

Disorders of the Nervous System

A Novel Neuroprotective Mechanism for Lithium That Prevents Association of the p75^{NTR}-Sortilin Receptor Complex and Attenuates proNGF-Induced Neuronal Death *In Vitro* and *In Vivo*

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Abstract

Neurotrophins play critical roles in the survival, maintenance and death of neurons. In particular, proneurotrophins have been shown to mediate cell death following brain injury induced by status epilepticus (SE) in rats. Previous studies have shown that pilocarpine-induced seizures lead to increased levels of proNGF, which binds to the p75^{NTR}-sortilin receptor complex to elicit apoptosis. A screen to identify compounds that block proNGF binding and uptake into cells expressing p75 and sortilin identified lithium citrate as a potential inhibitor of proNGF and p75^{NTR}-mediated cell death. In this study, we demonstrate that low, submicromolar doses of lithium citrate effectively inhibited proNGF-induced cell death in cultured neurons and protected hippocampal neurons following pilocarpine-induced SE *in vivo*. We analyzed specific mechanisms by which lithium citrate afforded neuroprotection and determined that lithium citrate prevented the association and internalization of the p75^{NTR}-sortilin receptor complex. Our results demonstrate a novel mechanism by which low-dose treatments of lithium citrate are effective in attenuating p75^{NTR}-mediated cell death *in vitro* and *in vivo*.

Key words: apoptosis; neuroprotection; neurotrophins; p75; proNGF; seizures

Significance Statement

Neuronal death occurs after prolonged severe seizures and is partially due to the induction of proNGF and its p75 neurotrophin receptor. The p75^{NTR} utilizes a coreceptor, sortilin, to bind proNGF and promote apoptotic signaling. We show here that submicromolar concentrations of lithium citrate prevented p75^{NTR}-mediated neuronal death by impairing the formation and internalization of the coreceptor complex. Although lithium has many neuroprotective functions that occur at millimolar concentrations, we demonstrate a novel mechanism for lithium citrate to afford neuroprotection from seizure-induced death at submicromolar doses.

Introduction

The neurotrophin growth factors regulate many aspects of neuronal function, including cell survival and death.

Neurotrophins are initially synthesized as precursor proneurotrophins and are cleaved to generate their C-terminal mature forms, which bind to the Trk family of receptor tyrosine

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kinases to enhance neuronal survival and differentiation (Huang and Reichardt, 2003; Reichardt, 2006). In contrast, proneurotrophins bind with high affinity to a receptor complex comprised of p75^{NTR} and sortilin, which can initiate apoptotic signaling (Lee et al., 2001; Nykjaer et al., 2004). Following injury, proneurotrophins and p75^{NTR} are upregulated and can play a significant role in promoting neuronal cell death. Previous studies have demonstrated that prolonged severe seizures, induced by either pilocarpine or kainic acid, promote neuronal death in rats mediated by p75^{NTR} (Troy et al., 2002). Seizures also elevate levels of proNGF, a potent ligand for the activation of p75^{NTR}-mediated cell death (Volosin et al., 2008), and prevent its cleavage by MMP7, leading to increased levels of intact proNGF in the extracellular environment (Le and Friedman, 2012). ProNGF induces neuronal death by interacting with a receptor complex consisting of p75^{NTR} and sortilin (Nykjaer et al., 2004; Hempstead, 2009). Moreover, the two receptors can be recruited to the cell surface by inflammatory cytokines, thereby increasing vulnerability to proNGF after brain injury (Choi and Friedman, 2014).

Recently, we performed a drug screen to identify compounds that block the binding of proNGF to cells expressing sortilin and p75^{NTR}, and lithium citrate was among these compounds. Lithium ion can inhibit apoptosis by a variety of different mechanisms (Wada et al., 2005), including increasing Akt activity, by phosphorylating and inactivating GSK3 β (Tajes et al., 2009; Pasquali et al., 2010), and promoting autophagy (Motoi et al., 2014; Del Grosso et al., 2016; Liu et al., 2017). Chronic treatment with lithium has also been shown to upregulate BDNF expression in the brain (Fukumoto et al., 2001) and retina (Wu et al., 2014), which provides another potential neuroprotective mechanism for lithium ion. However, since the drug screen identified lithium citrate by preventing the binding and uptake of proNGF to its p75^{NTR}-sortilin receptor complex, another potential target for lithium ion could be in altering this receptor complex.

In a rat experimental model of temporal lobe epilepsy (TLE), status epilepticus (SE) induced by pilocarpine causes a defined pattern of damage in the hippocampus with severe loss of neurons in the CA1 region as well as the hilus/dentate gyrus region (Turski et al., 1984, 1989). Previous studies have demonstrated that much of the seizure-induced neuronal loss is due to the upregulation of proNGF and p75^{NTR} (Roux et al., 1999; Troy et al., 2002; Volosin et al., 2008), and blocking proNGF-p75^{NTR}

signaling, using function-blocking polyclonal antibodies to the prodomain of proNGF, attenuates hippocampal cell death following seizures (Volosin et al., 2008; Song et al., 2010). Therefore, in these studies, we investigated the mechanisms by which lithium citrate affords neuroprotection from proNGF induced cell death, and tested the efficacy of lithium citrate in preventing p75^{NTR}-mediated cell death following pilocarpine-induced seizures. We demonstrate that low doses of lithium, well below the dose used for the standard lithium-pilocarpine model of epilepsy (Jope et al., 1986) and below the dose needed to phosphorylate GSK3 β or induce BDNF (Wada et al., 2005), decreased the association and internalization of the p75^{NTR}-sortilin receptor complex, and prevented proNGF-induced neuronal apoptosis in culture and *in vivo*.

Materials and Methods

Alexa Fluor 594 labeling of proNGF

Twenty micrograms of purified human proNGF, prepared in SF9 cells as described (Feng et al., 2010), was added to 10 μ l of PBS and 7 μ l of reconstituted Alexa Fluor 594 (excitation 590 nm, emission 617 nm; Invitrogen) according to the manufacturer's protocol (microscale protein labeling kit A30008) and incubated at room temperature, in the dark, for 15 min. Tris, pH 8.0, was added to obtain a final concentration of 50 mM to quench the labeling reaction. Labeled proNGF was then extensively dialyzed using PBS, pH 7.4, at 4°C in the dark. Alexa Fluor-labeled proNGF was used within 48 h. HT-1080 cells that stably expressed p75 and sortilin (generated as described; Feng et al., 2010), were cultured in 384 well flat bottom plates (CellBind) for 24 h. Cells were treated with one of 2560 compounds from the SpecPlus Collection (MicroSource Discovery Systems) at a concentration of 10 μ M, or 20 μ M neurotensin, or diluent control, using an automated robotics system, followed by addition of 200 nM Alexa Fluor-proNGF. Cells were incubated for 18 h, then rinsed with PBS, and fixed with 4% paraformaldehyde for 10 min, rinsed three times with PBS, and then counterstained using Hoechst at a final concentration of 10 μ g/ml for 2 h at room temperature. Cells were analyzed for uptake of Alexa Fluor 594-conjugated proNGF using a Discovery 1 automatic fluorescence microscope from Molecular Devices as described previously (Pipalia et al., 2006).

Images were acquired with a Photometrics Cool Snap HQ camera and analyzed using Metamorph Discovery 1 image analysis software. To correct for shading, an image was created by averaging all of the images from a plate and smoothing the averaged image with a low pass filter. Thresholding was performed, using a low threshold to include all areas occupied by cells. The outlines of cells were selected, as were the outlines of nuclei, assessed by Hoechst staining. The number of pixels in the area of the cell within two nuclear diameters of the nuclei were calculated, and the average proNGF intensity was calculated as the total intensity above the low threshold/number of pixels above low threshold (modified from Pipalia et al., 2006).

wrote the paper.

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Normalized values were obtained by dividing the values in the presence of each compound by the values obtained in the presence of solvent control in each plate. All compounds were tested in replicates of eight. Compounds from the SpecPlus Collection (MicroSource Discovery Systems) were assayed in cells that expressed p75^{NTR} and sortilin. The compounds in the collection are primarily Food and Drug Administration-approved compounds or natural products.

Primary hippocampal neuronal cultures

All animal studies were conducted using the National Institutes of Health guidelines for the ethical treatment of animals with approval of the Rutgers Institutional Animal Care and Facilities Committee.

Rat hippocampi were dissected from embryonic day 18 animals and dissociated as previously described (Friedman, 2010). Dissociated neurons were then plated on poly-D-lysine (0.1 mg/ml)-coated dishes maintained in serum free media. The media consisted of 1:1 MEM and F12, with glucose (6 mg/ml), insulin (2.5 mg/ml), putrescine (60 μ M), progesterone (20 nM), transferrin (100 μ g/ml), selenium (30 nM), penicillin (0.5 U/ml), and streptomycin (0.5 μ g/ml). Neuronal cultures were maintained in media for 5 d before treatment with proNGF and lithium citrate.

