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Inhibition of the Jak-STAT pathway prevents CNTF-mediated survival of axotomized oxytocinergic magnocellular neurons in organotypic cultures of the rat supraoptic nucleus

Jason M. Askvig^a, David Y. Lo^b, Adam W. Sudbeck^a, Kathryn E. Behm^a, Laura J. Leiphon^a, and John A. Watt^a

^a Department of Anatomy & Cell Biology, University of North Dakota School of Medicine and Health Sciences, Grand Forks, ND 58203, USA.

^b Laboratory of Neuronal Structure and Function, Salk Institute for Biological Studies ③ La Jolla, CA 92037-1099

Abstract

Previous studies have demonstrated that ciliary neurotrophic factor (CNTF) enhances survival and process outgrowth from magnocellular neurons in the paraventricular (PVN) and the supraoptic (SON) nuclei. However, the mechanisms by which CNTF facilitates these processes remain to be determined. Therefore, the aim of this study was to identify the immediate signal transduction events that occur within the rat SON following administration of exogenous rat recombinant CNTF (rrCNTF) and to determine the contribution of those intracellular signaling pathway(s) to neuronal survival and process outgrowth, respectively. Immunohistochemical and Western blot analysis demonstrated that axonal injury and acute unilateral pressure injection of 100 ng/μl of rrCNTF directly over the rat SON resulted in a rapid and transient increase in phosphorylated-STAT3 (pSTAT3) in astrocytes but not neurons in the SON *in vivo*. Utilizing rat hypothalamic organotypic explant cultures, we then demonstrated that administration of 25 ng/ml rrCNTF for 14 days significantly increased the survival and process outgrowth of OT magnocellular neurons. In addition, pharmacological inhibition of the Jak-STAT pathway via AG490 and cucurbitacin I significantly reduced the survival of OT magnocellular neurons in the SON and PVN; however, the contribution of the Jak-STAT pathway to CNTF-mediated process outgrowth remains to be determined. Together, these data indicate that CNTF-induced survival of OT magnocellular neurons is mediated indirectly through astrocytes via the Jak-STAT signaling pathway.

Keywords

axonal sprouting; CNTF; CNTFR α ; infundibular nerve crush; Jak-STAT; magnocellular neurosecretory system; PVN; STAT3

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Corresponding Author: John Watt, Room 1701 Stop 9037, 501 N Columbia Road, Grand Forks, ND 58203; phone: 701-777-6225; fax: 701-777-2477; john.watt@med.und.edu, jason.askvig@my.und.edu, dlo@salk.edu, asudbeck@medicine.nodak.edu, kathryn.behm@my.und.edu, laura.leiphon@med.und.edu.

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Introduction

Neuronal survival and structural reorganization of damaged neuronal circuitry following injury is key to functional recovery. However, the limited capacity for proper elaboration of neural processes inherent to the central nervous system limits the degree to which functional recovery may occur. Clearly, developing strategies through which cell survival and neurite outgrowth can be promoted and directed, respectively, holds great therapeutic potential. Long recognized for its robust capacity for structural reorganization, the magnocellular neurosecretory system provides an excellent model system with which to investigate the cellular mechanisms which underlie post-injury survival and axonal reorganization (Moll, 1957, Raisman, 1973, Silverman and Zimmerman, 1982, Watt, et al., 1999, Watt and Paden, 1991). Toward this end, we utilize a unilateral lesion of the hypothalamo-neurohypophyseal tract in which the neurosecretory axons in the animal's right hemisphere paraventricular (PVN) and supraoptic (SON) nuclei are severed while the contralateral SON and PVN nuclei are spared (Watt and Paden, 1991). The lesion results in the loss of 42% of the neurosecretory axons in the neural lobe (NL) followed by a return to control levels by four weeks post-lesion (Watt and Paden, 1991). The axonal recovery results from a collateral sprouting response arising from the non-injured, contralateral magnocellular neurons in the absence of a functional deficit (Watt and Paden, 1991). Coincident with the sprouting response, ciliary neurotrophic factor (CNTF) and the CNTF specific receptor, CNTF receptor alpha (CNTFR α), levels increase in the intact contralateral SON from which the sprouting response originates (Askvig, et al., 2012, Watt, et al., 2006, Watt, et al., 2009). CNTF has been previously implicated in hypothalamic magnocellular neuron sprouting *in vitro* (Vutskits, et al., 1998) and has been demonstrated to promote motor neuron sprouting *in vivo* (Gurney, et al., 1992, Guthrie, et al., 1997, Kwon and Gurney, 1994, Oyesiku and Wigston, 1996, Siegel, et al., 2000, Simon, et al., 2010, Ulenkate, et al., 1994, Wright, et al., 2007, Xu, et al., 2009). Moreover, multiple reports have demonstrated that CNTF promotes survival of magnocellular neurons in the PVN and SON following axotomy in organotypic cultures (House, et al., 2009, Rusnak, et al., 2002, Rusnak, et al., 2003, Vutskits, et al., 1998, Vutskits, et al., 2003). However, the specific intracellular signal transduction pathway(s) which mediate these distinct responses have not yet been elucidated.

Intracellular CNTF signal transduction is mediated by the tripartite CNTF receptor complex comprised of CNTFR α , LIF receptor β (LIFR β), and glycoprotein 130 (gp130) (Davis, et al., 1993, Ip, et al., 1992). The Jak-STAT (Janus kinase-signal transducer and activator of transcription) intracellular signal transduction pathway is considered the canonical intracellular signaling pathway utilized by CNTF (Bonni, et al., 1993, Bonni, et al., 1997, Darnell, et al., 1994). After ligand binding and receptor dimerization the LIFR β and gp130 receptor components and their constitutively bound Jak molecules, which includes the ubiquitously expressed Jak1, Jak2, and Tyk2, are tyrosine phosphorylated (Stahl and Yancopoulos, 1994). The resultant phosphotyrosines on LIFR β and gp130 serve as docking sites for STAT molecules (Segal and Greenberg, 1996, Stahl, et al., 1994, Stahl and Yancopoulos, 1994). Subsequently, STAT3, which has been demonstrated to be preferentially phosphorylated by CNTF at tyrosine 705 (Tyr⁷⁰⁵) (Bonni, et al., 1997, Darnell, et al., 1994, Wegenka, et al., 1993), is phosphorylated by Jak molecules (Lutticken, et al., 1994). The phosphorylated STAT molecules then dimerize and translocate to the nucleus and are capable of directly activating gene transcription by binding to DNA in a sequence specific fashion (Bonni, et al., 1993, Boulton, et al., 1995, Stahl, et al., 1995). Our working hypothesis is that CNTF signals through the Jak-STAT intracellular signal transduction pathway in the SON to promote neuronal survival. In order to test this hypothesis, we utilized acute unilateral pressure injections of rat recombinant CNTF (rrCNTF) into the SON *in vivo* to determine the spatial and temporal activation of the Jak-STAT pathway in the SON. In addition, the contribution of the Jak-STAT pathway to

magnocellular neuron survival in the SON and PVN were assessed using stationary hypothalamic organotypic cultures as initially developed by Stoppini et al. (1991). Organotypic cultures exhibit several advantages over other *in vitro* culture systems primarily because of the preservation of the *in vivo* cytoarchitecture and the use of fully differentiated neurons (House, et al., 1998, Vutskits, et al., 1998). Furthermore, the ability to directly manipulate the culture media with growth factors and pharmacological agents and assess magnocellular neuron survival in hypothalamic organotypic cultures facilitates analysis of pathway-mediated cellular events more rapidly than can be achieved using our *in vivo* injury model system.

