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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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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*

Abbreviated title: Lithium blocks p75-sortilin interactions

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The authors report no conflict of interest.

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 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, sub-micromolar doses of lithium citrate effectively inhibited proNGF-induced cell death in cultured neurons, and protected hippocampal neurons following pilocarpine-induced status epilepticus *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*.

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62 **Significance Statement**

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

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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 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 in by a variety of different mechanisms (Wada et al., 2005), including increasing Akt activity, by phosphorylating and inactivating GSK-3β (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, 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; Turski et al., 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 GSK-3ß 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*.

Methods

Alexa 594 labeling of proNGF

Twenty micrograms of purified human proNGF, prepared in SF9 cells as described (Feng et al, 2010) was added to 10 µl of phosphate-buffered saline (PBS) and 7 µl of reconstituted AlexaFluor 594 (excitation 590 nM, emission 617 nM) (Molecular Probes) 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-labeled proNGF was utilized 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 hours. Cells were treated with one of 2560 compounds from the SpecPlus Collection (Microsource Discovery Sytems, Groton, CT) at a concentration of 10 micromolar, or 20 micromolar neurotensin, or diluent control, utilizing an automated robotics system, followed by addition of 200nM Alexa-proNGF. Cells were incubated for 18 hours, 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 µg/ml for 2h at room temperature. Cells were analyzed for uptake of Alexa 594-conjugated proNGF using a Discovery – 1 automatic fluorescence microscope from Molecular Devices as described (Pipalia et al, 2006).

Images were acquired with a Photometrics Cool Snap HQ camera and analyzed using Metapmorph 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 Hoecht 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).

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, Groton, CT) were assayed in cells that expressed $p75^{NTR}$ and sortilin. The compounds in the collection are primarily Food and Drug Administration (FDA)-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, 2000). Dissociated neurons were then plated on poly-D-lysine (0.1mg/ml)-coated dishes maintained in serum free media. The media consisted of 1:1 MEM and F12, with glucose (6mg/ml), insulin (2.5mg/ml), putrescine (60 µM), progesterone (20nM), transferrin (100µg/ml), selenium (30nM), penicillin (0.5U/ml) and streptomycin (0.5µg/ml). Neuronal cultures were maintained in media for 5 days 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 pre-screen testing and was used here at doses ranging from 10 nM to 100 μM. 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, 2000). 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.

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

For Western blot analysis, hippocampal neurons plated in 6 well dishes ($1x10^6$ cells/well) were treated as indicated, washed in sterile PBS (pH 7.4) and lysed in buffer containing 120mM 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 GSK-3β, p-GSK-3β, BDNF and actin overnight. After washing 3 times with TBST for 15 minutes each, the blots were incubated with appropriate secondary antibodies for 1 hour at room temperature. The membrane was washed 3 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 reprobed with actin. All analyses were performed at least three times in independent experiments.

Pilocarpine induced seizures

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

To assess the effects of lithium citrate on neuronal death *in vivo*, animals were treated with lithium citrate (6mg/kg, s.c.) 30 minutes prior to receiving pilocarpine or saline. To assess whether lithium could provide effective neuroprotection 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 hours for 3 days to maintain relatively constant levels of lithium (Malhi and Tanious, 2011). Levels of lithium ion that entered the brain were measured in cerebrospinal fluid (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 two hours and cyroprotected in 30% sucrose overnight. Sections (12µm) 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 one hour in the dark. Sections were washed again three times, with DAPI (4',6' - diamidino-2-phenylindole) (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 (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-$p75^{NTR}$.

Co-Immunoprecipitation and Western Blotting

Cultured hippocampal neurons were treated with lithium citrate for 30 minutes followed by a 30 minute 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 0.6M octylglucoside, 10% Triton X-100, 10X TNE with a phosphatase inhibitor cocktail tablet (Roche). Whole cell lysates were precleared with protein G Sepharose beads (Pierce) at 4°C for 60 minutes. The cleared lysates were incubated overnight at 4°C with α-$p75^{NTR}$ (192 IgG, Millipore) followed by a 2 hour 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 days 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 2 hr incubation with Protein G-Sepharose beads. Samples were analyzed by Western blot 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 100nM glycine to quench remaining biotin, and were then incubated at 37°C for 10 minutes 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, 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 $p75^{NTR}$/cleaved caspase-3 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 $p75^{NTR}$/fluorojade double-labeled cells in the hilus and CA1 regions of the

hippocampus. The number of labeled cells is expressed as percent control relative to the number of labeled cells in control brains. Statistical analysis was performed using ANOVA with Tukey's posthoc 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 posthoc analysis and $p<0.05$ was considered significant.

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) 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 co-expression 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-proNGF was inhibited by >90% upon concomitant treatment with 10 μM neurotension, 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 micromolar concentration) that exhibited a 66% reduction in uptake, and resulted in greater

than 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 (less than 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 protective dose of 100 nM (Figure 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 GSK3ß, therefore we investigated whether the doses at which lithium citrate prevented proNGF-induced death were sufficient to phosphorylate GSK3ß. Interestingly, the lowest protective doses of 100 nM and 1 µM were insufficient to phosphorylate GSK3ß (Figure 1B). In addition, a different method of inactivating GSK3ß using 6-bromoindirubin-3'-oxime (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 GSK3ß phosphorylation and inactivation. Additionally, 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. 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 μM, a dose at least 1000-fold higher than the dose required for protection from proNGF (Figure 1C). These data indicate that the protection afforded by lithium citrate was due neither to phosphorylation of GSK-3ß 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 re-probed 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 (figure 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 internalized biotinylated proteins, which were probed for $p75^{NTR}$ (figure 3A, D) and sortilin (figure 3A, C). Pretreatment with 100 nM lithium citrate significantly reduced the internalization of sortilin and $p75^{NTR}$ (figure 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 days after seizure pilocarpine elicited extensive neuronal death in the hilus and CA1 regions of the hippocampus. Pretreatment with lithium citrate followed by twice daily i.p. injections showed significant

