whether Li^+ administration augments neurotrophin therapy of the damaged SGNs. Experiments with additional drugs and molecular-genetic tools are needed to test whether GSK3 indeed plays a role in SGN neurite regeneration, possibly by integrating neurotrophin and Wnt signals at the growth cone.

Acknowledgments

We thank Ms. Soon Ki for technical assistance; Dr. William Lytton for access to and Mr. Larry Eberle for system administration on the SUNY Downstate high-performance computing cluster, which is supported by research grant R01 MH086638 from the National Institute of Mental Health to W.L.; and Ms. Olipriya Das, Ms. Roza George, Dr. Byron Kemper, and Dr. Mark Stewart for comments on the manuscript. This work was supported by training grant T32 DC006612 from the National Institute on Deafness and Other Communication Disorders, a medical student training grant from the American Otological Society, and a grant from the Charles M. Goodenberger Fund at the University of Illinois to S.M.S.; by grants from the Campus Research Board and the Mary Jane Neer Fund at the University of Illinois to A.S.F.; and by a research award from the National Organization for Hearing Research to R.K.

Abbreviations

APC	adenomatosis polyposis coli
C-domain	central domain
CRMP2	collapsin response mediator protein 2
DAPI	4 ,6-diamidino-2-phenylindole
GSK3	glycogen synthase kinase 3
IC_{50}	half-maximal inhibitory concentration
MAP1B	microtubule-associated protein 1B
PBS	phosphate-buffered saline

RT-PCR	reverse transcription and polymerase chain reaction
SGN	spiral ganglion neuron
Wnt	wingless-related MMTV integration site

References

- Aletsee C, Beros A, Mullen L, Palacios S, Pak K, Dazert S, Ryan AF. Ras/MEK but not p38 signaling mediates NT-3-induced neurite extension from spiral ganglion neurons. *J Assoc Res Otolaryngol.* 2001; 2:377–387. [PubMed: 11833610]
- Aletsee C, Brors D, Mlynski R, Ryan AF, Dazert S. Wortmannin, a specific inhibitor of phosphatidylinositol-3-kinase influences neurotrophin-induced spiral ganglion neurite growth. *Laryngorhinootologie.* 2002a; 81:189–195. [PubMed: 11967771]
- Aletsee C, Brors D, Palacios S, Pak K, Mullen L, Dazert S, Ryan AF. The effects of laminin-1 on spiral ganglion neurons are dependent on the MEK/ERK signaling pathway and are partially independent of Ras. *Hear Res.* 2002b; 164:1–11. [PubMed: 11950519]
- Atkinson PJ, Cho CH, Hansen MR, Green SH. Activity of all JNK isoforms contributes to neurite growth in spiral ganglion neurons. *Hear Res.* 2011; 278:77–85. [PubMed: 21554942]
- Bodmer D, Gloddek B, Ryan AF, Huverstuhl J, Brors D. Inhibition of the c-Jun N-terminal kinase signaling pathway influences neurite outgrowth of spiral ganglion neurons in vitro. *Laryngoscope.* 2002; 112:2057–2061. [PubMed: 12439181]