Materials and Methods

Animals

Male Sprague-Dawley rats used in the *in vivo* studies were purchased from Charles River Laboratories (Wilmington, MA) while pregnant female Sprague Dawley rats (E15) were purchased from Harlan (Minneapolis, MN). All rats were housed in the University of North Dakota Center for Biomedical Research Facility, an AAALAC accredited facility, under a 12L:12D light cycle with ad lib access to lab chow and tap water throughout the investigations. Experimental protocols utilized in these studies followed the guidelines in the NIH guide for the care and use of laboratory animals and were approved by the UND Institutional Animal Care and Use Committee (protocol #0704-2c). All efforts were made to minimize the numbers of animals used in this study and their suffering.

Infundibular nerve crush

Male Sprague-Dawley rats (250-400g) were secured in a stereotaxic apparatus and kept under constant isoflurane anesthesia (2.5%; Abbott Laboratories, Abbott Park, IL) using a tabletop anesthesia apparatus (Matrx Quantiflex Low Flow V.M.C.; Matrx, Orchard Park, NY) equipped with an isoflurane vaporizer (Matrx VIP 3000; Matrx). A Dremel drill was used to remove a window of skull 3 mm in length and 1.5 mm in width at -3.1 AP in relation to bregma. A triangular blade (2 mm) constructed from stainless steel music wire (0.015 mm diameter) supported by a hypotube (ID of 0.017 mm; Small Parts Inc, Logansport, IN) was lowered at the coordinates -3.1 AP until it flexed at the base of the skull, and the blade was left in place for 5 minutes to complete the infundibular nerve crush. The blade was then removed, the wound was sutured shut, and the animal was returned to its cage. Animals were sacrificed at 3 hrs post injury. Sham operated controls were prepared exactly as described except that the blade was lowered 5 mm ventral to the skull's surface to avoid damaging the axons of the hypothalamo-neurohypophysial tract. Completeness of the hypothalamic lesion was verified histologically in all animals included in this study.

Acute rrCNTF injections

For acute injection of rrCNTF into the rat SON, male Sprague-Dawley rats (250-400g) were secured in a stereotaxic apparatus and kept under constant isoflurane anesthesia (2.5%) using a tabletop anesthesia apparatus equipped with an isoflurane vaporizer. Lyophilized carrier-free rrCNTF (#C3835, lot #080M1730, Sigma; St. Louis, MO) solubilized in artificial cerebral spinal fluid (aCSF, 290 mOsm/l) to a final concentration of 100 ng/ μ l was drawn into a 10 μ l syringe (26 gauge; Hamilton, Reno, NV). The syringe was placed in a stereotaxic injector (Quintessential Stereotaxic Injector; Stoelting, Wood Dale, IL) mounted onto a stereotaxic apparatus (Stoelting) and 1 μ l rrCNTF, or vehicle (aCSF), was pressure injected immediately dorsal to the right SON (coordinates: AP -0.9 mm, ML -2.3 mm, DV -9.5 mm) at a rate of 0.2 μ l/minute over a 5 minute period. Following injection, the syringe was left in place for 5 minutes to allow for diffusion away from the injection site. Precise targeting of the cannula tip was confirmed by immunohistochemical confirmation of

the syringe tract with goat anti-rat CNTF (1:100; #AF-557-NA, R&D Systems, Minneapolis, MN). We observed a 90% success rate in cannula targeting and only those animals with a confirmed syringe tract and CNTF-immunoreactive injection site were used in these studies. The following groups were prepared for Western blot analysis following CNTF injection; 1 hr post-vehicle injected SON; non-infused contralateral control SON from 1 hr post-CNTF injection; 1 hr post-CNTF injected SON; and 3 hrs post-CNTF injected SON. The pharmacological inhibitor of Jak2, AG490 (#658401, Calbiochem, Gibbstown, NJ), was dissolved in dimethylsulfoxide (DMSO) and administered (10 mg/kg, intraperitoneally) 1 hr prior to rCNTF injection. These rats were sacrificed at 1 hr post-CNTF injection (AG490 + 1 hr CNTF-injected SON).

Immunohistochemistry

Following experimental periods, animals were anesthetized with isoflurane before being perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate buffer. Following perfusion fixation, brains were removed, post-fixed overnight, cryoprotected in 20% sucrose in phosphate buffered saline (PBS) then snap frozen in isopentane chilled in dry ice. Serial cryosections (14 μ m) were thaw-mounted on gelatin coated slides and stored at -20°C until further use.

For single-label peroxidase immunohistochemistry, slide mounted sections were washed with PBS containing 0.3% Triton X-100 (PBS-T; Sigma) in 3 $\times$ 10 minute intervals before and after processing and in between incubations. Sections were pretreated with 0.3% hydrogen peroxide (in PBS) for 30 minutes at room temperature to inhibit endogenous peroxidase activity followed by treatment with 5% of the appropriate normal serum (Vector, Burlingame, CA) in PBS-T (blocking buffer) for 1 hour at room temperature to reduce non-specific staining. Tissue immunoreactivity was detected by sequential incubation with primary antibodies (in blocking buffer) overnight at 4°C, secondary biotinylated antibodies (1:500 in blocking buffer; Vector) for 1 hour at room temperature, followed by avidin-biotin complex (10 μ l/ml in PBS-T; Vector ABC *Elite* kit) for 1 hour at room temperature. Bound antibodies were visualized using glucose oxidase (0.3%; Sigma) to generate hydrogen peroxide (Itoh, et al., 1979) with either 0.05% 3, 3' diaminobenzidine (DAB, Sigma) in PBS to obtain a brown reaction product or a 1% nickel chloride (Sigma) enhanced DAB (Ni-DAB) to obtain a black/purple reaction product. For dual-label peroxidase immunohistochemistry, the process described above was repeated using the same section, but with the application of a different primary antibody. The initial primary antibody was visualized using either DAB or Ni-DAB, followed by the subsequent primary antibody being visualized with the opposite color reaction. Following DAB treatment, sections were then dehydrated in a graded series of ethanol, cleared in xylene, and cover-slipped with Permount (Sigma).

For dual-label fluorescence immunohistochemistry, all steps were the same as listed above unless otherwise noted. Tissue immunoreactivity was detected by incubation in a cocktail containing either rabbit anti-phosphorylated-STAT3 (pSTAT3; Tyr⁷⁰⁵; 1:300; #9131, Cell Signaling, Danvers, MA) or rabbit anti-total-STAT3 (tSTAT3; 1:500; #9132, Cell Signaling) with either mouse anti-glial fibrillary acidic protein (GFAP; 1:1000; #G-3893, Sigma), guinea pig anti-oxytocin (OT; 1:1000; #T-5021, Peninsula Laboratories, San Carlos, CA), or guinea pig anti-vasopressin (VP; 1:1000; #T-5048, Peninsula Laboratories) in blocking buffer overnight at 4°C followed by species-specific secondary IgG conjugated to either Alexa Fluor 488 or Alexa Fluor 594 (1:100; Molecular Probes, Eugene, OR). Fluorescent sections were coverslipped with Vectashield mounting medium containing DAPI (Vector) and examined using an Olympus BX-51 fluorescent microscope with attached DP-71 color camera and dedicated software. Images were prepared for reproduction using Adobe Photoshop CS2.

All of the primary antibodies utilized in the immunohistochemistry experiments have been previously characterized either within our lab or by other labs. The mouse anti-GFAP, guinea pig anti-OT, and guinea pig anti-VP antibodies specifically label astrocytes and magnocellular neurons within the SON, respectively (Askvig, et al., 2012). The anti-pSTAT3 antibody does not cross react with other STAT molecules when phosphorylated on the corresponding residue (manufacturer's technical information). Moreover, others have utilized the pSTAT3 and tSTAT3 antibodies for immunohistochemistry (Sriram, et al., 2004).