Cell culture treatments and survival assay

Lithium citrate doses in the nanomolar and micromolar ranges did not show toxicity in prescreen testing and was used here at doses ranging from 10 nM to 100 μ M. Following proNGF (2-5 ng/ml) and lithium citrate treatment, hippocampal neurons were lysed and healthy nuclei were counted using a hemocytometer to assess cell viability (Friedman, 2010). To distinguish between nuclei of healthy cells and those of dead cells, pyknotic and irregular membrane shapes common to cells dying via apoptosis were assessed and excluded. Cell counts were performed in triplicate.

6-Bromindirubin-3'-oxime (BIO) was purchased from Sigma and dissolved in DMSO for a stock concentration of 3 mM.

For Western blot analysis, hippocampal neurons plated in six-well dishes (1×10^6 cells/well) were treated as indicated, washed in sterile PBS (pH 7.4) and lysed in buffer containing 120 mM Tris, 2% SDS, 10% glycerol and protease inhibitors. Equal amounts of protein were subjected to PAGE, transferred to nitrocellulose membrane and blocked with 5% nonfat milk. Blots were incubated in primary antibodies to GSK3 β , p-GSK3 β , BDNF, and actin overnight. After washing three times with TBST for 15 min each, the blots were incubated with appropriate secondary antibodies for 1 h at room temperature. The membrane was washed three times with TBST before being visualized using either ECL (Pierce) or scanned with the Odyssey infrared imaging system (LI-COR Bioscience). To ensure equal protein levels, blots were stained with Ponceau and re probed with actin. All analyses were performed at least three times in independent experiments.

Pilocarpine induced seizures

Adult male Sprague Dawley rats (250-350 g) were pre-treated with methyl-scopolamine (1 mg/kg, s.c.; Sigma) to prevent peripheral effects 30 min before giving pilocarpine (350-380 mg/kg) to induce SE. One hour following the onset of SE (Racine scale stage 5 behavior), animals were treated with diazepam (10 mg/kg) and phenytoin (50 mg/kg) to reduce the seizures. Control animals received the same treatments except they received saline instead of pilocarpine. Animals were given Hartmann's solution (130 mM NaCl, 4 mM KCl, 3 mM CaCl₂, and 28 mM lactate; 1 ml/100 g) daily until they were capable of eating and drinking ad libitum and monitored for 3 d.

To assess the effects of lithium citrate on neuronal death *in vivo*, animals were treated with lithium citrate (6 mg/kg, s.c.) 30 min before receiving pilocarpine or saline. To assess whether lithium could provide effective neuro-protection if given after the seizures, a different cohort of animals was given lithium citrate after the seizures were stopped with diazepam and phenytoin. In each experiment, animals were designated as controls, pilocarpine, pilocarpine + lithium, and lithium alone. Rats treated with lithium citrate were given injections every 12 h for 3 d to maintain relatively constant levels of lithium (Malhi and Tanious, 2011). Levels of lithium ion that entered the brain were measured in CSF collected at the time of euthanasia.

Immunohistochemistry

Animals were anesthetized with ketamine/xylazine and perfused transcardially with saline followed by 4% paraformaldehyde. The brains were removed and postfixed in 4% paraformaldehyde for 2 h and cyroprotected in 30% sucrose overnight. Sections (12 μ m) were cut on a cryostat (Leica) and mounted onto charged slides. Sections were blocked in PBS/5% BSA and permeabilized with PBS/0.3% Triton X-100, and then exposed to primary antibodies overnight at 4°C in PBS/1% BSA. Slides were then washed three times in PBS, exposed to secondary antibodies coupled to different fluorophores at room temperature for 1 h in the dark. Sections were washed again three times, with 4',6'-diamidino-2-phenylindole (DAPI; Sigma; 1:10,000) present in the final wash. Sections were coverslipped with antifading medium (ProLong Gold; Invitrogen) and analyzed by fluorescence microscopy (Nikon). Primary antibodies used are as follows: anti-p75 (1:500; R&D Systems, RRID:AB_2298561) and anti-cleaved caspase-3 (CC3; 1:1000; Cell Signaling Technology, RRID:AB_2069869).

Fluoro-Jade C labeling

The number of dying neurons following pilocarpine induced seizures was assessed by labeling with Fluoro-Jade C (Millipore) according to the manufacturer's protocol. Sections were then immunostained with anti-p75^{NTR}.

Coimmunoprecipitation and Western blotting

Cultured hippocampal neurons were treated with lithium citrate for 30 min followed by a 30-min treatment with proNGF and compared with neurons treated with proNGF alone, lithium citrate alone and untreated control neurons. Cells were harvested in a buffer containing 0.6 M octylglucoside, 10% Triton X-100, 10 \times TNE with a phosphatase

inhibitor cocktail tablet (Roche). Whole-cell lysates were precleared with protein G-Sepharose beads (Pierce) at 4°C for 60 min. The cleared lysates were incubated overnight at 4°C with α -p75^{NTR} (192 IgG, Millipore) followed by a 2-h incubation at 4°C with protein G-Sepharose beads. Finally, the beads were washed five times with the buffer described above, eluted by boiling in loading buffer for SDS-PAGE. Equal amounts of protein were separated by 8% PAGE, transferred to nitrocellulose membranes, and probed for sortilin (diluted 1:500, BD Sciences) and p75^{NTR} (diluted 1:500, Cell Signaling). All Western blot analyses were performed at least three times with samples from independent experiments.

For the *in vivo* experiments, hippocampi were dissected 3 d after the seizures and homogenized in RIPA buffer. Lysates were cleared with protein G-Sepharose and incubated overnight with anti-sortilin (BD Science), followed by a 2-h incubation with protein G-Sepharose beads. Samples were analyzed by Western blotting for p75^{NTR} (Millipore).

Biotinylation assays

Cell surface receptor biotinylation assays were performed using cultured hippocampal neurons. Cultures were rinsed with PBS and subsequently washed with PBS containing magnesium chloride and calcium chloride. Cultures were then biotinylated with sulfo-NHS-S-S-biotin (Pierce). Cells were rinsed with 100 nM glycine to quench remaining biotin, and were then incubated at 37°C for 10 min in media alone (control) or in media containing proNGF (3 ng/ml), lithium citrate (100 nM), or proNGF + lithium citrate. Remaining cell surface biotin was cleaved with 50 mM glutathione, 75 mM NaCl, 75 mM NaOH, 0.01 g/ml BSA, and 10 mM EDTA, and cells were lysed in RIPA buffer with protease inhibitors. Biotinylated proteins were precipitated with streptavidin beads to pull down internalized receptors, followed by immunoblotting for p75^{NTR} and sortilin. Each experiment was repeated at least three times.

Quantification and statistical analysis

For quantification of immunostaining analysis, every 8th section throughout the hippocampus was processed for p75^{NTR}/CC3 double immunocytochemistry. Double-labeled cells from the hilus and CA1 regions, areas susceptible to pilocarpine-induced damage, were counted on both sides of the hippocampus. Adjacent sections were taken for analysis of p75^{NTR}/fluor Jade double-labeled cells in the hilus and CA1 regions of the hippocampus. The number of labeled cells is expressed as percentage control relative to the number of labeled cells in control brains. Statistical analysis was performed using ANOVA with Tukey's *post hoc* analysis, and $p < 0.05$ was considered significant.

For quantification of immunoprecipitation and biotinylation analysis, bands were quantified densitometrically and are shown as the mean of three independent experiments. Statistical analysis was performed using ANOVA with Tukey's *post hoc* analysis and $p < 0.05$ was considered significant.

Table 1. List of compounds from the screen of the SpecPlus Collection that blocked proNGF uptake

Compound
Levodopa
2',2'-Bisepigallocatechin monogallate
Meclizine hydrochloride
Suramin
Atorvastatin calcium
Miglitol
Acetyl tyrosine ethyl ester
Prazosin hydrochloride
Lovastatin
Glyburide
Quercetin pentamethyl ether
Perindopril erbumine
Almotriptan
Oxapropin
Lithium citrate

Fifteen compounds were identified from the 2560 screened that that exhibited a 66% reduction in proNGF uptake at 10 μ M concentration and resulted in >85% viability as compared to control.

Results

Screen to identify inhibitors of proNGF binding and uptake to p75^{NTR}-sortilin expressing cells

We performed a drug screen to identify compounds that blocked the binding and internalization of fluorescently-labeled (Alexa Fluor) proNGF using cells stably expressing p75^{NTR} and sortilin. Stable clones of human fibrosarcoma HT-1080 cells expressing p75^{NTR}, sortilin, or both receptors were generated, and the receptor expression was confirmed by Western blot analysis. Our prior studies using these conditions documented that coexpression of p75^{NTR} and sortilin led to enhanced uptake of labeled proNGF, as compared to cells expressing comparable levels of p75^{NTR} or sortilin alone (Feng et al., 2010). Binding and uptake of Alexa Fluor-proNGF was inhibited by >90% on concomitant treatment with 10 μ M neurotensin, consistent with prior studies in which neurotensin impaired the crosslinking of proNGF to p75/sortilin complexes, and impaired proNGF-induced apoptosis (Nykjaer et al., 2004). Compounds from a commercially available library were screened for reduction in proNGF-uptake. Drugs (at 10 μ M concentration) that exhibited a 66% reduction in uptake, and resulted in >85% viability as compared to control, were considered potential antagonists. Fifteen compounds which met these criteria, and have been used in humans are listed in Table 1. Lithium citrate was among the 15 candidate compounds identified, inhibiting proNGF uptake by 66.4% as compared to diluent control, and exhibited no significant toxicity (<5% cell loss as compared to control). Therefore, lithium citrate was further evaluated using cultured neurons.