neuroprotection in both the hilus (figure 4) and CA1 (figure 5), evaluated by counting cells double-labeled for $p75^{NTR}$ and cleaved caspase-3 (figure 4A, B and 5A, B) or double-labeled for $p75^{NTR}$ and fluorojade C (figure 4C and 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 GSK-3ß (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 GSK3ß *in vivo* (figure 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. Cerebrospinal fluid (CSF) was collected from the cisterna magna of each animal before being euthanized, and assayed for the level of lithium. Since only 50-70 µl of CSF can be obtained from each animal, samples were pooled from 5 rats in each of the 4 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 i.p. 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 13µM 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 were treated with lithium citrate delivered intraperitoneally after one

hr of SE, at the time the seizures were terminated with diazepam and phenytoin, followed by twice daily i.p. 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 (figure 7) and CA1 (figure 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 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 temporal lobe epilepsy (TLE), severe continuous seizures defined as status epilepticus (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 status epilepticus (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 $p75^{NTR}$ colocalized with cleaved caspase 3, the main executor protein in the apoptotic pathway, which was maximal by three days after the seizures in the CA1 and dentate hilus regions, consistent with previous studies (Roux et al., 1999; Troy et al., 2002). Fluorojade 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 cleaved caspase 3 and

fluorojade 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 would be neuroprotective if provided after SE rather than prior to 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 status epilepticus *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 GSK3ß, 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 GSK-3ß 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 GSK-3ß 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 GSK3ß or upregulation of BDNF, suggesting that neither phosphorylation of GSK3β 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

461 that the mechanism of protection might be at the level of the membrane receptors. We
462 investigated whether lithium at the neuroprotective nanomolar concentration was effective in
463 blocking either the association of $p75^{NTR}$ with sortilin, or the internalization of the two
464 receptors following proNGF treatment in culture. Co-immunoprecipitation analysis
465 demonstrated that lithium citrate decreased the association of $p75^{NTR}$ and sortilin both *in vitro*
466 and *in vivo*, thereby reducing formation of the requisite receptor complex for proNGF to
467 induce apoptosis. Using surface biotinylation assays to track the internalization of membrane
468 receptors, we also found that lithium reduced the internalization of $p75^{NTR}$ and sortilin into the
469 neurons. These data suggest that the neuroprotective effect of lithium citrate at these low doses
470 may be through disrupting the association and internalization of $p75^{NTR}$ and sortilin and
471 thereby preventing proNGF apoptotic signaling. Although lithium ion can exert
472 neuroprotection through multiple mechanisms, some of the well-established pathways of
473 lithium actions were not activated by this treatment paradigm. Other receptors and pathways
474 are likely to be affected by the lithium citrate treatment as well, however preventing the
475 activation of the $p75^{NTR}$/sortilin receptor complex may contribute to the neuroprotective
476 effects.

477 A previous study demonstrated that the binding of proNGF to the $p75^{NTR}$-sortilin
478 receptor complex is stabilized in the presence of calcium (Feng et al., 2010). It is well
479 established that the prodomain of proNGF is intrinsically disordered, and thus may adopt
480 distinct, transient conformational changes in response to environmental perturbations. Indeed,
481 addition of calcium at physiologic levels enhanced the interaction of the prodomain region
482 with sortilin, and stabilized the formation of a ~600kD proNGF/sortilin/p75 complex. In
483 contrast, calcium chelation significantly reduced the interaction of proNGF with its receptors.

484 One possible mechanism for the actions of lithium ion may be to alter the conformation of the
485 prodomain region of proNGF, possibly leading to the attenuation of the proNGF-induced
486 $p75^{NTR}$-sortilin association and/or internalization, and suggesting a novel mechanism for
487 neuroprotection by lithium ion.

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

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584

585

Figure Legends

Figure 1. Dose-dependent effects of lithium citrate on hippocampal neurons. **A**. E18 hippocampal neurons were cultured for 5 days and treated overnight with or without proNGF (3ng/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 GSK3ß *in vitro*. Cultured hippocampal neurons were treated with the indicated doses of lithium citrate and analyzed by Western blot for P-GSK3ß. Blots were stripped and re-probed for total GSK3ß. Quantification of 4 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 blot for BDNF. Blots were stripped and re-probed for actin. Quantification of 3 independent experiments is shown, * significant at $p<0.05$ by ANOVA.

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

609 Figure 3. Lithium citrate decreases the internalization of $p75^{NTR}$ and sortilin. **A**. E18
610 hippocampal neurons were cultured for 5 days, biotinylated, and treated for 10 min with
611 proNGF. After stripping remaining surface biotin with glutathione, streptavidin was used to
612 pull down the internalized, biotinylated proteins, which were analyzed by Western blot for
613 sortilin and $p75^{NTR}$. **B**. Lysates prior to streptavidin pulldown were also analyzed by Western
614 blot for sortilin and $p75^{NTR}$ to assess the input. Densitometric quantification of biotinylated
615 sortilin (**C**) and $p75^{NTR}$ (**D**) from 3 independent experiments. * different from Control at $p <$
616 0.05, ** different from proNGF at $p < 0.05$.
617
618 Figure 4. Lithium citrate pretreatment prevents neuronal death in the hilus following seizures
619 *in vivo*. Adult male rats were subjected to pilocarpine-induced seizures with or without 30 min
620 pretreatment with lithium citrate (6mg/kg) and injections twice daily for 3 days. **A**. Sections
621 through the hilus showing double labeling with anti-$p75^{NTR}$ and anti-cleaved caspase-3 (CC3).
622 **B**. Quantification of $p75^{NTR}$/CC3 double-labeled cells in the hilus with the different treatments.
623 Cells were counted in every 8^{th} section through the hippocampus. **C**. Quantification of
624 $p75^{NTR}$/Fluorojade labeled cells in the hilus with the indicated treatments. Size bar in panel A
625 equals 10 μm and is the same for all panels. Quantification in B and C is expressed as percent
626 control values which are set at 100%.
627
628 Figure 5. Lithium citrate pretreatment prevents neuronal death in the CA1 region following
629 seizures in vivo. Adult male rats were subjected to pilocarpine-induced seizures with or
630 without 30 min pretreatment with lithium citrate (6mg/kg) and injections twice daily for 3
631 days. **A**. Sections through CA1 showing double labeling with anti-$p75^{NTR}$ and anti-cleaved