- Brors D, Aletsee C, Dazert S, Huverstuhl J, Ryan AF, Bodmer D. Clostridium difficile toxin B, an inhibitor of the small GTPases Rho, Rac and Cdc42, influences spiral ganglion neurite outgrowth. *Acta Otolaryngol.* 2003; 123:20–25. [PubMed: 12625568]
- Chiu CT, Chuang DM. Molecular actions and therapeutic potential of lithium in preclinical and clinical studies of CNS disorders. *Pharmacol Ther.* 2010; 128:281–304. [PubMed: 20705090]
- Clark, G. Cochlear Implants: Fundamentals and Applications. Springer; New York: 2003.
- da Silva JS, Dotti CG. Breaking the neuronal sphere: regulation of the actin cytoskeleton in neuritogenesis. *Nat Rev Neurosci.* 2002; 3:694–704. [PubMed: 12209118]
- Davis AC. The prevalence of hearing impairment and reported hearing disability among adults in Great Britain. *Int J Epidemiol.* 1989; 18:911–917. [PubMed: 2621028]
- Dill J, Wang H, Zhou F, Li S. Inactivation of glycogen synthase kinase 3 promotes axonal growth and recovery in the CNS. *J Neurosci.* 2008; 28:8914–8928. [PubMed: 18768685]
- Fantetti KN, Zou Y, Fekete DM. Wnts and Wnt inhibitors do not influence axon outgrowth from chicken statoacoustic ganglion neurons. *Hear Res.* 2011; 278:86–95. [PubMed: 21530628]
- Food and Drug Administration. Approved drug products with therapeutic equivalence evaluations. U.S. Dept. of Health and Human Services; Silver Spring, MD: 2012.
- Gillespie LN, Shepherd RK. Clinical application of neurotrophic factors: the potential for primary auditory neuron protection. *Eur J Neurosci.* 2005; 22:2123–2133. [PubMed: 16262651]
- Glueckert R, Bitsche M, Miller JM, Zhu Y, Prieskorn DM, Altschuler RA, Schrott-Fischer A. Deafferentation-associated changes in afferent and efferent processes in the guinea pig cochlea and afferent regeneration with chronic intrascalar brain-derived neurotrophic factor and acidic fibroblast growth factor. *J Comp Neurol.* 2008; 507:1602–1621. [PubMed: 18220258]
- Gordon-Weeks, PR. Neuronal Growth Cones. Cambridge University Press; Cambridge: 2000.
- Grandjean EM, Aubry JM. Lithium: updated human knowledge using an evidence-based approach: Part I: Clinical efficacy in bipolar disorder. *CNS Drugs.* 2009a; 23:225–240. [PubMed: 19320531]
- Grandjean EM, Aubry JM. Lithium: updated human knowledge using an evidence-based approach: part III: clinical safety. *CNS Drugs.* 2009b; 23:397–418. [PubMed: 19453201]
- Grandjean EM, Aubry JM. Lithium: updated human knowledge using an evidence-based approach. Part II: Clinical pharmacology and therapeutic monitoring. *CNS Drugs.* 2009c; 23:331–349. [PubMed: 19374461]