Low dose of lithium protects hippocampal neurons from proNGF-induced death in vitro

To analyze mechanisms by which lithium citrate could protect hippocampal neurons from proNGF-induced death, cultured neurons were treated with proNGF with or without lithium citrate pretreatment. A dose-response analysis demonstrated that lithium citrate protected neurons from proNGF-induced death, with the lowest protec-

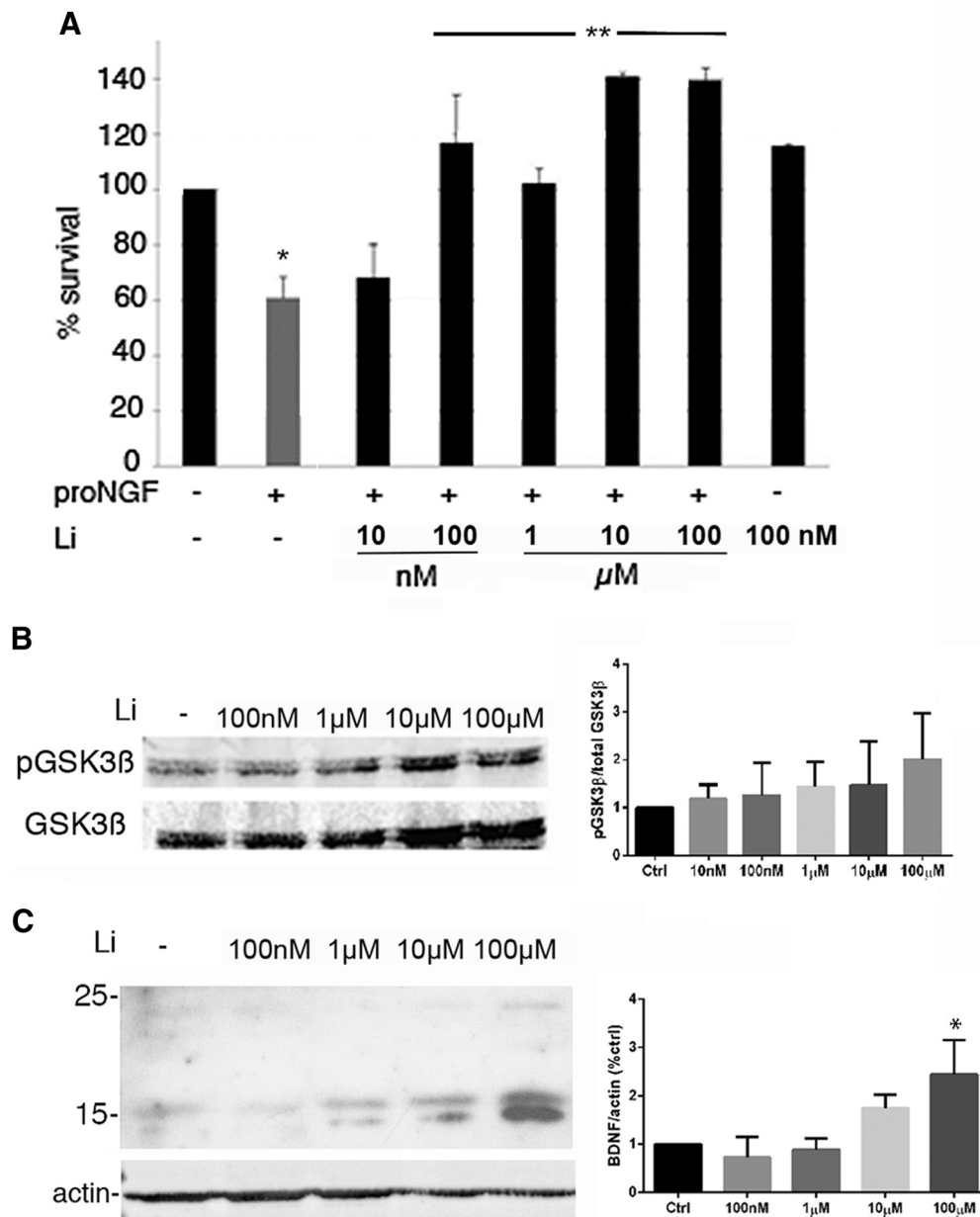
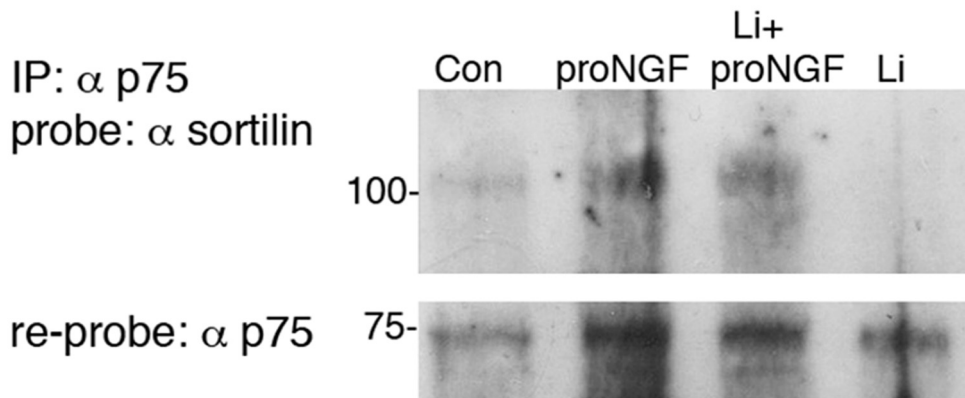


Figure 1. Dose-dependent effects of lithium citrate on hippocampal neurons. **A**, E18 hippocampal neurons were cultured for 5 d and treated overnight with or without proNGF (3 ng/ml) alone or in the presence of different doses of lithium citrate. Cells were then lysed and healthy nuclei of surviving neurons were counted; 100 nM lithium citrate was sufficient to protect neurons from proNGF. **B**, Dose response for lithium citrate to induce phosphorylation of GSK3β *in vitro*. Cultured hippocampal neurons were treated with the indicated doses of lithium citrate and analyzed by Western blotting for p-GSK3β. Blots were stripped and reprobed for total GSK3β. Quantification of four independent experiments is shown. **C**, Dose response for lithium citrate to induce BDNF expression. Cultured hippocampal neurons were treated with the indicated doses of lithium citrate and analyzed by Western blotting for BDNF. Blots were stripped and reprobed for actin. Quantification of three independent experiments is shown, * significant at $p < 0.05$ by ANOVA.

tive dose of 100 nM (Fig. 1A). Lithium ion is known to be neuroprotective by a variety of mechanisms (Wada et al., 2005; Young, 2009), including via phosphorylation and inactivation of GSK3β, therefore we investigated whether the doses at which lithium citrate prevented proNGF-induced death were sufficient to phosphorylate GSK3β. Interestingly, the lowest protective doses of 100 nM and 1 μM were insufficient to phosphorylate GSK3β (Fig. 1B). In addition, a different method of inactivating GSK3β using

BIO (Meijer et al., 2003), rather than lithium citrate was unable to protect hippocampal neurons from proNGF-induced apoptosis (not shown). These data indicate that the neuroprotective actions of lithium citrate were not mediated by GSK3β phosphorylation and inactivation. Additionally, chronic treatment with lithium has also been shown to upregulate BDNF expression in the brain (Fukushima et al., 2001) and retina (Wu et al., 2014), which provides another potential neuroprotective mechanism for