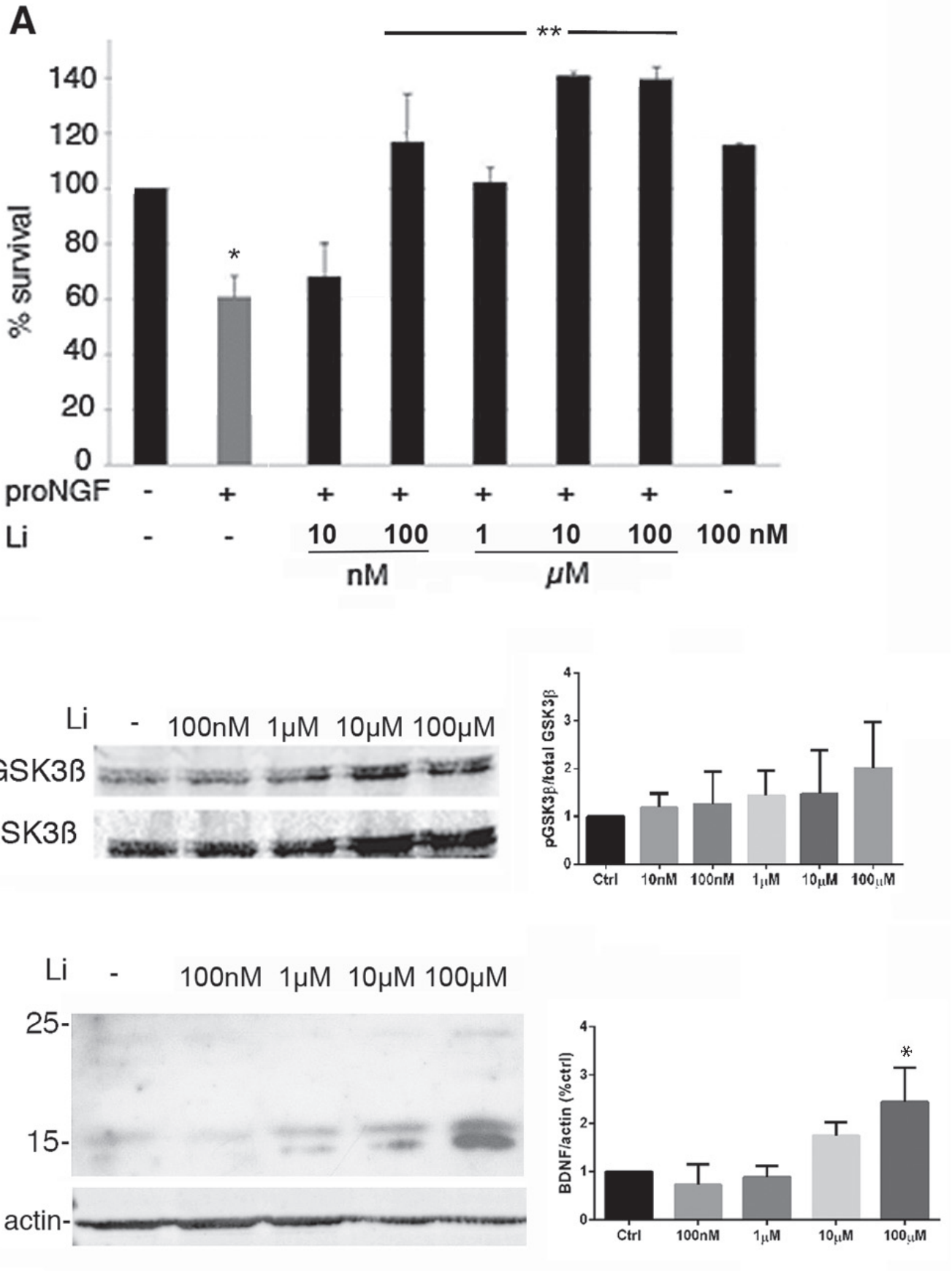
caspase-3 (CC3). **B**. Quantification of $p75^{NTR}$/CC3 double-labeled cells in the CA1 with the different treatments. Cells were counted in every 8^{th} section through the hippocampus. **C**. Quantification of $p75^{NTR}$/Fluorojade labeled cells in CA1 with the indicated treatments. Size bar in panel A equals 10 µm and is the same for all panels. Quantification in B and C is expressed as percent control values which are set at 100%.