- Hall AC, Brennan A, Goold RG, Cleverley K, Lucas FR, Gordon-Weeks PR, Salinas PC. Valproate regulates GSK-3-mediated axonal remodeling and synapsin I clustering in developing neurons. *Mol Cell Neurosci*. 2002; 20:257–270. [PubMed: 12093158]
- Hall AC, Lucas FR, Salinas PC. Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. *Cell*. 2000; 100:525–535. [PubMed: 10721990]
- Hallworth R, Luduena RF. Differential expression of beta tubulin isoforms in the adult gerbil cochlea. *Hear Res*. 2000; 148:161–172. [PubMed: 10978833]
- Horner KC, Higuieret D, Cazals Y. Efferent-mediated protection of the cochlear base from acoustic overexposure by low doses of lithium. *Eur J Neurosci*. 1998; 10:1524–1527. [PubMed: 9749806]
- Horner KC, Huang ZW, Higuieret D, Cazals Y. Reversible hearing impairment induced by lithium in the guinea pig. *Neuroreport*. 1997; 8:1341–1345. [PubMed: 9172132]
- Hur EM, Zhou FQ. GSK3 signalling in neural development. *Nat Rev Neurosci*. 2010; 11:539–551. [PubMed: 20648061]
- Jiang H, Guo W, Liang X, Rao Y. Both the establishment and the maintenance of neuronal polarity require active mechanisms: critical roles of GSK-3 β and its upstream regulators. *Cell*. 2005; 120:123–135. [PubMed: 15652487]
- Kim WY, Zhou FQ, Zhou J, Yokota Y, Wang YM, Yoshimura T, Kaibuchi K, Woodgett JR, Anton ES, Snider WD. Essential roles for GSK-3 α and GSK-3 β in neurotrophin-induced and hippocampal axon growth. *Neuron*. 2006; 52:981–996. [PubMed: 17178402]
- Klein PS, Melton DA. A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci U S A*. 1996; 93:8455–8459. [PubMed: 8710892]
- Krylova O, Herreros J, Cleverley KE, Ehler E, Henriquez JP, Hughes SM, Salinas PC. WNT-3, expressed by motoneurons, regulates terminal arborization of neurotrophin-3-responsive spinal sensory neurons. *Neuron*. 2002; 35:1043–1056. [PubMed: 12354395]
- Krylova O, Messenger MJ, Salinas PC. Dishevelled-1 regulates microtubule stability: a new function mediated by glycogen synthase kinase-3 β . *J Cell Biol*. 2000; 151:83–94. [PubMed: 11018055]
- Lallemend F, Hadjab S, Hans G, Moonen G, Lefebvre PP, Malgrange B. Activation of protein kinase C β 1 constitutes a new neurotrophic pathway for deafferented spiral ganglion neurons. *J Cell Sci*. 2005; 118:4511–4525. [PubMed: 16179609]
- Lie M, Grover M, Whitlon DS. Accelerated neurite growth from spiral ganglion neurons exposed to the Rho kinase inhibitor H-1152. *Neuroscience*. 2010; 169:855–862. [PubMed: 20478368]
- Lucas FR, Goold RG, Gordon-Weeks PR, Salinas PC. Inhibition of GSK-3 β leading to the loss of phosphorylated MAP-1B is an early event in axonal remodelling induced by WNT-7a or lithium. *J Cell Sci*. 1998; 111:1351–1361. [PubMed: 9570753]
- Lucas FR, Salinas PC. WNT-7a induces axonal remodeling and increases synapsin I levels in cerebellar neurons. *Dev Biol*. 1997; 192:31–44. [PubMed: 9405095]
- McKnight RF, Adida M, Budge K, Stockton S, Goodwin GM, Geddes JR. Lithium toxicity profile: a systematic review and meta-analysis. *Lancet*. 2012; 379:721–728. [PubMed: 22265699]
- Meijering E, Jacob M, Sarria JC, Steiner P, Hirling H, Unser M. Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. *Cytometry A*. 2004; 58:167–176. [PubMed: 15057970]
- Miller JM, Le Prell CG, Prieskorn DM, Wys NL, Altschuler RA. Delayed neurotrophin treatment following deafness rescues spiral ganglion cells from death and promotes regrowth of auditory nerve peripheral processes: effects of brain-derived neurotrophic factor and fibroblast growth factor. *J Neurosci Res*. 2007; 85:1959–1969. [PubMed: 17492794]
- Mullen LM, Pak KK, Chavez E, Kondo K, Brand Y, Ryan AF. Ras/p38 and PI3K/Akt but not Mek/Erk signaling mediate BDNF-induced neurite formation on neonatal cochlear spiral ganglion explants. *Brain Res*. 2012; 1430:25–34. [PubMed: 22119396]
- Owen R, Gordon-Weeks PR. Inhibition of glycogen synthase kinase 3 β in sensory neurons in culture alters filopodia dynamics and microtubule distribution in growth cones. *Mol Cell Neurosci*. 2003; 23:626–637. [PubMed: 12932442]
- Pasquali L, Busceti CL, Fulceri F, Paparelli A, Fornai F. Intracellular pathways underlying the effects of lithium. *Behav Pharmacol*. 2010; 21:473–492. [PubMed: 20700048]