As a control for the dual-label fluorescence immunohistochemistry, incubations with either rabbit anti-pSTAT3 or rabbit anti-tSTAT3 antibodies on separate sections of tissue were followed by an incubation with the species-specific fluorescent-conjugated secondary antibodies for anti-GFAP, -OT, and -VP. Similarly, after separate sections of tissue were incubated with the anti-GFAP, -OT, or -VP antibodies the tissue was exposed to the fluorescent-conjugated secondary antibodies for the rabbit anti-pSTAT3 and -tSTAT3. These controls demonstrated an absence of immunoreactivity in the rat SON, indicating that the fluorescent-conjugated secondary antibodies were specific for their appropriate primary antibody and there was no observable cross-reactivity between the secondary antibodies.

Gel electrophoresis and Western blot analysis

Following acute injection of rrCNTF, animals were anesthetized with isoflurane anesthesia and decapitated. SON samples were carefully collected under a dissecting microscope and pooled from 3 rats (18 total rats, n=6) in a solution of radioimmuno-precipitation assay (RIPA) buffer containing 20 mM Tris (pH 7.5; Sigma), 150 mM NaCl (Sigma), 1% nonidet P-40 (Roche Diagnostics; Indianapolis, IN), 0.5% sodium deoxycholate (Sigma), 1 mM EDTA (Sigma), 0.1% SDS (Pierce; Rockford, IL), 1% protease inhibitor (Protease Inhibitor Cocktail; Sigma) and 1% phosphatase inhibitor (Phosphatase Inhibitor Cocktail 2; Sigma). The SON samples were then homogenized in RIPA buffer and centrifuged at 10,000 rpm for 20 minutes at 4°C. Supernatant from each sample was stored at -80°C until needed. Each lane was loaded with 15µg of protein and separated by a 12% SDS-PAGE gel (Precise Protein Gels; Pierce) at 90V for approximately 1.25 hours and then electrophoretically transferred to a PVDF membrane (0.2µm; BioRad, Hercules, CA) at 70 V for 2 hours. Membranes were washed in PBS containing 0.5% Tween 20 (PBS-Tween; Sigma) for a minimum of 4×20 minute intervals between incubations. Membranes were then incubated in blocking solution (5% BSA in PBS-Tween; Sigma) for 1 hour at room temperature. Next, the membranes were incubated in either rabbit anti-pSTAT3 (Tyr⁷⁰⁵; 1:1000; #9131, Cell Signaling) or rabbit anti-phosphorylated-Jak2 (pJak2; Tyr^{1007/1008}; 1:2000; #ab58268, Abcam, Cambridge, MA) overnight at 4°C followed by incubation in donkey anti-rabbit IgG conjugated to horseradish peroxidase (1:100,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 hours at room temperature. Following PBS washes for 2 hours, the bands were subsequently visualized using the West Femto chemiluminescent detection kit (Pierce) with high performance chemiluminescence film (Amersham Hyperfilm ECL; GE Healthcare; VWR; West Grove, PA) developed using an AGFA CP1000 film processor. Subsequently, bound antibodies were stripped with a stripping buffer (pH 2.2; 15g glycine; Sigma, 1g SDS; Bio-Rad, 10ml Tween 20 in 1L ultrapure water) for 10 minutes and the blots were sequentially re-probed for rabbit anti-tSTAT3 (1:3,000; #9132, Cell Signaling) or rabbit anti-total-Jak2 (tJak2; 1:2000; #3230, Cell Signaling) and mouse anti-β-actin (1:50,000; #A2228, Sigma).

All of the primary antibodies utilized in the Western blot analysis have been previously characterized either within our lab or by other labs. The mouse anti-β-actin antibody recognizes an epitope located on the N-terminal end of the rat β-isoform of actin (Gimona, et al., 1994). β-actin was used as a tissue loading control for our Western blot analyses and

the anti- β -actin antibody consistently recognized a band at approximately 42 kDa in all tissue samples analyzed in the present study. Furthermore, others have utilized the pSTAT3, tSTAT3, pJak2, and tJak2 antibodies for Western blot analysis (Mitsui, et al., 2003, Sriram, et al., 2004) and we consistently observed STAT3 and Jak2 bands at their expected molecular weights of 90 and 130 kDa, respectively.

Densitometric analysis of immunoblot signals was performed using MCID image analysis software (version 7.0, Imaging Research Inc.). Band intensities were expressed as relative optical density (ROD) units. To obtain accurate density measurements band areas were multiplied by the ROD value and the background was subtracted. In order to determine the amount of total protein that is phosphorylated, the ROD of the phosphorylated protein was normalized to the ROD of the total protein, as opposed to the ROD of β -actin, to obtain a ratio used for statistical analysis. Analysis was repeated on 3 separate samples per group resulting in mean ratio values for each group that were used for statistical analysis as described below.

Stationary hypothalamic organotypic cultures

Organotypic cultures were prepared using a modification of techniques described previously (House, et al., 2009, House, et al., 2006, House, et al., 1998, Rusnak, et al., 2002, Rusnak, et al., 2003, Shahar, et al., 2004). Briefly, 6-day-old Sprague Dawley rat pups were decapitated and their brains were removed and placed in chilled Geys Balanced Salt Solution (Gibco, Grand Island, NY) enriched with glucose (5 mg/ml; Sigma). The brains were trimmed to remove exterior cortical material and 350 μ m coronal sections were obtained using a McIlwain Tissue Chopper (Stoelting). The sections containing the magnocellular neurosecretory system nuclei were placed in chilled Geys Balanced Salt Solution and then trimmed dorsal to the third ventricle and lateral to the SON under a dissecting microscope. Sections from each animal were then placed on a single Millicell-CM filter insert (pore size 0.4 μ m, 30mm diameter; Millipore, Bedford, MA) and each filter insert was then placed in a 35 $\times$ 10 mm Petri dish containing 1.1-1.2ml of culture media for the experimental period.

Media and incubations

The standard culture media was made fresh at the beginning of every experiment and consisted of Eagle's Basal Medium with Earle's salts (50%; Gibco), heat inactivated horse serum (25%; Gibco), Hank's balanced salt solution (25%; Gibco), glucose (0.5%; Sigma), penicillin/streptomycin (25 units/ml; Gibco), and glutamine (1.0 mM; Gibco). The osmolality and pH of the culture media were measured from the stock media solution every 48 hours using a Wescor vapor pressure osmomoter (Wescor 5500; Logan, UT) and a mini pH meter (IQ Scientific Instruments, Loveland, CO), respectively. Our analysis demonstrated that the media osmolality was maintained at 310.5 ± 0.45 mOsm/l and the pH was at 8.2 ± 0.03 throughout the experimental period. Incubation of the cultures was stationary in 5% CO₂-enriched air at 35°C for the entire experimental period.

In the current study, hypothalamic slices were cultured in the presence or absence of rrCNTF (#C3835, lot #080M1730, Sigma) for 14 days. All groups had their culture media replaced every 48 hours and always received fresh additions of rrCNTF. Inhibition experiments were performed by administering AG490 or cucurbitacin I (JSI-124; #238590, Calbiochem) in the absence of rrCNTF for 1 hour prior to treatment of the cultures followed by replacement with media containing the inhibitor plus rrCNTF for the duration of the experimental period. Additional control cultures received only the inhibitor for the entire experimental period.