A



B

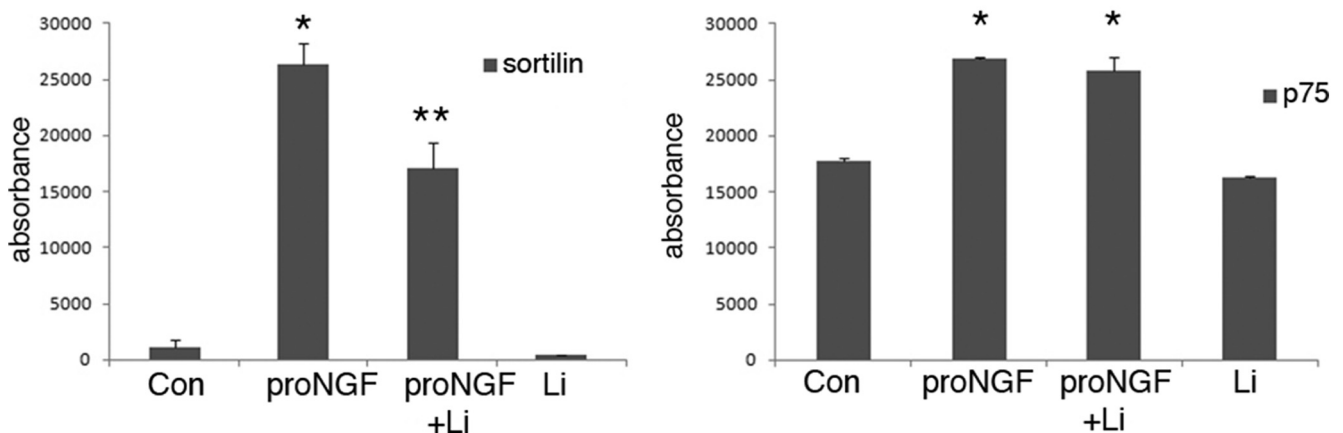


Figure 2. Lithium citrate decreases the association between p75^{NTR} and sortilin. E18 hippocampal neurons were cultured for 5 d and treated with vehicle or proNGF (3 ng/ml) for 30 min, with or without a 30-min pretreatment with 100 nM lithium citrate. Cells were lysed, immunoprecipitated with anti-p75^{NTR}, and probed with anti-sortilin. Blots were reprobed with anti-p75^{NTR}. **A**, Representative blot showing co-IP of p75^{NTR} and sortilin and reprob for p75^{NTR}. **B**, Densitometric quantification of sortilin and p75^{NTR} bands from three independent experiments; *different from control $p < 0.05$, **different from proNGF $p < 0.05$.

lithium. Therefore, we compared the dose of lithium citrate that was required for BDNF induction with the dose that protected hippocampal neurons from proNGF-induced apoptosis. Robust induction of BDNF was observed at 100 μ M, a dose at least 1000-fold higher than the dose required for protection from proNGF (Fig. 1C). These data indicate that the protection afforded by lithium citrate was due neither to phosphorylation of GSK3 β nor to induction of BDNF, and might represent a novel mechanism.

Lithium citrate prevents the association and internalization of the p75^{NTR}-sortilin receptor complex

The initial identification of lithium citrate as being protective from proNGF-induced apoptosis was based on a screen that blocked binding to the p75^{NTR}-sortilin receptor complex and subsequent internalization, therefore we assessed whether lithium citrate could act at the level of the membrane receptors to prevent proNGF actions by interfering with the formation of the p75^{NTR}-sortilin receptor complex, or internalization of the receptors. Cultured

hippocampal neurons were treated for 30 min with proNGF with or without a 30-min pretreatment with 100 nM lithium citrate and compared to untreated neurons or treatment with lithium citrate alone. Cell lysates were immunoprecipitated with anti-p75^{NTR}, probed for sortilin and reprob for p75^{NTR}. Levels of p75^{NTR} were increased by proNGF treatment, which was not affected by the lithium citrate pretreatment, however the amount of co-immunoprecipitated sortilin was significantly reduced by the lithium citrate pretreatment (Fig. 2), suggesting that lithium citrate attenuated the association between sortilin and p75^{NTR}.

Treatment with proNGF elicits internalization of the p75^{NTR}-sortilin receptor complex, therefore we also investigated whether lithium citrate treatment could affect receptor internalization. Cell surface biotinylation experiments investigated the internalization of the receptors after proNGF treatment. Cultured hippocampal neurons were biotinylated and then incubated with proNGF for 10 min with or without pretreatment with lithium citrate. Surface biotin was then stripped off and streptavidin was used to pull down internal-

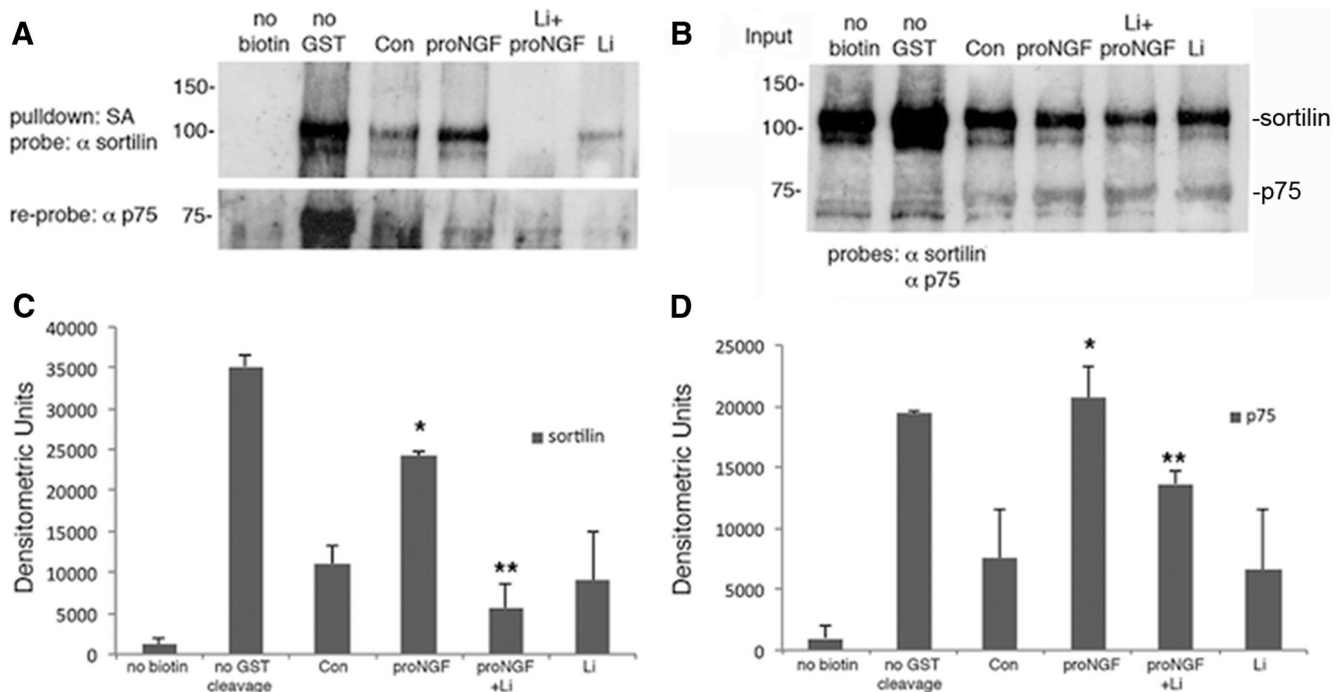


Figure 3. Lithium citrate decreases the internalization of p75^{NTR} and sortilin. **A**, E18 hippocampal neurons were cultured for 5 d, biotinylated, and treated for 10 min with proNGF. After stripping remaining surface biotin with glutathione, streptavidin was used to pull down the internalized, biotinylated proteins, which were analyzed by Western blotting for sortilin and p75^{NTR}. **B**, Lysates before streptavidin pull-down were also analyzed by Western blotting for sortilin and p75^{NTR} to assess the input. Densitometric quantification of biotinylated sortilin (**C**) and p75^{NTR} (**D**) from three independent experiments; *different from control at $p < 0.05$, **different from proNGF at $p < 0.05$.

ized biotinylated proteins, which were probed for p75^{NTR} (Fig. 3A,D) and sortilin (Fig. 3A,C). Pretreatment with 100 nM lithium citrate significantly reduced the internalization of sortilin and p75^{NTR} (Fig. 3). Thus, the low dose of lithium citrate (100 nM) that was protective from proNGF-induced neuronal death attenuated the association and internalization of the p75^{NTR}-sortilin receptor complex.

Lithium citrate protects hippocampal neurons from pilocarpine-induced neuronal loss

Pilocarpine-induced seizures elicit neuronal apoptosis in the CA1 and hilus regions of the rat hippocampus at least in part by increasing the level of extracellular proNGF and activating p75^{NTR} apoptotic signaling (Troy et al., 2002; Volosin et al., 2008; Le and Friedman, 2012). Since lithium citrate was able to prevent proNGF binding to the p75^{NTR}-sortilin receptor complex, we evaluated whether treatment with a low dose of lithium was able to attenuate neuronal loss in the hippocampus induced by seizures. Adult male rats were pretreated for 30 min with 6 mg/kg lithium citrate given by intraperitoneal injection, and then given pilocarpine to induce seizures. As previously shown, by 3 d after seizure pilocarpine elicited extensive neuronal death in the hilus and CA1 regions of the hippocampus. Pretreatment with lithium citrate followed by twice daily intraperitoneal injections showed significant neuroprotection in both the hilus (Fig. 4) and CA1 (Fig. 5), evaluated by counting cells double-labeled for p75^{NTR} and cleaved caspase-3 (CC3) (Figs. 4A,B, 5A,B) or double-labeled for p75^{NTR} and fluorojade C (Figs. 4C, 5C). The dose of lithium was at least 5-fold lower than doses

used in other paradigms of neuroprotection that involved BDNF upregulation (Lauterbach, 2013; Wu et al., 2014) or inhibition of GSK3 β (Diniz et al., 2013). We confirmed that this dose of lithium citrate reduced the interaction of p75^{NTR} and sortilin and did not elicit phosphorylation of GSK3 β *in vivo* (Fig. 6). The dose was also lower than used in the lithium-pilocarpine model of SE, which uses 127 mg/kg lithium (Hillert et al., 2014) compared with 6 mg/kg used here.