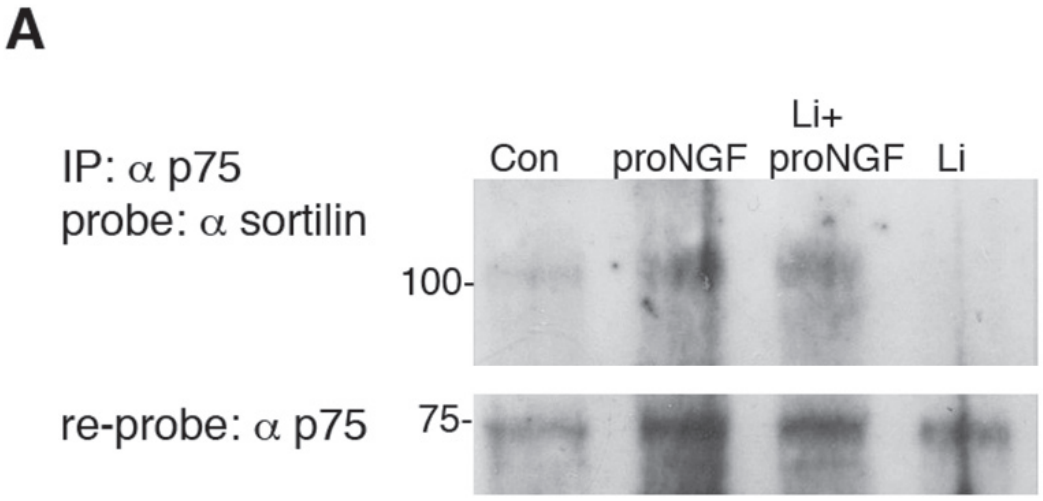
Figure 6. Treatment with lithium citrate reduces $p75^{NTR}$/sortilin interaction *in vivo* after pilocarpine-induced seizures. A. Hippocampal lysates were immunoprecipitated with anti-sortilin and probed for $p75^{NTR}$. B. Quantification of 2 independent cohorts expressed as percent control value which is set at 100%. ** indicates different from control at $p<0.05$, and * indicates different from pilo at $p<0.05$. C. Hippocampal lysates were probed for P-GSK3ß and total GSK3ß. D. Quantification of P-GSK3ß relative to total GSK3ß shows no effect of lithium citrate treatment *in vivo*.

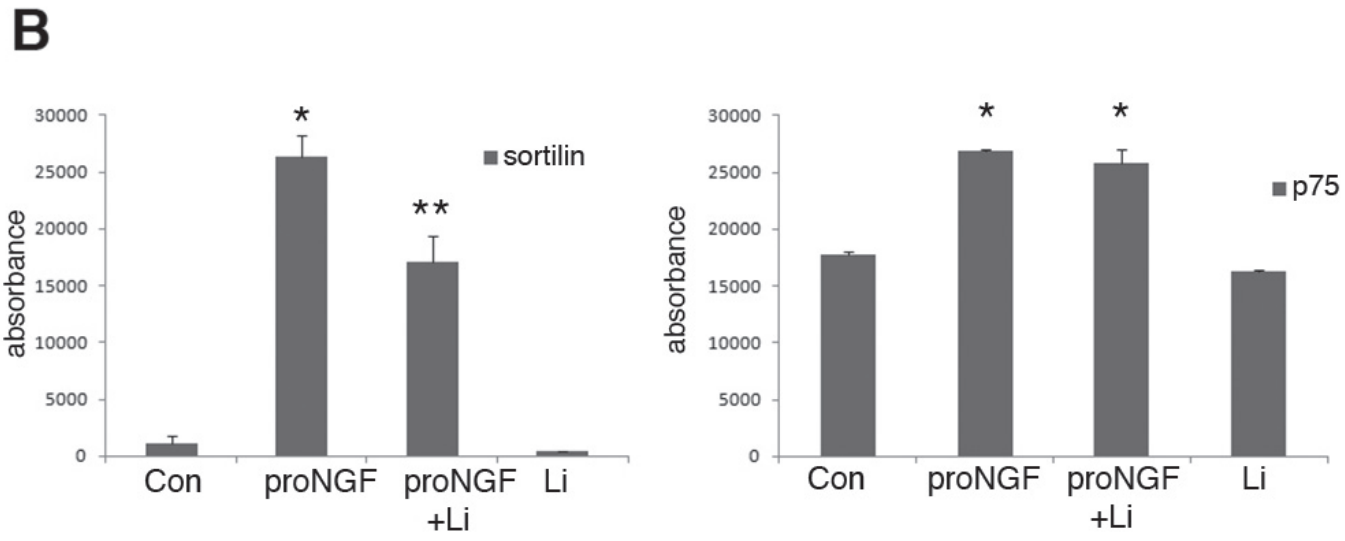
Figure 7. Treatment with lithium citrate after seizures prevents neuronal death in the hilus. Adult male rats were subjected to pilocarpine-induced seizures. Treatment with lithium citrate (6mg/kg) was initiated after the seizure was terminated and injected twice daily for 3 days. **A**. Sections through the hilus showing double labeling with anti-$p75^{NTR}$ and anti-cleaved caspase-3 (CC3). **B**. Quantification of $p75^{NTR}$/CC3 double-labeled cells in the hilus with the different treatments. Cells were counted in every 8^{th} section through the hippocampus. **C**. Quantification of $p75^{NTR}$/Fluorojade labeled cells in the hilus with the indicated treatments. Size bar in panel A equals 10 µm and is the same for all panels. Quantification in B and C is expressed as percent control values which are set at 100%.