As a control for the rCNTF protein, we had a construct generated for the reverse sequence of the rat CNTF sequence (NEO Group, Inc., Cambridge, MA). The construct contained a His-tag and was generated in *E. Coli* BL21 (*DE3*) strain using the protein sequence:

¹MNHKVHHHHH HMKKNNAIYH SGRAPIGPQH SSAFRLDHS RVTWQSLEQL
⁵¹VKLGWLKKEF LGGDGVNIPM GDAENRPIKY ELLIMLEEIQ YAFAAVQLLL
¹⁰¹THIAQHFDGE TPTFHVQQDE LLRALLVHFT RYAQLNEQLR EAETLESWQD
¹⁵¹TSAVPMGDAS DLNINKNLGQ HKVYSETLAT LDSRLKRALW ISRSCLDRRH
²⁰¹PTLPSHETFA M.

The reverse sequence CNTF construct was estimated to be 80% pure by a Coomassie blue-stained SDS-PAGE gel (NEO Group, Inc.). Subsequently, a group of hypothalamic organotypic explant cultures received 25 ng/ml reverse CNTF for 14 days.

Organotypic culture immunohistochemistry

Following experimental periods, the explants were prepared for immunohistochemistry with fixation in 4% paraformaldehyde (Sigma) in 0.1 M phosphate buffer for 1.5 hours. For immunohistochemical analysis, sections were washed with PBS in 3×10 minute intervals before and after all incubations. For single-label peroxidase immunohistochemistry, endogenous peroxidase activity and non-specific staining were prevented by treatment with 0.3% H₂O₂ (Sigma) followed by incubation in blocking buffer (10% normal horse serum containing 0.3% Triton X-100) for 1.5 hours. The explants were then incubated for 36 hours at 4°C in highly specific monoclonal mouse antibodies against OT-neurophysin (PS 38, 1:500; a gift from Dr. Harold Gainer) or VP-neurophysin (PS 41, 1:500; a gift from Dr. Harold Gainer). These antibodies were characterized by Ben-Barak et al. (1985) and have been consistently utilized to label OT and VP neurons and their processes within organotypic cultures (House, et al., 2009, House, et al., 2006, House, et al., 1998, Rusnak, et al., 2002, Rusnak, et al., 2003, Shahar, et al., 2004, Vutskits, et al., 1998, Vutskits, et al., 2003). Next the cultures were incubated in horse anti-mouse biotinylated secondary antibody (1:500; Vector), followed by avidin-biotin complex (ABC; 10 µl/ml in PBS; Vector ABC *Elite* kit) for 1 hour at room temperature. Bound antibodies were visualized using 0.05% diaminobenzidine (DAB, Sigma) in PBS developed through the glucose-oxidase method (Itoh, et al., 1979). The hypothalamic slices were then removed from their filters and placed directly on gelatin coated slides. All slides were then dehydrated in increasing concentrations of alcohol followed by xylene rinses and coverslipped with Permount (Fisher, Pittsburgh, PA). Images were prepared for reproduction using Adobe Photoshop CS2.

Magnocellular neuronal counts

The slides containing the immunoreactive explant culture slices were coded by a third party blind to the experimental conditions. In order to obtain the total number of neurons in the PVN and SON, immunoreactive cells were counted using a drawing tube attached to an Olympus BX51 microscope. The values used in statistical analysis represent the total number of immunoreactive neurons for each nuclei of one neonatal hypothalami (i.e. one filter insert) and it was the mean of two individual's independent neuronal counts that were used as the group mean for statistical analysis as described below.

Statistical analysis

Distribution normality of each group of data was tested using the Kolmogorov-Smirnov test (GraphPad InStat, version 3.06 for Windows; San Diego California) and all groups were normally distributed. Statistical differences between groups were compared using one-way

ANOVA with Tukey's post *hoc* test (GraphPad InStat) with $p < 0.05$ considered statistically significant. Results are expressed as the group means $\pm$ SEM.

Results

Injury and exogenous rrCNTF transiently activates STAT3 in astrocytes and not magnocellular neurons of the SON *in vivo*

To determine the distribution of tSTAT3 within the SON, dual-label immunofluorescence experiments pairing anti-tSTAT3 with the neuronal markers anti-OT and anti-VP and the astrocytic marker anti-GFAP were performed. We observed robust tSTAT3-immunoreactivity distributed throughout the SON indicating its constitutive expression. Subsequent dual-label immunofluorescence pairing anti-tSTAT3 (Fig. 1A, D) with anti-OT (Fig. 1B) and anti-VP (Fig. 1E) confirmed the presence of tSTAT3 within OT- (Fig. 1C) and VP- (Fig. 1F) immunoreactive magnocellular neurons. Subsequent co-localization of anti-tSTAT3 (Fig. 1G) with anti-GFAP (Fig. 1H) confirmed the presence of tSTAT3-immunoreactivity within GFAP-immunoreactive astrocytes of the ventral glial limitans (Fig. 1I). Together, these results demonstrate that magnocellular neurons and astrocytes of the SON have the potential for signal transduction through the Jak-STAT pathway in the rat SON.

Axotomy of neurons leads to the phosphorylation and nuclear translocation of STAT3 (Haas, et al., 1999, Xia, et al., 2002). Furthermore, STAT3 activation is mediated by endogenous CNTF (Bonni, et al., 1997, Darnell, et al., 1994, Wegenka, et al., 1993), and has been shown to be involved in regenerative axon growth (Liu and Snider, 2001). Thus, activation of STAT3 is considered to be part of the molecular machinery used by some neurons in response to injury, and is most likely associated with post-lesion reorganization processes. To investigate injury-induced STAT3 signaling, immunostaining with an antibody specific for STAT3 phosphorylated at Tyr⁷⁰⁵ was performed in the SON of animals having undergone infundibular nerve crush. Immunoperoxidase labeling with the pSTAT3 antibody demonstrated a number of nuclear pSTAT3 immunoreactive profiles localized to the SON and the ventral glial limitans of the SON (SON-VGL) 3 hrs after infundibular nerve crush (Fig. 2A). The immunoreactivity pattern of pSTAT3 was consistent with its presence on astrocytes. Subsequent dual immunoperoxidase labeling with anti-pSTAT3 and anti-GFAP further demonstrated that the nuclear pSTAT3 was present within astrocytes (Fig. 2B) in the SON.

We next determined the specific cellular phenotypes in which exogenous rrCNTF-induced STAT3 activation occurred. At 1 hr post-pressure injection of 100 ng/ μ l rrCNTF, dual-label immunofluorescent experiments pairing anti-pSTAT3 (Fig. 3A, D) with either anti-OT (Fig. 3B), anti-VP (not shown), or anti-GFAP (Fig. 3E) revealed STAT3 activation within astrocytes (Fig. 3F, arrows), but not OT (Fig. 3C) or VP magnocellular neurons (data not shown). Consistent with the injury-induced activation of STAT3, the inset in Fig. 3F clearly demonstrated that pSTAT3-immunoreactivity is exclusively localized within the GFAP-immunoreactive astrocytes in the SON following exogenous rrCNTF injection.

Quantitative Western blot analysis demonstrated that exogenous rrCNTF (100ng/ μ l, pressure injected) induced a statistically significant increase in pSTAT3 levels at 1 hr post injection (Fig. 4A; one-way ANOVA, $F=44.77$, $p < 0.0001$). At 3 hrs post injection, pSTAT3 levels remained significantly elevated over control values (Fig. 4A; one-way ANOVA, $F=44.77$, $p < 0.0001$); but had decreased significantly from the peak level observed at 1 hr post injection (Fig. 4A; one-way ANOVA, $F=44.77$, $p=0.0285$). Intraperitoneal (IP) administration of 10 mg/kg AG490 1 hr before rrCNTF injection significantly suppressed the phosphorylation of STAT3 (Fig. 4A; one-way ANOVA, $F=44.77$, $p < 0.0001$) and Jak2

(Fig. 4B; one-way ANOVA, $F=12.38$, $p<0.001$) compared to the levels at 1 hr post-CNTF injection without affecting protein levels of tSTAT3 or tJak2, demonstrating that AG490 inhibits activation of the Jak-STAT pathway. Together, these data indicate a rapid and transient activation of STAT3 in astrocytes in the SON following exogenous rrCNTF administration and infundibular nerve crush.