Given the many potential actions of lithium ion, it was important to use the lowest effective dose, and to determine the amount of lithium that gains access to the brain. CSF was collected from the cisterna magna of each animal before being euthanized, and assayed for the level of lithium. Since only 50–70 μ l of CSF can be obtained from each animal, samples were pooled from five rats in each of the four treatment categories for analysis of lithium content. Animals with no injected lithium citrate had minimal levels of lithium detected in the CSF (0.84 ng/ml for controls without pilocarpine, and 1.55 ng/ml for animals with pilocarpine). Rats with no pilocarpine, but with 6 mg/kg lithium citrate injected intraperitoneally had 49.34 ng/ml lithium in the CSF, and rats with pilocarpine-induced seizures and injections of 6 mg/kg lithium had 81.1 ng/ml lithium in the CSF, corresponding to 13 μ M as an effective neuroprotective dose. This analysis showed that the lithium injected peripherally was able to access the brain, and that this low dose was sufficient to afford neuroprotection.

To determine whether lithium could also afford neuroprotection if provided after the seizures, adult male rats

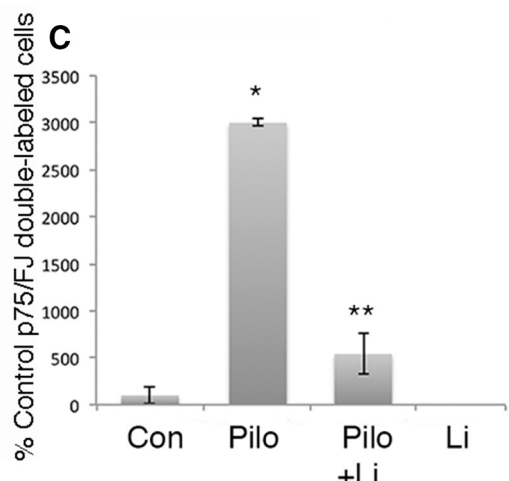
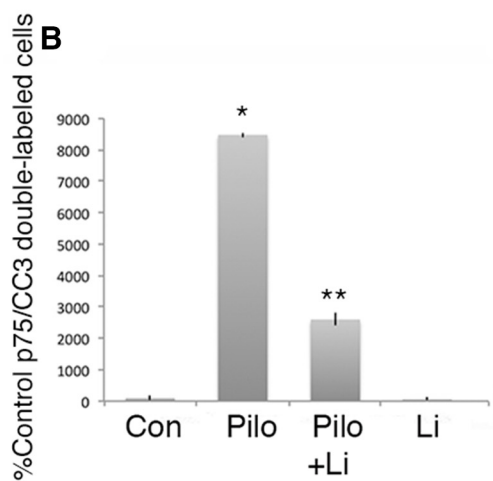
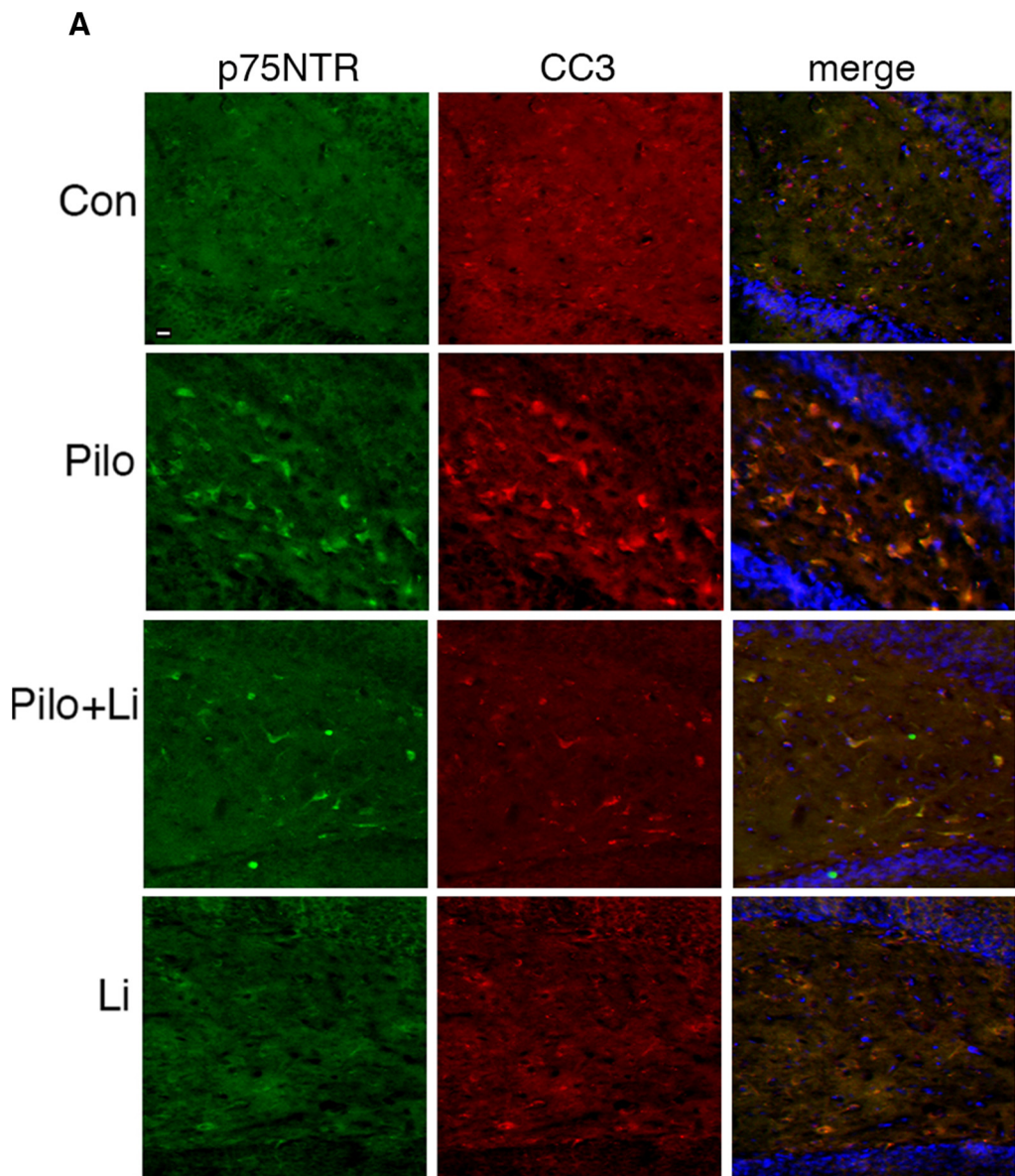


Figure 4. Lithium citrate pretreatment prevents neuronal death in the hilus following seizures *in vivo*. Adult male rats were subjected to pilocarpine-induced seizures with or without a 30-min pretreatment with lithium citrate (6 mg/kg) and injections twice daily for 3 d. **A**, Sections through the hilus showing double labeling with anti-p75^{NTR} and anti-CC3. **B**, Quantification of p75^{NTR}/CC3 double-labeled

continued

cells in the hilus with the different treatments. Cells were counted in every 8th section through the hippocampus. **C**, Quantification of p75^{NTR}/fluorojade-labeled cells in the hilus with the indicated treatments. Scale bars: 10 μ m. Quantification in **B**, **C** is expressed as percentage control values which are set at 100%. * different from control at $p < 0.05$, ** different from Pilo at $p < 0.05$.

were treated with lithium citrate delivered intraperitoneally after 1 h of SE, at the time the seizures were terminated with diazepam and phenytoin, followed by twice daily intraperitoneal injections. Even when initially provided after the termination of the seizures, treatment with lithium citrate dramatically reduced the number of apoptotic neurons in both the hilus (Fig. 7) and CA1 (Fig. 8).