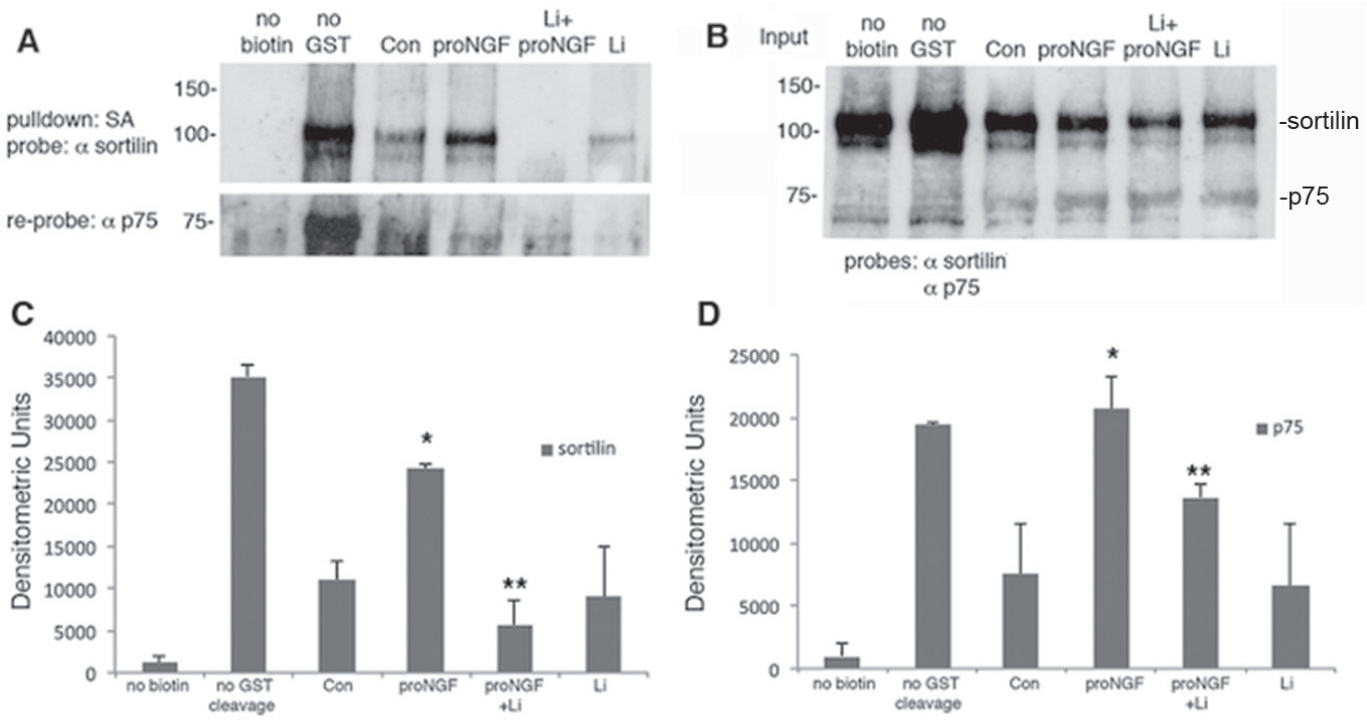
Figure 8. Treatment with lithium citrate after seizures prevents neuronal death in the CA1 region. Adult male rats were subjected to pilocarpine-induced seizures. Treatment with lithium citrate (6mg/kg) was initiated after the seizure was terminated and injected twice daily for 3 days. A. Sections through CA1 showing double labeling with anti-$p75^{NTR}$ and anti-cleaved caspase-3 (CC3). **B**. Quantification of $p75^{NTR}$/CC3 double-labeled cells in the CA1 with the different treatments. Cells were counted in every 8^{th} section through the hippocampus. **C**. Quantification of $p75^{NTR}$/Fluorojade labeled cells in CA1 with the indicated treatments. Size bar in panel A equals 10 μm and is the same for all panels. Quantification in B and C is expressed as percent control values which are set at 100%.

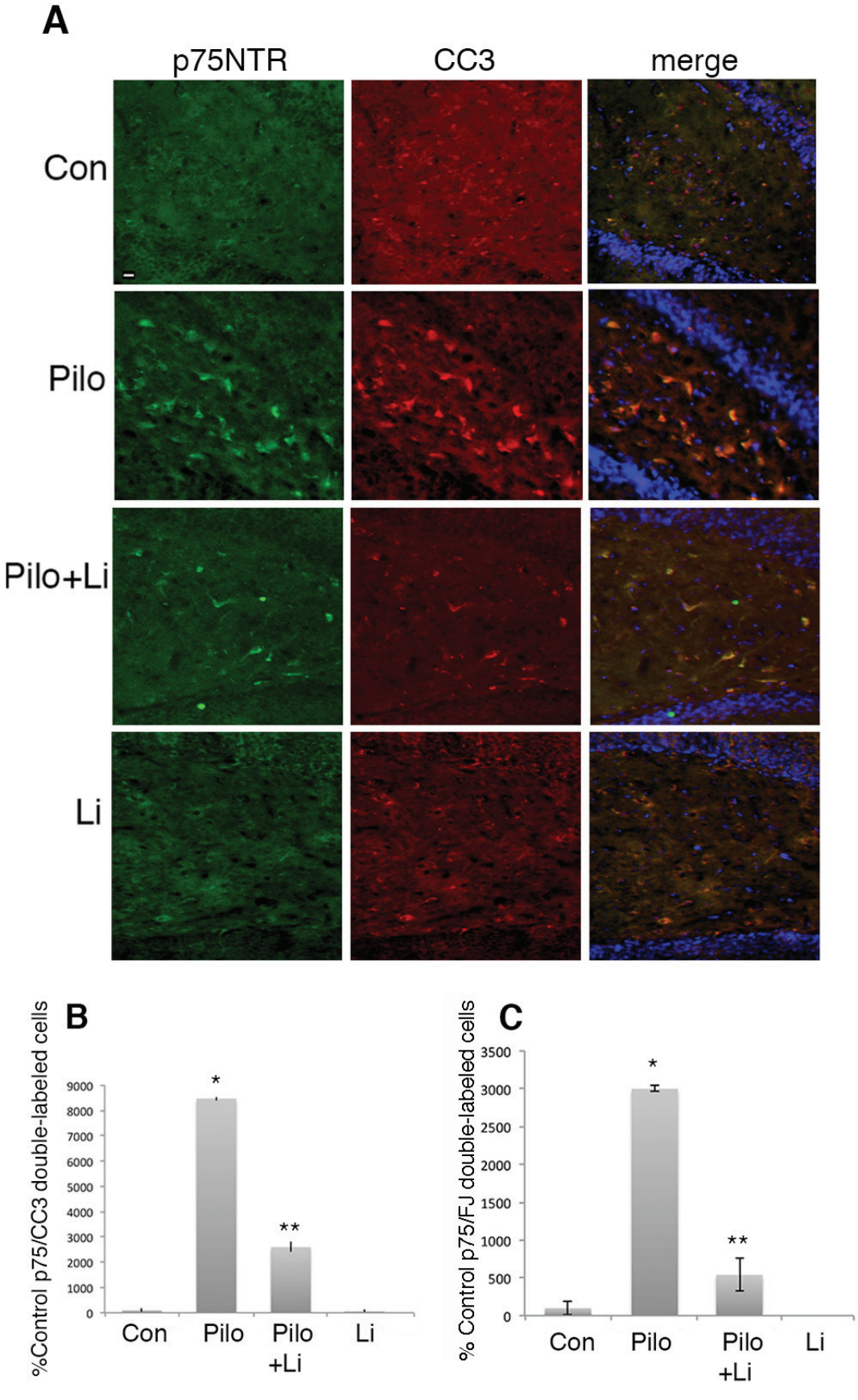
Table 1. List of compounds from the screen of the SpecPlus Collection that blocked proNGF uptake. Fifteen compounds were identified from the 2560 screened that that exhibited a 66% reduction in proNGF uptake at 10 micromolar concentration, and resulted in greater than 85% viability as compared to control.