Exogenous rrCNTF promotes the survival of oxytocinergic magnocellular neurons in stationary hypothalamic organotypic explant cultures

We next determined the neuroprotective influence of exogenous rrCNTF on magnocellular neuron survival. To do so we used stationary hypothalamic organotypic cultures that contained intact PVN and SON nuclei and the associated accessory nuclei (ACC). Organotypic cultures exhibit several advantages over other *in vitro* culture systems primarily because of the preservation of the *in vivo* cytoarchitecture and the use of fully differentiated neurons (House, et al., 1998, Vutsits, et al., 1998). We first established the optimum concentration of rrCNTF that significantly increased neuronal survival (Table 1). Our results show that addition of 10 ng/ml rrCNTF to control media for 14 days did not increase the survival of OT magnocellular neurons over control values in either the SON (Table 1; one-way ANOVA, $F=31.64$, $p=0.0768$) or PVN (Table 1; one-way ANOVA, $F=25.45$, $p=0.1798$). However, addition of 25 ng/ml rrCNTF for 14 days increased significantly the number of OT magnocellular neurons in the SON by 587% (Table 1; one-way ANOVA, $F=31.64$, $p<0.0001$) and in the PVN by 83.85% (Table 1; one-way ANOVA, $F=25.45$, $p=0.003$) over control values. Treatment with 50 ng/ml rrCNTF for 14 days did not promote survival above that observed in the 25 ng/ml rrCNTF treatment group in the SON (Table 1; one-way ANOVA, $F=31.64$, $p=0.1260$). Neuronal cell counts demonstrated that administration of 10 or 25 ng/ml rrCNTF for 14 days did not promote the survival of VP neurons in the SON (Table 1; one-way ANOVA, $F=3.059$, $p=0.0804$, $p=0.0588$) or PVN (Table 1; one-way ANOVA, $F=5.822$, $p=0.6083$, $p=0.0159$) of hypothalamic organotypic explant cultures. However, administration of 50 ng/ml rrCNTF for 14 days resulted in a small, but statistically significant, increase in VP magnocellular neurons in the SON (Table 1; one-way ANOVA, $F=3.059$, $p=0.0027$); although, there was a significant reduction in the amount of VP neurons seen in the PVN following exogenous rrCNTF (25 and 50 ng/ml) treatment (Table 1; one-way ANOVA, $F=5.822$, $p=0.0159$, $p=0.0004$). Analysis of control explant culture media demonstrated that the osmolality and pH did not change during the 14 day experimental period, with levels maintained around 310 mmol/kg and 8.1, respectively. Therefore, due to the more robust survival of OT magnocellular neurons following 25 ng/ml rrCNTF administration, all future experiments utilized a concentration of 25 ng/ml rrCNTF.

Figure 5A is a micrograph montage of OT magnocellular neurons from a representative explant culture slice, fixed and immunohistochemically stained, at the time of sacrifice (post-natal day 6). The organization of the OT magnocellular neurons into their respective magnocellular neurosecretory system nuclei (PVN and SON) is typical of what is observed in adult rat magnocellular neurosecretory system nuclei. In addition, Figure 5A also demonstrates the hypothalamo-neurohypophysial tract (arrows), which contains the magnocellular neuron axons that project to the NL. Figure 5B illustrates OT magnocellular neurons from a representative explant slice that received only control media for 14 days. These hypothalamic organotypic explant cultures still exhibit easily distinguishable OT magnocellular neuron cell bodies and processes located within the PVN, SON, and ACC; however, due to the inherent neuronal death associated with the axotomy, there are significantly fewer OT magnocellular neurons present when compared to Figure 5A. Figure 5C illustrates a representative explant slice that received 25 ng/ml rrCNTF for 14 days. When compared to Figure 5B, the substantial increase in the survival of OT magnocellular neurons in the magnocellular neurosecretory system following exogenous rrCNTF

administration is apparent, as well as a prominent increase in OT magnocellular neuron process density throughout all of the magnocellular neurosecretory system nuclei, particularly the PVN (Fig. 5C, arrows), indicating that exogenous rrCNTF may be promoting process outgrowth from OT magnocellular neurons.

In order to confirm that the neuroprotective effects of exogenous rrCNTF were due to the specific protein sequence and tertiary structure of the rrCNTF protein, we had a reverse sequence rat CNTF construct generated. Incubation of hypothalamic organotypic explant cultures in 25 ng/ml reverse rrCNTF for 14 days resulted in OT magnocellular neuron numbers in the SON (Table 2; one-way ANOVA, $F=42.72$, $p=0.2175$) and PVN (Table 2; one-way ANOVA, $F=11.8$, $p=0.8791$) that were not significantly different than what was observed following 14 days of control media treatment. These data are the first reports utilizing a reverse sequence construct of rrCNTF in order to validate the pro-survival effects that exogenous rrCNTF has on injured magnocellular neurons and our results demonstrate that it is the specific protein sequence and tertiary structure of rrCNTF that resulted in the survival of injured OT magnocellular neurons in the SON and PVN.

Pharmacological inhibition of the Jak-STAT pathway prevents rrCNTF-induced oxytocinergic magnocellular neuron survival in hypothalamic organotypic explant cultures

Intracellular signaling pathways known to be activated by CNTF include MAPK-ERK, Jak-STAT and PI3K-AKT pathways (Bonni, et al., 1993, Dolcet, et al., 2001, Park, et al., 2004, Sango, et al., 2008). However, the Jak-STAT intracellular signal transduction pathway is considered the canonical intracellular signaling pathway utilized by CNTF. We demonstrated that pressure injection of exogenous rrCNTF into the SON resulted in a robust increase in STAT3 activation in astrocytes, indicating that CNTF utilizes the Jak-STAT pathway in the SON. Therefore, we determined the contribution of the Jak-STAT pathway to rrCNTF-induced OT magnocellular neuron survival in the SON and PVN (Table 2). We utilized two pharmacological inhibitors of the Jak-STAT pathway that act on two separate and distinct signaling components: AG490, a protein tyrosine kinase inhibitor of Jak2 (Ozog, et al., 2004, Park, et al., 2004), and cucurbitacin I (JSI-124), a selective protein tyrosine kinase inhibitor of STAT3 (Blaskovich, et al., 2003). When the hypothalamic organotypic cultures were treated with 25 ng/ml rrCNTF plus 50 μ M AG490 there was a small, but statistically significant reduction of OT magnocellular neurons by 39% in the SON (Fig. 6A; one-way ANOVA, $F=42.72$, $p=0.0002$) compared to the 25 ng/ml rrCNTF treated group. Within the PVN, the amount of OT neurons in the 25 ng/ml rrCNTF plus 50 μ M AG490 group was not significantly different from the 25 ng/ml rrCNTF treated group (Fig. 6B; one-way ANOVA, $F=11.8$, $p>0.05$); although, subsequent statistical analysis directly comparing the two groups with a Student's t-test demonstrated a slight significant decrease ($p=0.0230$). However, incubation with 25 ng/ml rrCNTF plus 100 μ M AG490 resulted in a statistically significant decrease in OT neurons in the SON (Fig. 6A; one-way ANOVA, $F=42.72$, $p<0.0001$) and PVN (Fig. 6B; one-way ANOVA, $F=11.8$, $p=0.0054$) to control levels. Similarly, inhibition of STAT3 activation via 10 μ M cucurbitacin I in the presence of 25 ng/ml rrCNTF resulted in a statistically significant decrease in OT neurons in the SON (Fig. 6A; one-way ANOVA, $F=42.72$, $p<0.0001$) and PVN (Fig. 6B; one-way ANOVA, $F=11.8$, $p<0.0001$) to control levels. When the hypothalamic organotypic explant cultures were treated with inhibitors alone there was not a statistical difference in the number of OT neurons in the SON or PVN compared to the control media-treated group (Fig. 6A, B; one-way ANOVA), demonstrating that in the absence of rrCNTF, the concentrations of the inhibitors used did not adversely affect OT neuron survival. Altogether, these data demonstrate that pharmacological inhibition of the Jak-STAT pathway prevented CNTF-induced survival of OT neurons in the SON and PVN.