Discussion

Low dose of lithium citrate is neuroprotective in vivo

Lithium ion has been used as an effective therapy in many models of disease, particularly as a mood-stabilizing drug, as well as to treat brain and spinal cord injury (Young, 2009), where it can protect neurons from

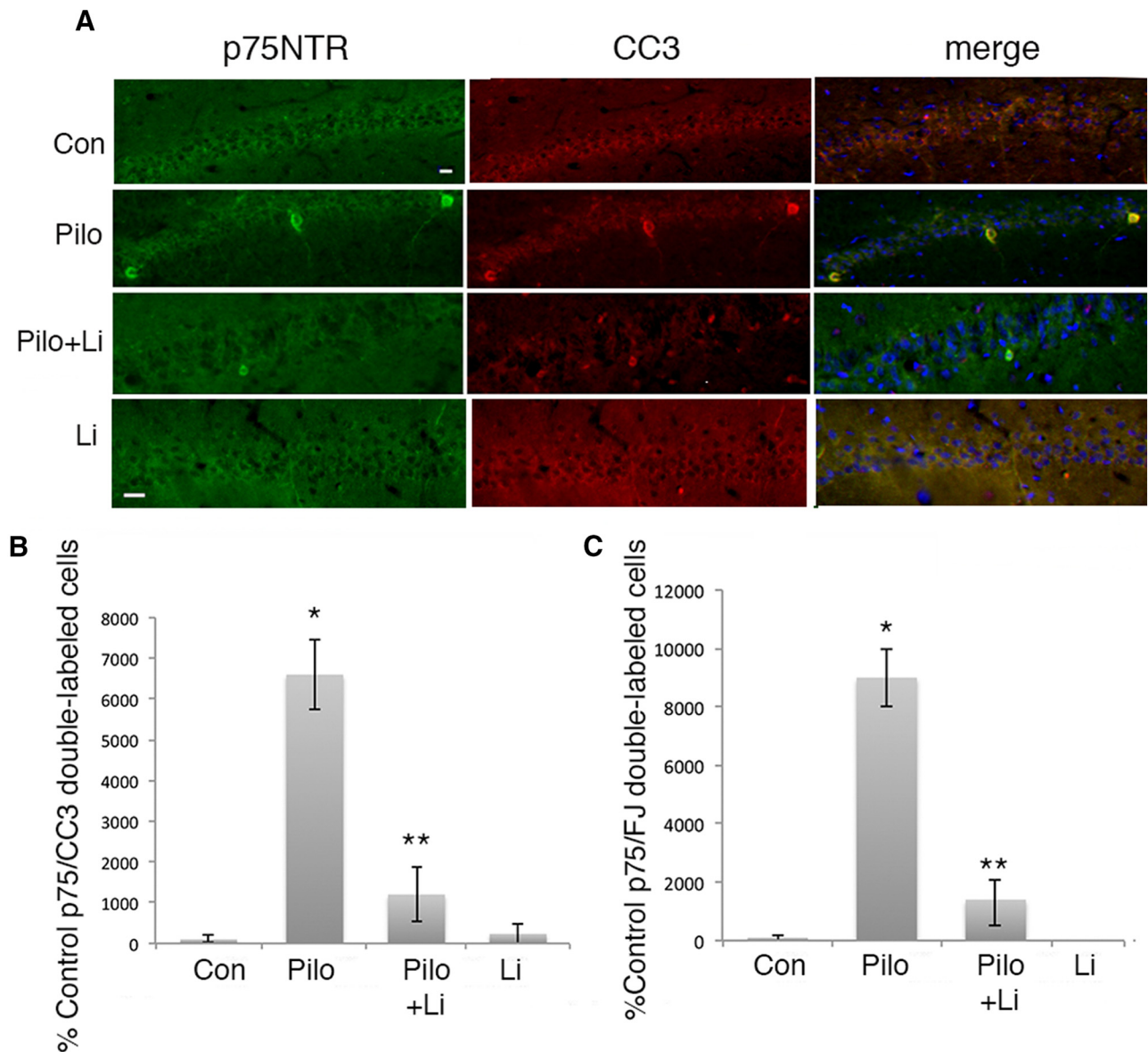


Figure 5. Lithium citrate pretreatment prevents neuronal death in the CA1 region following seizures *in vivo*. Adult male rats were subjected to pilocarpine-induced seizures with or without a 30-min pretreatment with lithium citrate (6 mg/kg) and injections twice daily for 3 d. **A**, Sections through CA1 showing double labeling with anti-p75^{NTR} and anti-CC3. **B**, Quantification of p75^{NTR}/CC3 double-labeled cells in the CA1 with the different treatments. Cells were counted in every 8th section through the hippocampus. **C**, Quantification of p75^{NTR}/fluorojade-labeled cells in CA1 with the indicated treatments. Scale bars: 10 μ m. Quantification in **B**, **C** is expressed as percentage control values which are set at 100%. * different from control at $p < 0.05$, ** different from Pilo at $p < 0.05$.

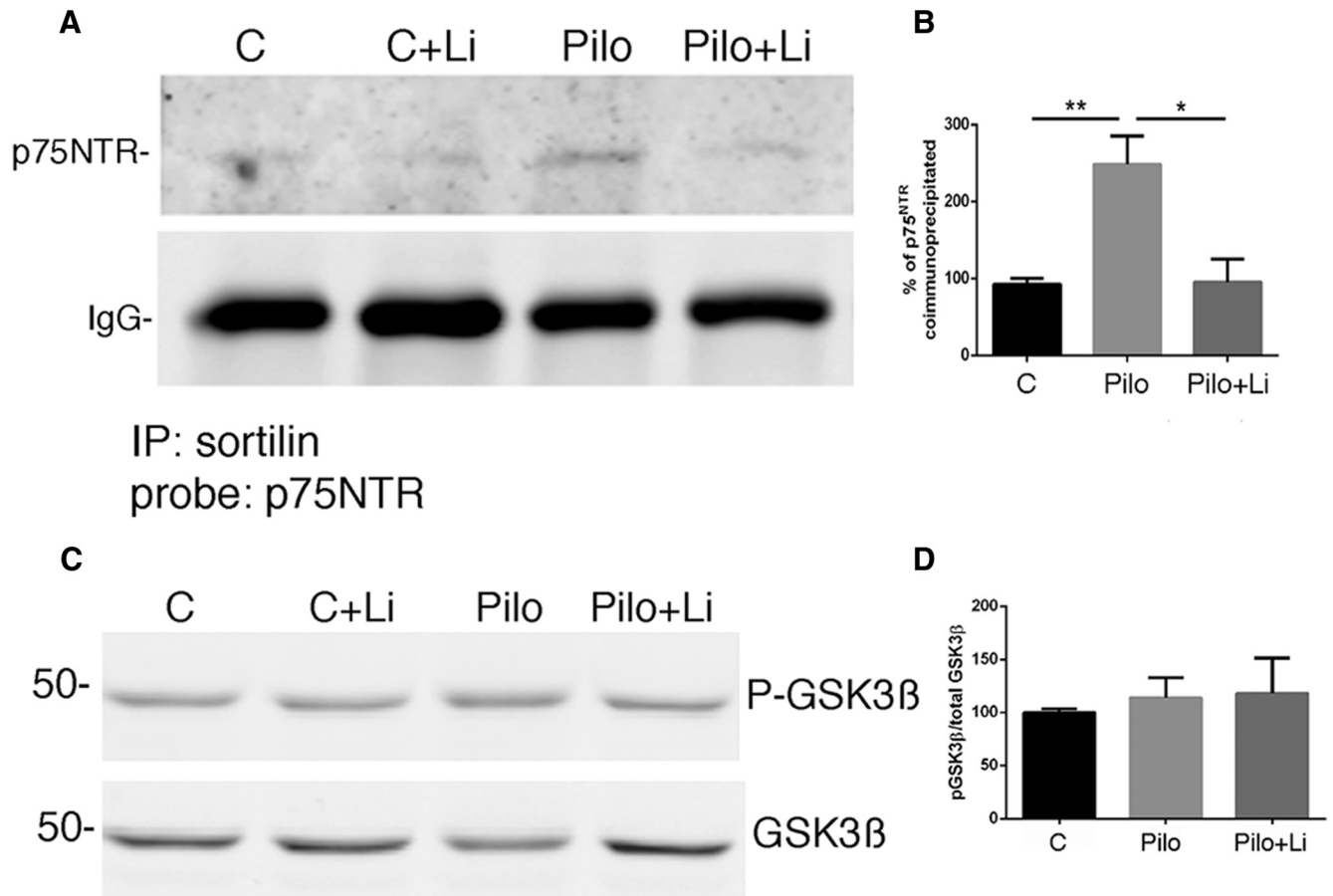


Figure 6. Treatment with lithium citrate reduces p75^{NTR}/sortilin interaction *in vivo* after pilocarpine-induced seizures. **A**, Hippocampal lysates were immunoprecipitated with anti-sortilin and probed for p75^{NTR}. **B**, Quantification of two independent cohorts expressed as percentage control value which is set at 100%; **different from control at $p < 0.05$, *different from pilo at $p < 0.05$. **C**, Hippocampal lysates were probed for p-GSK3 β and total GSK3 β . **D**, Quantification of p-GSK3 β relative to total GSK3 β shows no effect of lithium citrate treatment *in vivo*.

death and promote axon sprouting (Dill et al., 2008). Doses of lithium used in these studies range from the micromolar to millimolar range. At these doses, lithium ion has been shown to have many biological activities that can be neuroprotective (Wada et al., 2005; Young, 2009). Among the most well-established functions of lithium ion is the phosphorylation and inhibition of GSK3 β and as well as upregulating levels of BDNF and stimulating anti-apoptotic signaling (Rowe and Chuang, 2004; Wada et al., 2005; Young, 2009; Pasquali et al., 2010). Additionally, lithium ion can promote autophagy, which can be neuroprotective, although this was observed at millimolar doses *in vitro* (Motoi et al., 2014; Del Grosso et al., 2016; Liu et al., 2017), and at 50 mg/kg *in vivo* (Liu et al., 2017), doses much higher than those used here. Lithium ion has also been shown to delay disease progression of amyotrophic lateral sclerosis (ALS) in humans and in the mouse G93A model of the disease (Fornai et al., 2008). Since lithium ion can activate so many different neuroprotective signaling pathways, it has many potential therapeutic properties. The effective clinical dose for lithium ion is generally in the range of 0.6–1.0 mM (Young, 2009; Liu et al., 2017). In the current study, we demonstrate a

neuroprotective effect of lithium citrate from seizure-induced neuronal loss at a dose several orders of magnitude lower than the established clinical range.