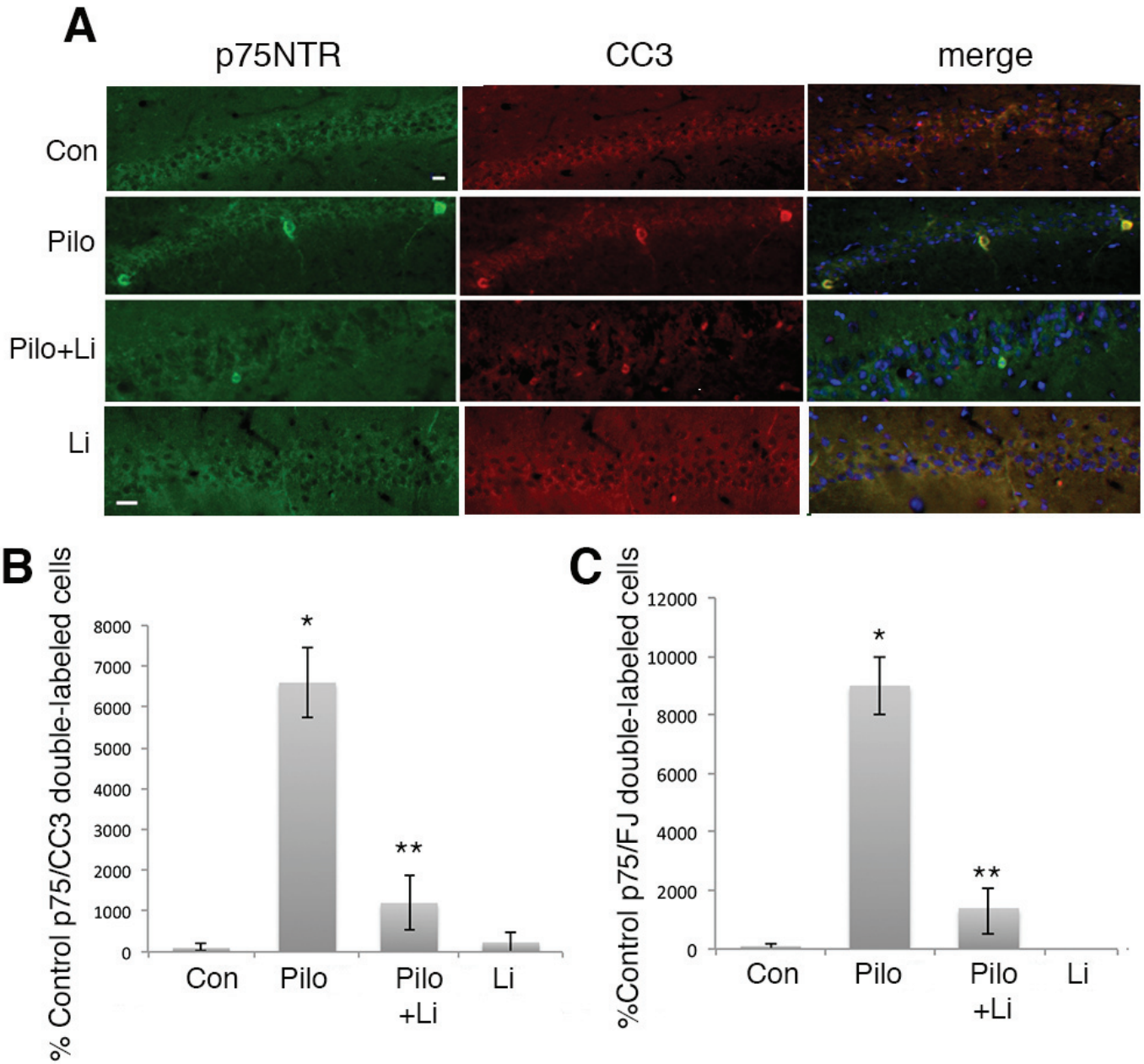


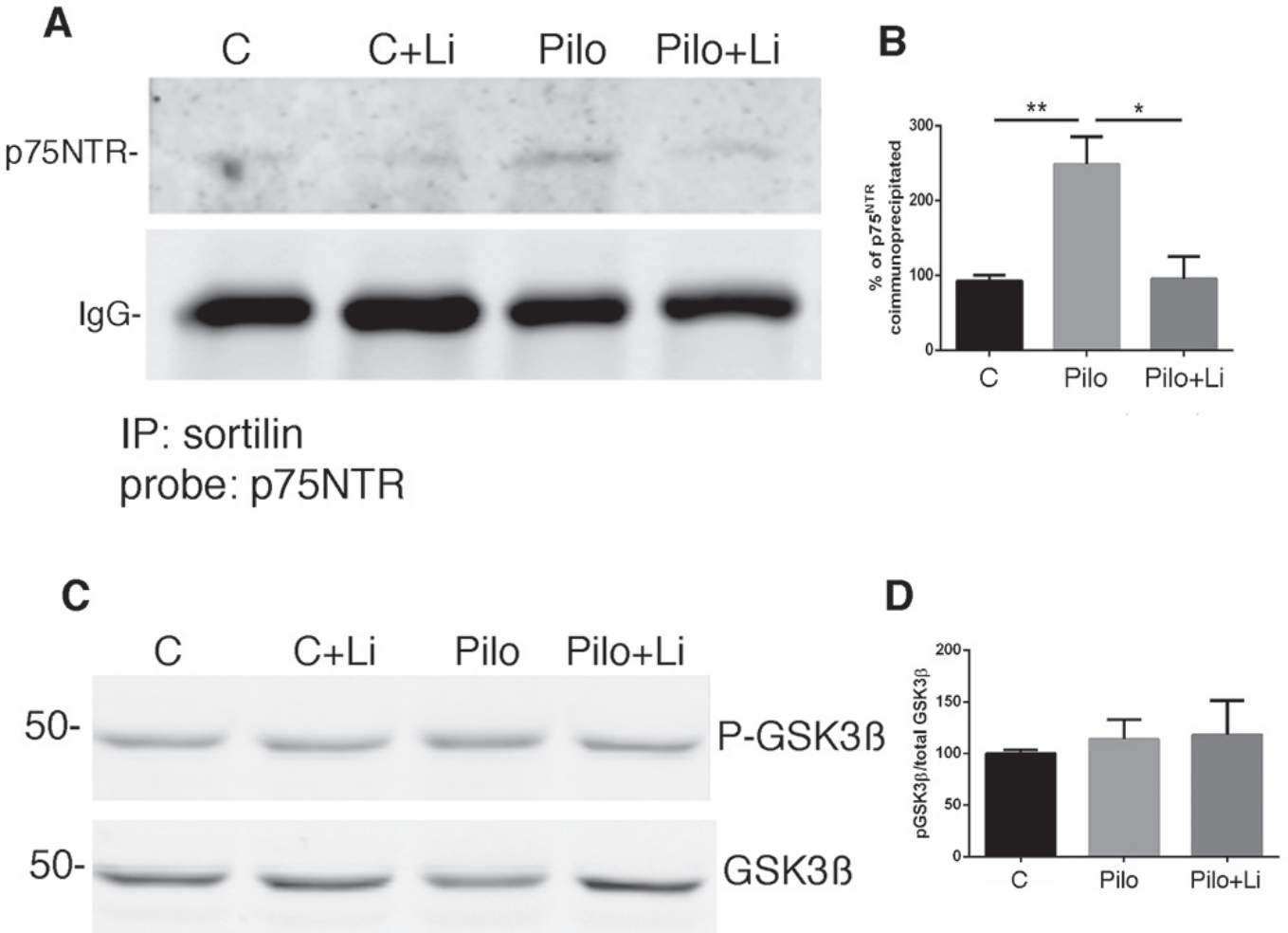