Representative micrographs of the SON further illustrate these results. Although the tissue thickness and complexity of the processes prevented quantitative stereologic analysis, we consistently observed an overall increase in process outgrowth density in the SON following 14 days of 25 ng/ml rrCNTF administration (Fig. 6F) compared to control media treated cultures (Fig. 6C), suggesting that exogenous CNTF may have a role in mediating process outgrowth of OT magnocellular neurons. Moreover, no visual difference was observed in magnocellular neuron process density following inhibition of the Jak-STAT pathway with AG490 (Fig. 6D) or cucurbitacin I (Fig. 6E) compared to control SON (Fig. 6C). Similar results were observed when analyzing individual PVN following pharmacological inhibition of the Jak-STAT pathway (not shown).

Discussion

Injury and CNTF-induced activation of the Jak-STAT pathway in the SON

Specific intracellular phosphorylation cascades mediate responses to distinct extracellular stimuli, such as the response to growth factors or cytokines. Ultimately, an injured cell's ability to grow, survive, differentiate, or die is controlled by the integrated action of numerous phosphorylation cascades. The Jak-STAT intracellular signal transduction pathway is considered the canonical intracellular signaling pathway utilized by CNTF and CNTF has been demonstrated to preferentially phosphorylate STAT3 at Tyr⁷⁰⁵ (Bonni, et al., 1997, Darnell, et al., 1994, Wegenka, et al., 1993). In the current study we identified tSTAT3-immunoreactivity within the neurons and astrocyte cell bodies and processes throughout the SON. Moreover, tSTAT3-immunoreactivity was highly localized to the SON and not within the adjacent optic chiasm or surrounding hypothalamic region. These findings demonstrate specific localization of the Jak-STAT pathway to the SON.

We previously hypothesized that CNTF plays a role in the axonal reorganization of magnocellular neurons in the SON following injury (Askvig, et al., 2012, Lo, et al., 2008, Watt, et al., 2006, Watt, et al., 2009). In support of this hypothesis we demonstrated that following axotomy CNTF and the CNTF receptor components are increased in the SON both during the period of axonal sprouting and neuronal injury (Askvig, et al., 2012). Others have shown that injury to the lens of the eye resulted in STAT3 activation in retinal ganglion cells along with elevated levels of endogenous CNTF in retinal astrocytes during the period of axonal regeneration in the rat retina (Muller, et al., 2007). Therefore, it has been postulated that axonal sprouting is promoted by CNTF-mediated activation of the Jak-STAT pathway. Consistent with those reports, we demonstrated that axonal injury resulted in activation of STAT3 in astrocytes in the SON. Consequently, these data, in conjunction with our previous reports demonstrating the post-injury increases in CNTF expression, are supportive of our hypothesis that CNTF is involved in the post-injury response, via astrocytic STAT3 activation, in the SON. However, to date, CNTF-induced activation of the Jak-STAT pathway in the SON has not been reported. Thus, in order to assess the activation of the Jak-STAT pathway, we pressure injected exogenous rrCNTF directly over the SON.

CNS cells in general have comparable levels of tSTAT3, but they display quantitatively different levels of pSTAT3 following activation of the STAT3 pathway (MacLennan, et al., 2000). A single intravitreal injection of Axokine (Regeneron), a synthetic analog of CNTF, induced rapid and persistent activation of STAT3 in retinal Müller cells, astrocytes, and retinal ganglion cells (Peterson, et al., 2000). A rapid and transient activation of STAT3 was also observed in facial motor neurons, spinal motor neurons, and cranial motor neurons following a single injection of rrCNTF (MacLennan, et al., 2000). In accordance with these findings, our data revealed that a single pressure injection of rrCNTF directly over the SON resulted in a pronounced, but transient activation of STAT3 in astrocytes of the SON. We did not observe pSTAT3-immunoreactivity in magnocellular neurons following rrCNTF

injection. This is in accordance with our previous observation that magnocellular neurons in the SON are not immunoreactive for LIFR β (Askvig, et al., 2012), which CNTF requires to mediate an intracellular signaling cascade (Davis, et al., 1993). Consistent with our results, neurons of the hippocampus, neocortex, cerebellar cortex, and olfactory bulb express CNTFR α and tSTAT3, yet CNTF administration does not lead to STAT3 activation within these neurons (MacLennan, et al., 2000). Similarly, it was recently demonstrated that intrahippocampal injection of exogenous CNTF activated STAT3 in astrocytes and not neurons (Bechstein, et al., 2012). Altogether, these results indicate a cell-type-dependent regulation of CNTF-mediated STAT3 activation in the SON.

The role of the Jak-STAT pathway in CNTF-induced neuronal survival

Stationary hypothalamic organotypic cultures exhibit several advantages over other *in vitro* culture systems primarily because of the preservation of the *in vivo* cytoarchitecture and the use of fully differentiated neurons (House, et al., 1998, Vutskits, et al., 1998). Moreover, the ability to directly manipulate the culture media with growth factors and pharmacological agents and assess magnocellular neuron survival in hypothalamic organotypic cultures provides our lab several advantages over *in vivo* injury model systems. Exogenous rrCNTF has been shown to promote the survival of injured magnocellular neurons in hypothalamic organotypic explant cultures (House, et al., 2009, Rusnak, et al., 2002, Rusnak, et al., 2003, Vutskits, et al., 1998, Vutskits, et al., 2003). In the present study we demonstrated that hypothalamic organotypic explant cultures exposed to 25 ng/ml rrCNTF for 14 days had significantly more OT magnocellular neurons in the SON and PVN compared to explant cultures not treated with rrCNTF. Furthermore, the effects that exogenous rrCNTF has on injured magnocellular neurons was validated by the use of a reverse sequence construct of rrCNTF, which demonstrated that it is the specific protein sequence and tertiary structure of rrCNTF that resulted in the survival of injured OT magnocellular neurons in the SON and PVN.

Others have reported that exogenous rrCNTF has a greater effect on VP magnocellular neuron survival in the SON (Rusnak, et al., 2002) and the PVN (Vutskits, et al., 1998) compared to the observed effect on OT magnocellular neurons. However, in our hands VP magnocellular neurons did not survive in the presence of 25 ng/ml rrCNTF in the SON or PVN. It should be noted that, unlike Vutskits et al. (1998), we did not differentiate between parvocellular and magnocellular neurons within the PVN, therefore our analysis of the PVN contains a heterogeneous population of neurons. Kusano et al. (1999) demonstrated that VP magnocellular neurons survived at 255 mOsm/l but not at 300 and 330 mOsm/l culture media, while the survival of OT magnocellular neurons did not change from 255 to 330 mOsm/l culture media. The osmolality of the culture media used in the current study was maintained at 310 mOsm/l. Therefore, future studies utilizing 255 mOsm/l culture media will be performed to promote VP magnocellular neuron survival in the SON and PVN.