In addition to its neuroprotective function, lithium is often used together with pilocarpine to generate a model of epileptic seizures, however the dose of lithium used in that model is a significantly higher [3 mEq (Jope et al., 1986) or 127 mg/kg (Hillert et al., 2014)] than the protective dose used here (6 mg/kg).

In injury models of TLE, severe continuous seizures defined as SE lead to increased expression and stabilization of proNGF, which binds with selective high affinity to the p75^{NTR}-sortilin receptor complex and elicits apoptosis (Friedman, 2010). p75^{NTR} is widely expressed in the CNS during development, but in the adult hippocampus, this receptor is expressed primarily following injury. Previous studies have demonstrated that proNGF and p75^{NTR} play a major role in mediating neuronal death after SE (Troy et al., 1997; Volosin et al., 2008; Le and Friedman, 2012), we anticipated that disrupting this association may prevent neuronal death after SE. In these studies, pilocarpine was used to induce seizures. Animals treated with pilocarpine showed increased expression of p75^{NTR} colocal-

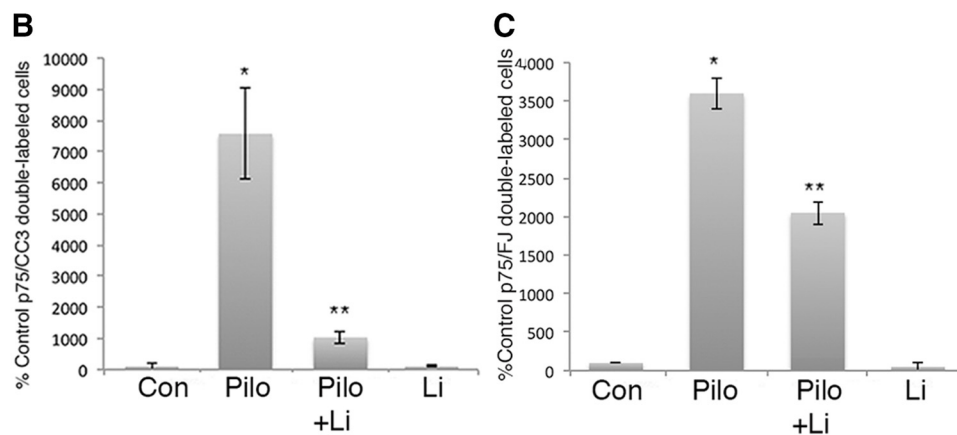
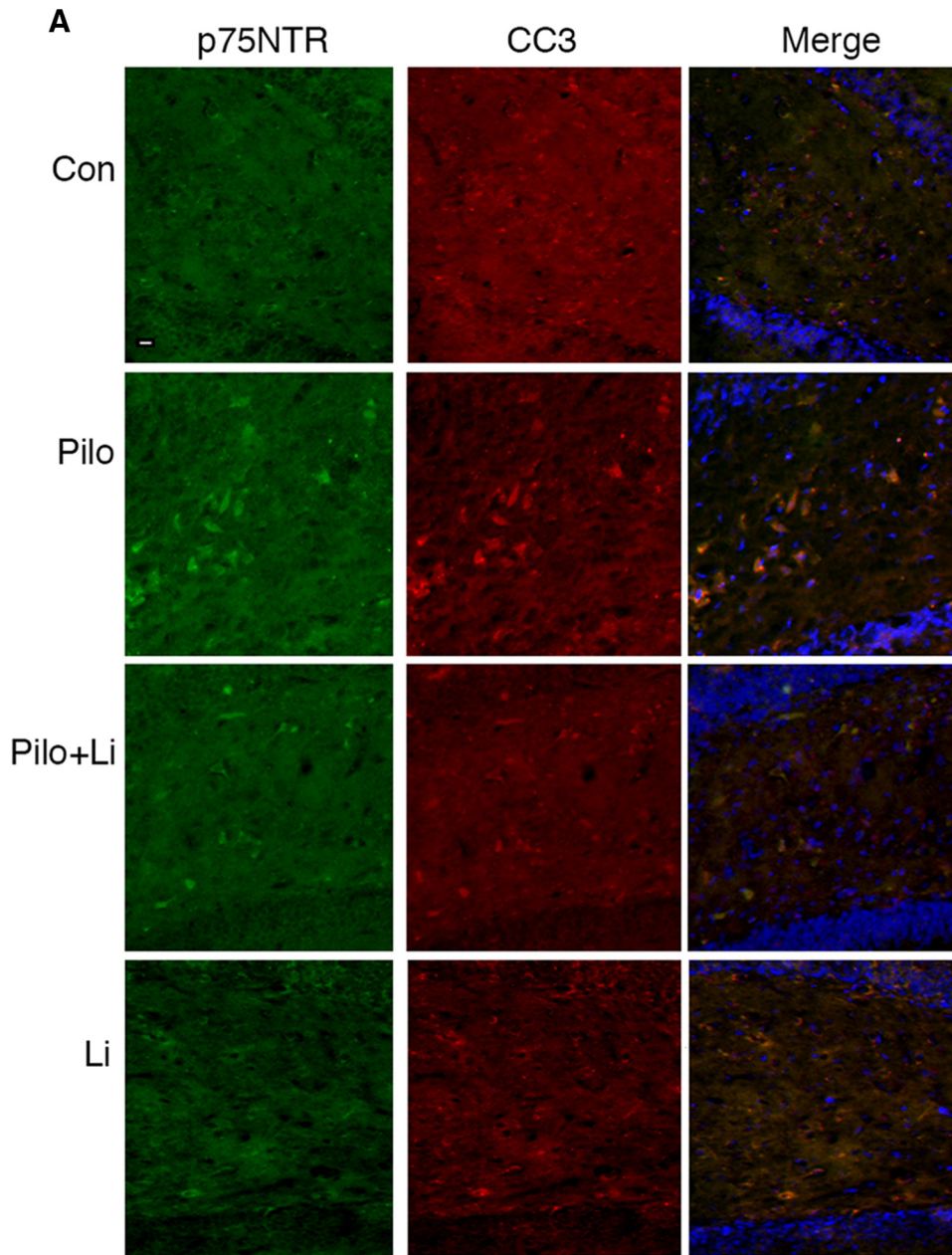


Figure 7. Treatment with lithium citrate after seizures prevents neuronal death in the hilus. Adult male rats were subjected to pilocarpine-induced seizures. Treatment with lithium citrate (6 mg/kg) was initiated after the seizure was terminated and injected twice

continued

daily for 3 d. **A**, Sections through the hilus showing double labeling with anti-p75^{NTR} and anti-CC3. **B**, Quantification of p75^{NTR}/CC3 double-labeled cells in the hilus with the different treatments. Cells were counted in every 8th section through the hippocampus. **C**, Quantification of p75^{NTR}/fluorograde-labeled cells in the hilus with the indicated treatments. Scale bars: 10 μm. Quantification in **B**, **C** is expressed as percentage control values which are set at 100%. * different from control at $p < 0.05$, ** different from Pilo at $p < 0.05$.

ized with CC3, the main executor protein in the apoptotic pathway, which was maximal by 3 d after the seizures in the CA1 and dentate hilus regions, consistent with previous studies (Roux et al., 1999; Troy et al., 2002). Fluorograde C was additionally used to identify dying neurons, and also demonstrated increased double-labeling with p75^{NTR} after pilocarpine treatment. Pretreatment and twice daily injections with the low dose of lithium (6 mg/kg) resulted in decreased p75^{NTR} expression and reduced neuronal death compared to pilocarpine alone, evaluated by CC3 and fluorograde C labeling, in both the CA1 and hilus. Moreover, this treatment elicited a reduction in the association of the p75^{NTR}-sortilin receptor complex *in*

vivo. The amount of lithium measured in the CSF corresponded to 13 μM. As is common for drugs administered *in vivo*, this amount of lithium is higher than the lowest protective dose shown *in vitro*, but is well below the usual clinical dose (Young, 2009). These results demonstrated that a low dose of lithium can afford neuroprotection from seizure-induced neuronal death. The identification of lithium ion as an inhibitor of proNGF binding to its p75^{NTR}-sortilin receptor complex suggested that this may represent a novel mechanism for neuroprotection.