- Phiel CJ, Klein PS. Molecular targets of lithium action. *Annu Rev Pharmacol Toxicol.* 2001; 41:789–813. [PubMed: 11264477]
- Poulain FE, Sobel A. The microtubule network and neuronal morphogenesis: Dynamic and coordinated orchestration through multiple players. *Mol Cell Neurosci.* 2010; 43:15–32. [PubMed: 19660553]
- Purro SA, Ciani L, Hoyos-Flight M, Stamatakou E, Siomou E, Salinas PC. Wnt regulates axon behavior through changes in microtubule growth directionality: a new role for adenomatous polyposis coli. *J Neurosci.* 2008; 28:8644–8654. [PubMed: 18716223]
- R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing; Vienna, Austria: 2012. <http://www.R-project.org> [last accessed October 4, 2012]
- Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods.* 2012; 9:671–675. [PubMed: 22930834]
- Shah SM, Kang YJ, Christensen BL, Feng AS, Kollmar R. Expression of Wnt receptors in adult spiral ganglion neurons: frizzled 9 localization at growth cones of regenerating neurites. *Neuroscience.* 2009; 164:478–487. [PubMed: 19716861]
- Shibata SB, Budenz CL, Bowling SA, Pfingst BE, Raphael Y. Nerve maintenance and regeneration in the damaged cochlea. *Hear Res.* 2011; 281:56–64. [PubMed: 21596129]
- Shibata SB, Cortez SR, Beyer LA, Wiler JA, Di Polo A, Pfingst BE, Raphael Y. Transgenic BDNF induces nerve fiber regrowth into the auditory epithelium in deaf cochleae. *Exp Neurol.* 2010; 223:464–472. [PubMed: 20109446]
- van Amerongen R, Nusse R. Towards an integrated view of Wnt signaling in development. *Development.* 2009; 136:3205–3214. [PubMed: 19736321]
- Vieira M, Christensen BL, Wheeler BC, Feng AS, Kollmar R. Survival and stimulation of neurite outgrowth in a serum-free culture of spiral ganglion neurons from adult mice. *Hear Res.* 2007; 230:17–23. [PubMed: 17521837]
- Voth DE, Ballard JD. Clostridium difficile toxins: mechanism of action and role in disease. *Clin Microbiol Rev.* 2005; 18:247–263. [PubMed: 15831824]
- Waites CL, Craig AM, Garner CC. Mechanisms of vertebrate synaptogenesis. *Annu Rev Neurosci.* 2005; 28:251–274. [PubMed: 16022596]
- Webber A, Raz Y. Axon guidance cues in auditory development. *Anat Rec A Discov Mol Cell Evol Biol.* 2006; 288:390–396. [PubMed: 16550548]
- Wilson DH, Walsh PG, Sanchez L, Davis AC, Taylor AW, Tucker G, Meagher I. The epidemiology of hearing impairment in an Australian adult population. *Int J Epidemiol.* 1999; 28:247–252. [PubMed: 10342686]
- Wise AK, Richardson R, Hardman J, Clark G, O’leary S. Resprouting and survival of guinea pig cochlear neurons in response to the administration of the neurotrophins brain-derived neurotrophic factor and neurotrophin-3. *J Comp Neurol.* 2005; 487:147–165. [PubMed: 15880560]
- Xu N, Engbers J, Khaja S, Xu L, Clark JJ, Hansen MR. Influence of cAMP and protein kinase A on neurite length from spiral ganglion neurons. *Hear Res.* 2012; 283:33–44. [PubMed: 22154930]
- Yick LW, So KF, Cheung PT, Wu WT. Lithium chloride reinforces the regeneration-promoting effect of chondroitinase ABC on rubrospinal neurons after spinal cord injury. *J Neurotrauma.* 2004; 21:932–943. [PubMed: 15307905]
- Zimmermann CE, Burgess BJ, Nadol JBJ. Patterns of degeneration in the human cochlear nerve. *Hear Res.* 1995; 90:192–201. [PubMed: 8974997]