In the present study we utilized the pharmacological inhibitor, AG490, which specifically inhibits the tyrosine phosphorylation of Jak2, and cucurbitacin I, which specifically inhibits the tyrosine phosphorylation of STAT3. Our results demonstrated that in the presence of AG490 and cucurbitacin I the pro-survival responses of rrCNTF on OT neurons were completely abolished. These results are in agreement with others that have demonstrated that CNTF promotes neuronal survival through the Jak-STAT pathway (Bonni, et al., 1993, Dolcet, et al., 2001, Lutticken, et al., 1994, Muller, et al., 2009, Park, et al., 2004, Peterson, et al., 2000, Rhee, et al., 2004, Sango, et al., 2008, Symes, et al., 1994). In conjunction with our *in vivo* observations, these data indicate a paracrine mechanism of CNTF-mediated magnocellular neuron survival through associated astrocytes, which was initially hypothesized by Rusnak et al. (2003). This conclusion is supported by the observations that while CNTF promotes survival of magnocellular neurons in organotypic cultures (House, et

al., 2009, Rusnak, et al., 2002, Rusnak, et al., 2003, Vutskits, et al., 1998, Vutskits, et al., 2003), exogenous CNTF did not promote survival of cultured magnocellular neurons that are dissociated from astrocytes (Rusnak, et al., 2003). However, the astrocytic factor or factors that CNTF produces via the Jak-STAT pathway remain to be determined.

Various reports have demonstrated that neuronal survival can be stimulated by an astrocytic response. For example, Müller and Seifert (1982) demonstrated that a neurotrophic factor is produced and released by primary astrocyte cultures that promotes the survival of cultured hippocampal neurons. Furthermore, the combination of neurotrophic factors produced by astrocytes and membrane-bound molecules that mediate cell-to-cell interactions resulted in the long-term survival of neurons in culture (Schmalenbach and Muller, 1993). Consistent with our findings, others have demonstrated that CNTF promotes the survival of hippocampal (Bechstein, et al., 2012) and retinal neurons (Peterson, et al., 2000) via activation of the Jak-STAT pathway in glia cells; however, the CNTF-induced glia-derived factor(s) directly affecting neuronal survival are not known. CNTF has been demonstrated to increase the production of multiple neuroprotective proteins in glial cells including; vasoactive intestinal peptide (VIP) (Jones and Symes, 2000, Pitts, et al., 2001, Symes, et al., 1997, Symes, et al., 1994), connexin 43 (Ozog, et al., 2004, van Adel, et al., 2005), fibroblast growth factor-2 (FGF-2) (Albrecht, et al., 2002, Wang, et al., 2008), FGF receptor 1 (FGFR-1) (Jiang, et al., 1999), insulin-like growth factor type 1 receptor (IGF-IR) (Jiang, et al., 1999), nerve growth factor (NGF), p75 low-affinity receptor (Semkova and Kriegstein, 1999), and glial cell-line derived neurotrophic factor (GDNF) (Kradly, et al., 2008). CNTF also increases the function of glial glutamate transporters (GT), glutamate/aspartate transporter-1 (GLAST-1), and glutamine synthetase, which protected striatal neurons (Beurrier, et al., 2010) and retinal ganglion cells (van Adel, et al., 2005) against insult. In addition, the cell adhesion molecule polysialic acid neural cell adhesion molecule (PSA-NCAM), which is localized to magnocellular neuron dendrites and axons and astrocytes in the SON (Theodosios, et al., 1991), was demonstrated to be necessary for CNTF-mediated survival of OT and VP magnocellular neurons (Vutskits, et al., 2003), possibly through the mediation of astrocyte-neuronal interactions. Interestingly, while the CNTF-family member leukemia inhibitory factor (LIF) also promotes the survival of OT and VP magnocellular neurons, PSA-NCAM is only involved in CNTF-mediated magnocellular neuron survival (Vutskits, et al., 2003). Therefore, in order to better understand the mechanism of CNTF-induced magnocellular neuron survival in the SON, studies are currently underway to determine possible transcriptional products (i.e. growth factors, cellular adhesion molecules, and/or transcription factors) of the CNTF-induced astrocytic Jak-STAT pathway.

It was initially hypothesized that CNTF functions as a protective factor that is activated after an injury (Sendtner, et al., 1990). Siegel et al. (2000) hypothesized that since axonal sprouting also occurs as a result of neuronal injury, a factor that becomes active upon injury, such as CNTF, might also be involved in a sprouting response. Numerous reports have demonstrated that CNTF promotes motoneuron sprouting (Gurney, et al., 1992, Guthrie, et al., 1997, Kwon and Gurney, 1994, Oyesiku and Wigston, 1996, Siegel, et al., 2000, Simon, et al., 2010, Ulenkate, et al., 1994, Wright, et al., 2007, Xu, et al., 2009) and process outgrowth of retinal ganglion cells (Leibinger, et al., 2009, Muller, et al., 2007, Muller, et al., 2009). Furthermore, CNTF has been implicated in hypothalamic magnocellular neuron sprouting *in vitro* (Vutskits, et al., 1998). In the present study, our data suggests that CNTF induces process outgrowth from OT magnocellular neurons, but it remains to be determined if the Jak-STAT pathway is involved in CNTF-induced process outgrowth. However, in response to CNTF, others have demonstrated differential effects of the Jak-STAT, PI3-AKT, and MAPK-ERK pathways in mediating cell survival versus process outgrowth (Alonzi, et al., 2001, Ozog, et al., 2008, Sango, et al., 2008). Thus, CNTF may mediate differential

neuroprotective responses via different intracellular signal transduction pathways. Accordingly, studies utilizing pharmacological inhibitors of other intracellular signal transduction pathways in hypothalamic organotypic cultures are currently underway.

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Abbreviations

CNTF	ciliary neurotrophic factor
CNTFRα	ciliary neurotrophic factor receptor alpha
GFAP	glial fibrillary acidic protein
gp130	glycoprotein 130
Jak-STAT	janus kinase-signal transducer and activator of transcription
LIFRβ	leukocyte inhibitory factor receptor beta
OT	oxytocinergic
PVN	paraventricular nucleus
ROD	relative optical density
SON	supraoptic nucleus
VP	vasopressinergic

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Highlights

> Injury and exogenous rrCNTF activates STAT3 in astrocytes of the SON. > Exogenous rrCNTF promotes survival and process outgrowth of magnocellular neurons. > The Jak-STAT pathway mediates CNTF-induced neuronal survival in the SON and PVN.

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Fig. 1. Localization of tSTAT3 in the SON

Dual-label immunofluorescence experiments pairing anti-tSTAT3 (A, D, and G) with anti-OT (B), anti-VP (E), and anti-GFAP (H) revealed tSTAT3-immunoreactivity within OT (C) and VP (F) neurons and GFAP-immunoreactive astrocytes (I) of the SON (arrows). GFAP, glial fibrillary acidic protein; OC, optic chiasm; OT, oxytocin; tSTAT3, total STAT3; VP, vasopressin. Scale bar A-F = 75 μ m; G-I = 150 μ m.

Fig.2. pSTAT3 immunoreactivity in SON following infundibular nerve crush

(A) pSTAT3 immunoreactivity in the SON 3 hours post infundibular nerve crush demonstrated an increase in circular pSTAT3 immunoreactive profiles, confined to the SON-VGL and the SON (arrows). (B) Dual-label immunoperoxidase pairing anti-pSTAT3 (purple) with anti-GFAP (brown) confirmed the presence of pSTAT3 within GFAP-immunoreactive astrocytes in the parenchyma of the SON (arrows). Although obscured, pSTAT3-immunoreactivity can be discerned in the SON-VGL. Scale bar = 100 μ m.