Since there is a time lag after seizures for the induction of p75^{NTR} and proNGF to occur before the time of maximal neuronal loss, we evaluated whether lithium citrate

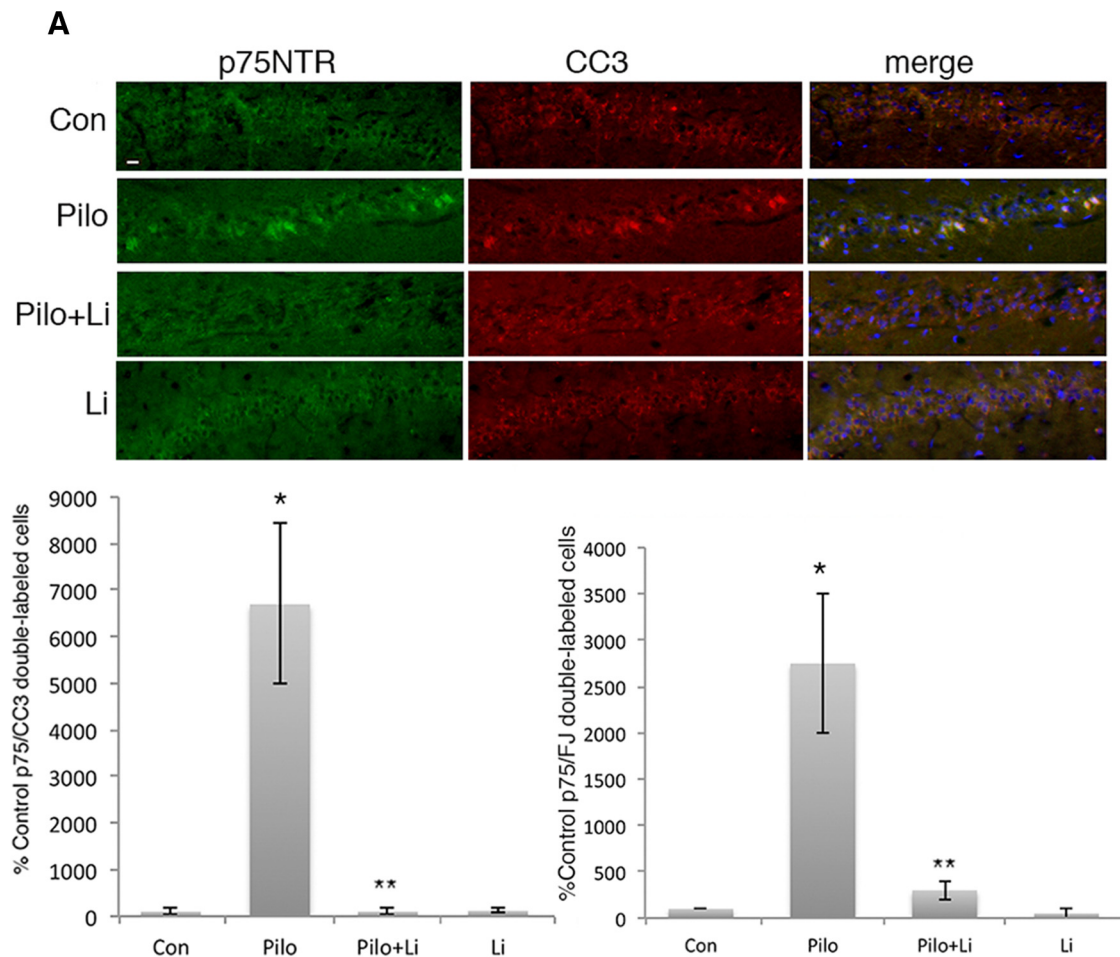


Figure 8. Treatment with lithium citrate after seizures prevents neuronal death in the CA1 region. Adult male rats were subjected to pilocarpine-induced seizures. Treatment with lithium citrate (6 mg/kg) was initiated after the seizure was terminated and injected twice daily for 3 d. **A**, Sections through CA1 showing double labeling with anti-p75^{NTR} and anti-CC3. **B**, Quantification of p75^{NTR}/CC3 double-labeled cells in the CA1 with the different treatments. Cells were counted in every 8th section through the hippocampus. **C**, Quantification of p75^{NTR}/fluorograde-labeled cells in CA1 with the indicated treatments. Scale bars: 10 μm. Quantification in **B**, **C** is expressed as percentage control values which are set at 100%. * different from control at $p < 0.05$, ** different from Pilo at $p < 0.05$.

would be neuroprotective if provided after SE rather than before the seizures. Rats were therefore given the first dose of lithium when the seizures were terminated with diazepam. In these experiments lithium citrate still afforded significant neuroprotection and reduced cell death in the CA1 and hilus, indicating that lithium citrate can be given after seizures and still prevent neuronal loss. The extent of the time window for neuroprotection after seizures remains to be determined, however being able to provide a neuroprotective agent after the seizure event may be of potential therapeutic value.

We show that lithium citrate has neuroprotective effects *in vivo* following seizures using doses well below the clinical range used to treat other disorders. The pattern of damage seen in animals treated with both pilocarpine and lithium citrate illustrates that the drug disrupts the well-described pattern of damage following SE. Measurement of lithium levels in the CSF demonstrated that peripherally-injected lithium citrate at this low dose (6 mg/kg) gained access to the brain, and provided a significant protective effect on hippocampal neurons following SE *in vivo*.

Mechanisms of neuroprotection by lithium

Cultured hippocampal neurons were used to investigate mechanisms by which lithium ion could afford neuroprotection, since Li^+ is known to prevent neuronal death through multiple pathways (Pasquali et al., 2010). Lithium ion can induce phosphorylation and inactivation of GSK3 β , a Ser/Thr kinase that is abundant in CNS neurons and promotes cell death by blocking the nuclear translocation of beta catenin. Lithium ion can also induce an increase in BDNF levels (Fukumoto et al., 2001; Wu et al., 2014), which can promote neuronal survival by activating the TrkB receptor and downstream Akt and Erk signaling. To assess whether lithium citrate may protect neurons by phosphorylation and inactivation of GSK3 β or by upregulating BDNF expression, we compared the doses of lithium citrate required for protection from proNGF-induced apoptosis with those required for activation of GSK3 β in cultured neurons and *in vivo*. We found that the doses of lithium that protected neurons from proNGF-induced death *in vitro*, or SE-induced death *in vivo*, were insufficient to elicit phosphorylation of GSK3 β or upregulation of BDNF, suggesting that neither phosphorylation of GSK3 β nor induction of BDNF was responsible for preventing neuronal death in these paradigms.

Lithium citrate prevents association and internalization of the p75^{NTR}-sortilin receptor complex

The original identification of lithium as a potential inhibitor of proNGF-induced neuronal death was based on its ability to prevent binding to the receptor complex, suggesting that the mechanism of protection might be at the level of the membrane receptors. We investigated whether lithium at the neuroprotective nanomolar concentration was effective in blocking either the association of p75^{NTR} with sortilin, or the internalization of the two receptors following proNGF treatment in culture. Coimmunoprecipitation analysis demonstrated that lithium citrate decreased the associ-

ation of p75^{NTR} and sortilin both *in vitro* and *in vivo*, thereby reducing formation of the requisite receptor complex for proNGF to induce apoptosis. Using surface biotinylation assays to track the internalization of membrane receptors, we also found that lithium reduced the internalization of p75^{NTR} and sortilin into the neurons. These data suggest that the neuroprotective effect of lithium citrate at these low doses may be through disrupting the association and internalization of p75^{NTR} and sortilin and thereby preventing proNGF apoptotic signaling. Although lithium ion can exert neuroprotection through multiple mechanisms, some of the well-established pathways of lithium actions were not activated by this treatment paradigm. Other receptors and pathways are likely to be affected by the lithium citrate treatment as well, however preventing the activation of the p75^{NTR}-sortilin receptor complex may contribute to the neuroprotective effects.

A previous study demonstrated that the binding of proNGF to the p75^{NTR}-sortilin receptor complex is stabilized in the presence of calcium (Feng et al., 2010). It is well established that the prodomain of proNGF is intrinsically disordered, and thus may adopt distinct, transient conformational changes in response to environmental perturbations. Indeed, addition of calcium at physiologic levels enhanced the interaction of the prodomain region with sortilin, and stabilized the formation of a ~600 kDa proNGF/sortilin/p75 complex. In contrast, calcium chelation significantly reduced the interaction of proNGF with its receptors. One possible mechanism for the actions of lithium ion may be to alter the conformation of the prodomain region of proNGF, possibly leading to the attenuation of the proNGF-induced p75^{NTR}-sortilin association and/or internalization, and suggesting a novel mechanism for neuroprotection by lithium ion.

In summary, we demonstrated in this study that low doses of lithium citrate were able to prevent proNGF-induced apoptosis of cultured hippocampal neurons, and provided significant neuroprotection from seizure-induced neuronal loss, even when provided after termination of the seizures. These data suggest a novel therapeutic use for lithium ion to provide neuroprotection from seizures at nanomolar doses, well below the dose currently in clinical use, thereby minimizing potential side effects from the multiple actions of lithium ion that occur at higher micromolar doses.

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