Highlights

- Li^+ altered the morphology of adult spiral ganglion neurons regenerating in culture.
- Low $[\text{Li}^+]$ enhanced neurite sprouting and branching; high $[\text{Li}^+]$ slowed elongation.
- Microtubules became more disorganized and growth cones arborized at higher $[\text{Li}^+]$.
- Li^+ effects on spiral ganglion neurites differ from those of most other drugs.
- The results are consistent with glycogen synthase kinase 3 as the target of Li^+ .

Fig. 1.

Imaging and analyzing cultured SGNs from adult mice. (A) Example of a stitched fluorescence image of an entire 40-mm² well containing multiple SGNs with regenerated neurites as well as non-neuronal cells; blue, nuclei labeled with DAPI; red, neuronal microtubules labeled with the TuJ1 antibody. (B) A single neuritic tree from the yellow square in A traced with NeuronJ; green, primary neurite; purple, secondary neurites; yellow, tertiary neurites; arrowheads, growth cones. (C) Example of a single growth cone imaged at higher magnification and measured with ImageJ; white, perimeter of C-domain; blue, length; green, width. Scale bar in C, 1 mm for A, 100 μ m for B, 11 μ m for C.

Fig. 2.

Enhanced neurite sprouting and branching at low $[Li^+]$ *versus* diminished elongation at high $[Li^+]$. Adult SGNs in culture were exposed to the indicated $[Li^+]$ for three days, and all surviving neurons were analyzed. (A) Number of neurons per well; overall likelihood-ratio test for Li^+ treatment as fixed effect: $\chi^2 = 5.0$, 5 df, $P = 0.42$; pseudo- $R^2 = 0.97$. (B) Cumulative fractions of neurons with zero, one, two, or three primary neurites; $\chi^2 = 27$, 5 df, $P = 7.1 \cdot 10^{-5}$; pseudo- $R^2 = 0.90$. (C) Number of branch points per neuritic tree; $\chi^2 = 21$, 5 df, $P = 6.6 \cdot 10^{-4}$; pseudo- $R^2 = 0.39$. (D) Lengths of primary neurites; $\chi^2 = 22$, 5 df, $P = 5.3 \cdot 10^{-4}$; pseudo- $R^2 = 0.16$. (E) Total lengths of neuritic trees; $\chi^2 = 36$, 5 df, $P = 1.2 \cdot 10^{-6}$; pseudo- $R^2 = 0.94$. Means of three replicate experiments with internal duplicates; error bars,

95% confidence intervals; shaded bands, 95% confidence intervals at 0 mM Li⁺ extended; asterisks, significant differences in pairwise comparisons with the control mean at 0 mM Li⁺ ($P \leq 0.05$; see Results); black bar above x-axis, range of Li⁺ IC₅₀ values for GSK3 (Phiel and Klein, 2001).

Fig. 3.

Microtubule disarrangement and growth-cone arborization with rising $[\text{Li}^+]$. (A) Immunofluorescence images of distal neurites from cultures treated with the indicated $[\text{Li}^+]$; arrowheads, growth cones; note the increasing thickness of the neurites (* in the 1.5-mM panel). (B) Enlargements of one representative growth cone for each $[\text{Li}^+]$ value; only the C-domains containing neuronal β -III-tubulin are visible; note the shape transformation from 'paintbrush' over 'teardrop' to arborization and the loss of microtubule organization. Scale bar in A, 60 μm for A and 19 μm for B.

Fig. 4.

Increasing growth-cone spreading, splaying, and asymmetry with higher $[Li^+]$. Dimensions and shape descriptors of 85 randomly-chosen C-domains ($n = 7$ to 21 per treatment): (A) Length and width; one-way analyses of variance: $F(5, 79) = 8.4$, $P = 2.2 \cdot 10^{-6}$, adj. $R^2 = 0.30$ and $F(5, 79) = 12$, $P = 1.3 \cdot 10^{-8}$, adj. $R^2 = 0.39$. (B) Width-to-length ratio; $F(5, 79) = 5.9$, $P = 1.1 \cdot 10^{-4}$, adj. $R^2 = 0.23$. (C) Area; $F(5, 79) = 9.5$, $P = 4.2 \cdot 10^{-7}$, adj. $R^2 = 0.34$. (D) Circularity; $F(5, 79) = 28$, $P = 3.5 \cdot 10^{-16}$, adj. $R^2 = 0.62$. (E) Fraction of asymmetric C-domains; $\chi^2 = 35$, 5 df, $P = 1.6 \cdot 10^{-6}$, pseudo- $R^2 = 0.99$. Means of three replicate experiments with internal duplicates; error bars, shaded bands, asterisks, and black bar above x-axis as in Fig. 2.