Fig. 3. Localization of pSTAT3 in the SON following exogenous rrCNTF injection

At 1 hr post-pressure injection of 100 ng/ μ l rrCNTF, dual-label immunofluorescent experiments pairing anti-pSTAT3 (A, D) with anti-OT (B) and anti-GFAP (E) revealed complete co-localization of pSTAT3 with GFAP-immunoreactive astrocytes (F) in the ventral glial limitans of the SON (arrows) and the SON parenchyma (arrowheads), but not in OT (C, asterisks) or VP (not shown) magnocellular neurons. The inset in (F) clearly demonstrated pSTAT3-immunoreactivity within the GFAP-immunoreactive astrocytes in the SON. GFAP, glial fibrillary acidic protein; OC, optic chiasm; OT, oxytocin; pSTAT3, phosphorylated STAT3. Scale bar A-F = 25 μ m; inset in F = 10 μ m.

Fig. 4. Exogenous rrCNTF resulted in the rapid and transient activation of STAT3 in the rat SON that is inhibited by AG490

(A) Western blot analysis of dissected SON samples demonstrated that acute pressure injection of 100 ng/μl rrCNTF induced a statistically significant increase in pSTAT3 levels in the SON at 1 hr post-CNTF-injection. At 3 hrs post-CNTF-injection pSTAT3 levels still remained significantly elevated compared to contralateral control and vehicle-infused control SON; however, the pSTAT3 levels at 3 hrs post-CNTF-injection were significantly decreased from the pSTAT3 levels at 1 hr post-CNTF-injection. Administration of AG490 (10 mg/kg IP) 1 hour prior to rrCNTF injection significantly decreased the pSTAT3 levels to levels that are not significantly different from vehicle-infused control SON but still significantly elevated from contralateral control SON. (B) Moreover, Western blot analysis demonstrated the level of pJak2 following AG490 inhibition is significantly decreased from all other groups ($p < 0.001$), while the pJak2 levels in the control and rrCNTF injected SON do not differ significantly. Column bars and error bars represent the mean and SEM of 6 groups. Each group represents isolated SON pooled from three rats. In order to determine the amount of total protein that is phosphorylated, the ROD of the phosphorylated protein was normalized to the ROD of the total protein, as opposed to the ROD of β-actin, to obtain a ratio used for statistical analysis. pJak2, phosphorylated Jak2; pSTAT3, phosphorylated STAT3; ROD, relative optical density; tJak2, total Jak2; tSTAT3, total STAT3. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

Fig. 5. Micrograph montages of magnocellular neurosecretory system nuclei in hypothalamic organotypic cultures immunohistochemically stained for OT magnocellular neurons
 (A) Micrograph montage of magnocellular neurosecretory system nuclei of a representative explant slice fixed and immunohistochemically stained for OT magnocellular neurons at the time of sacrifice (post-natal day 6). Note the hypothalamo-neurohypophyseal tract (A, arrows), which contains the magnocellular neuron axons that project to the NL. (B) Micrograph of OT magnocellular neurons of a representative explant slice that received control media for 14 days. Note the preservation of magnocellular neurosecretory system nuclei organization. (C) Montage of OT magnocellular neurons from a representative explant slice that received 25 ng/ml rrCNTF treatment for 14 days. Note the substantial

increase in OT magnocellular neurons in the SON and PVN as well as the maintenance of process density following rrCNTF treatment (arrows) compared to control (B). Note that the representative images were obtained from approximately the same level of the magnocellular neurosecretory system, which is apparent when comparing the III ventricle (III) between the images. ACC, accessory nuclei; III, III ventricle; PVN, paraventricular nucleus; SON, supraoptic nucleus. Scale bar =500 μ m.

Fig. 6. The Jak-STAT pathway is necessary to mediate the CNTF-induced survival of OT neurons in the SON and PVN

Immunohistochemical neuronal cell counts demonstrated that exogenous rrCNTF promoted the survival of OT neurons while inhibition of the Jak-STAT pathway with AG490 or cucurbitacin I significantly reduced the number of surviving OT neurons in the SON (A) and PVN (B). Note the substantial increase in neuron numbers in the SON following rrCNTF administration (F) compared to the control media group (C), and the groups receiving the pharmacological inhibition of the Jak-STAT pathway (D, E). Column bars and error bars represent the mean and SEM of [n] groups. Cucu I, cucurbitacin I; PVN, paraventricular nucleus; SON, supraoptic nucleus. *** $p < 0.0001$. Scale bar = 100 μm.

Table 1
Survival of Rat Hypothalamic Oxytocinergic (OT) and Vasopressinergic (VP) Neurons in the SON and PVN *In Vitro*.

Nuclei/Phenotype	Intact P6	Control	10 ng/ml rrCNTF	25 ng/ml rrCNTF	50 ng/ml rrCNTF
SON/OT	878.6±78.8 (15)	28.85±6.75 (23)	62.4±10.51 (15)	198.3±17.13 (26) ***	156.9±19.71 (17) ***
SON/VP	529.1±72.5 (14)	4.62±0.99 (24)	8.33±2.10 (16)	10.97±3.53 (15)	14.24±2.71 (19) *
PVN/OT	1092±89.2 (15)	71.11±8.82 (23)	53.17±5.69 (15)	130.7±10.44 (26) ***	160.8±8.18 (17) ***
PVN/VP	267.8±32.9 (14)	37.92±5.02 (24)	31.36±6.41 (16)	19.78±3.50 (15) *	10.82±2.03 (19) **

Mean = cells/filter ±SEM (n)

n = number of filters which is equivalent to number of neonatal hypothalami.

Asterisks represent significant differences compared to control in same row

* p<0.05

** p<0.01

*** p<0.0001.

Table 2
Survival of Rat Hypothalamic Oxytocinergic (OT) Neurons Following Pharmacological Inhibition in the SON and PVN *In Vitro*.

Nuclei/Phenotype	Control	Reverse CNTF (25 ng/ml)	25 ng/ml rrCNTF	50 μ M AG490	25 ng/ml rrCNTF + 50 μ M AG490	100 μ M AG490	25 ng/ml rrCNTF + 100 μ M AG490	10 μ M Cucu I	25 ng/ml rrCNTF + 10 μ M Cucu I
SON/OT	35.6 $\pm$ 5.9 (15) [#]	45.4 $\pm$ 4.9 (20) [#]	176.6 $\pm$ 13.1 (21) ^{***}	39.5 $\pm$ 5.6 (16) [#]	107.3 $\pm$ 11.5 (26) ^{***#}	24.6 $\pm$ 3.1 (16) [#]	65.9 $\pm$ 5.9 (25) [#]	35.0 $\pm$ 5.3 (19) [#]	19.9 $\pm$ 1.6 (23) [#]
PVN/OT	75.3 $\pm$ 7.6 (15) [#]	73.8 $\pm$ 6.5 (20) [#]	120.8 $\pm$ 7.7 (21) ^{***}	80.5 $\pm$ 8.6 (16) [#]	93.7 $\pm$ 8.2 (26)	60.3 $\pm$ 8.1 (16) [#]	87.6 $\pm$ 8.1 (25) [#]	44.0 $\pm$ 3.7 (19) [#]	45.1 $\pm$ 3.3 (23) [#]

Mean = cells/filter $\pm$ SEM (n)

n = number of filters which is equivalent to number of neonatal hypothalami.

Asterisks represent significant differences compared to control

p<0.0001.

[#] significantly different from 25 ng/ml rrCNTF in the same column ($p<0.05$).