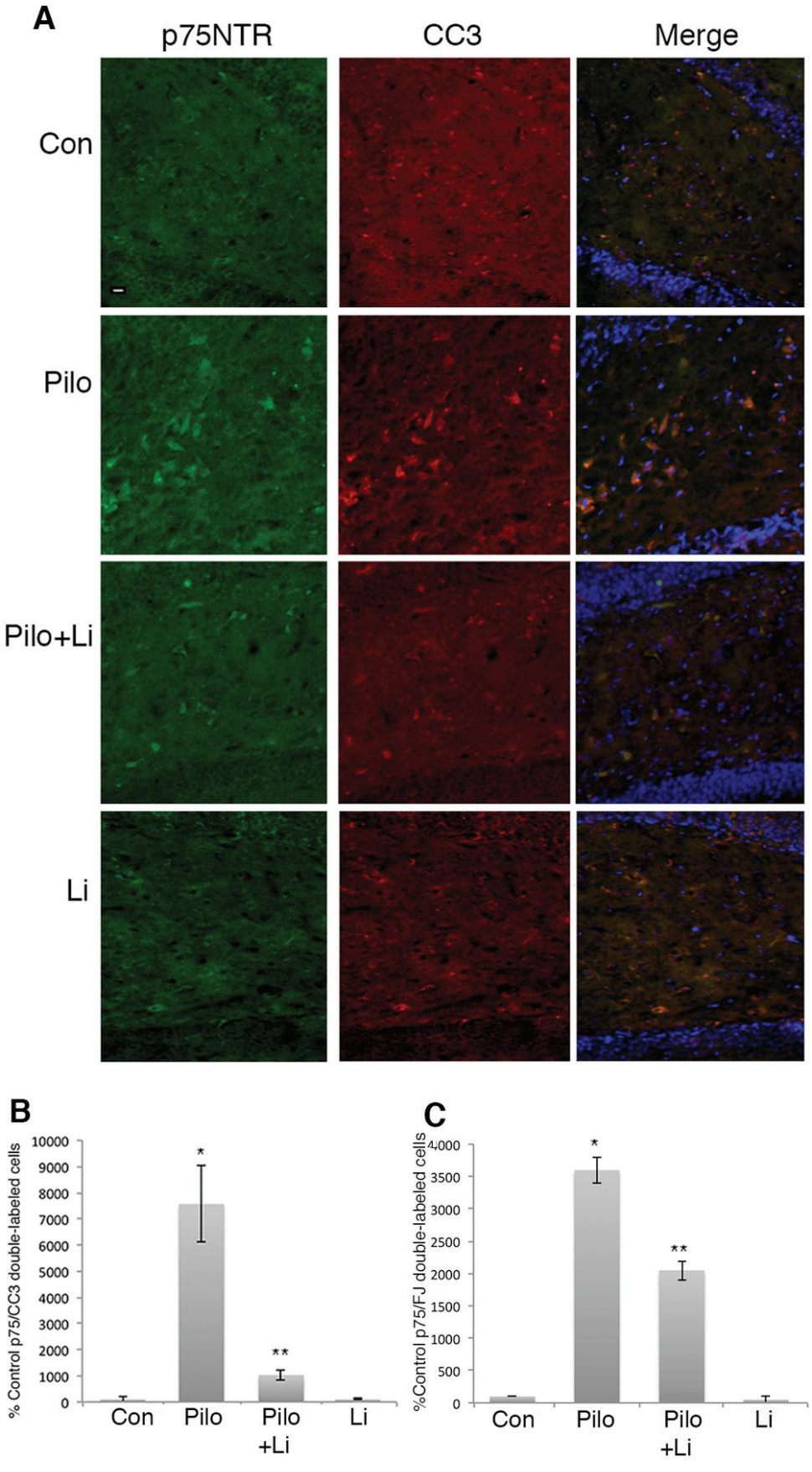












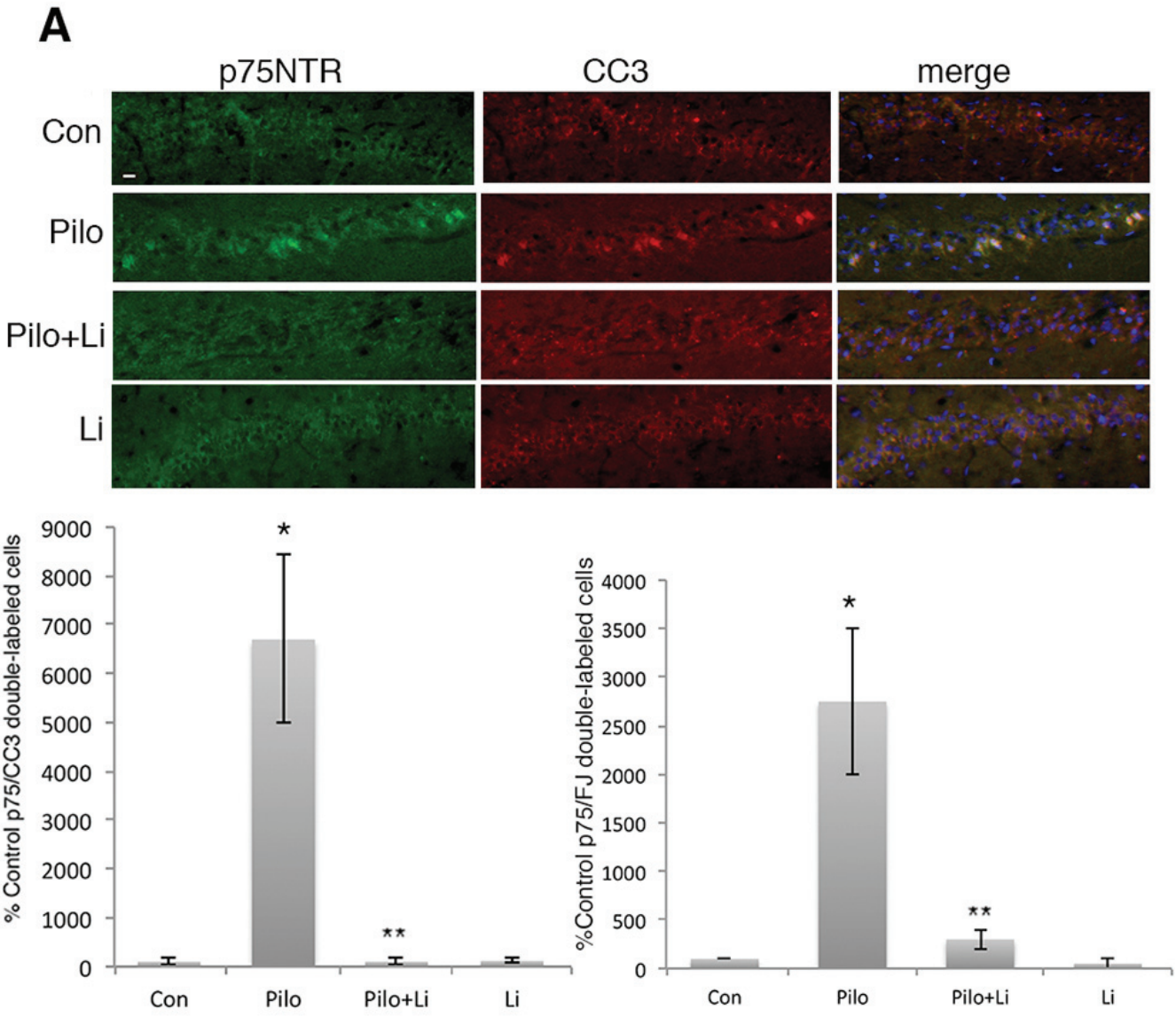


Table 1. List of compounds that blocked proNGF uptake

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
Oxaprozin
Lithium Citrate