Ciliary neurotrophic factor signaling in the magnocellular neurosecretory system: mechanisms of neuronal survival and sprouting

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CILIARY NEUROTROPHIC FACTOR SIGNALING IN THE MAGNOCELLULAR NEUROSECRETORY SYSTEM: MECHANISMS OF NEURONAL SURVIVAL AND SPROUTING

by

Jason Michael Askvig

Master of Science, the University of North Dakota, 2008

A Dissertation
Submitted to the Graduate Faculty
of the
University of North Dakota
In partial fulfillment of the requirements

for the degree of
Doctor of Philosophy

Grand Forks, North Dakota
December 2012
This dissertation, submitted by Jason M. Askvig in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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This dissertation meets the standards for appearance, conforms to the style and format requirements of the Graduate School of the University of North Dakota, and is hereby approved.

Wayne Swisher, PhD
Dean of the Graduate School

November 19, 2012
Date
PERMISSION

Title: Ciliary Neurotrophic Factor Signaling in the Magnocellular Neurosecretory System: Mechanisms of Neuronal Survival and Sprouting

Department: Anatomy and Cell Biology

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Jason M. Askvig
November 19, 2012
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For my best friend

Janelle
ABSTRACT

While it is well-known that ciliary neurotrophic factor (CNTF) promotes the survival of a myriad of neuronal phenotypes, little is understood on the specific CNTF-induced neuroprotective mechanisms in the central nervous system (CNS). Elucidation of these mechanisms may provide insight that would assist in the development of future therapeutic strategies. Therefore, the goal of my research is to determine the mechanisms underlying the neuroprotective effects of CNTF within the rat magnocellular neurosecretory system. The central hypothesis of my research is that CNTF acts as both a neuronal survival and sprouting factor for hypothalamic magnocellular neurons in vitro and in vivo. In order to determine the neuroprotective mechanisms that CNTF utilizes, we performed extensive animal work including, in vivo stereotaxic surgeries and in vitro organotypic explant cultures, in addition to Western blot and immunohistochemical analyses. Our analyses demonstrated that following injury to the magnocellular neurosecretory system, CNTF is up-regulated in the supraoptic nucleus (SON) that undergoes a collateral sprouting response and this increase is not due to heightened neurosecretory activity. Moreover, we demonstrated a reduction in the protein levels of the CNTF specific receptor, CNTFRα, in the mature SON. Thus, we believe that the inability of the mature rat to elicit the sprouting response is due to a deficit in CNTF signaling. CNTF has been demonstrated to promote survival and potentiate process outgrowth of...
magnocellular neurons. Utilizing hypothalamic organotypic cultures, we demonstrated that the Jak-STAT and MAPK-ERK\(\frac{1}{2}\) pathways are necessary for CNTF to mediate its pro-survival effects on oxytocinergic neurons. Conversely, we demonstrated that the PI3K-AKT pathway is necessary to promote CNTF-induced process outgrowth. Interestingly, our results show that the p38, JNK, and mTOR signaling components appear to mediate the post-axotomy apoptotic cascade that results in the significant loss of oxytocinergic neurons following the organotypic culture preparation. Altogether, these novel data provide evidence of the mechanisms that CNTF utilizes to elicit its neuroprotective actions. We submit that gaining a greater understanding of these mechanisms will lead to the development of future therapeutic strategies that will target nervous system injuries or disorders that result in neurodegenerative processes.
CHAPTER I
INTRODUCTION

Magnocellular Neurosecretory System

General Anatomy

The magnocellular neurosecretory system is characterized anatomically as being comprised of oxytocinergic and vasopressinergic magnocellular neurons in the supraoptic (SON) and paraventricular nuclei (PVN) of the hypothalamus, their non-myelinated axons that form the hypothalamo-neurohypophysial tract, and their axon terminals located in the neural lobe (NL; neurohypophysis, posterior pituitary) (Scharrer, 1954). Axons of the PVN and SON project ventromedially and dorsomedially, respectively, towards the dorsal part of the optic chiasm. From there the axons project posteromedially and travel through the median eminence, a structure located at the floor of the third ventricle. Within the median eminence, axons originating from the magnocellular neurons of the SON decussate in the internal zone of the median eminence, while axons from the PVN parvocellular neurons remain ipsilateral and terminate in the external zone of the median eminence (Silverman & Zimmerman, 1983). For the PVN and SON magnocellular axons continuing to the NL they course through the infundibulum, a stalk that connects the median eminence with the NL, and terminate in the NL where axon terminals, upon stimulation, release
oxytocin and vasopressin into surrounding fenestrated capillaries leading to the general circulation.

**Physiology of Magnocellular Neurons**

Most magnocellular neurons contain mRNA for both oxytocin and vasopressin, but in different amounts (Mezey & Kiss, 1991; Xi, Kusano, & Gainer, 1999). However, typically hypothalamic magnocellular neurons produce either oxytocin or vasopressin. Oxytocin and vasopressin are produced in the form of precursor molecules, prohormones, from which they are processed after translation to biologically active proteins by specific enzymatic cleavages. The prohormones also encode for the carrier protein neurophysin, which interacts with the biologically active hormones following post-translational processing (Brownstein, 1983). Following the production of the oxytocin and vasopressin prohormones in the neuronal somata, the prohormones are packaged in immature neurosecretory vesicles and transported down axons to the nerve varicosities and terminals in the NL. The specific enzymes necessary for prohormone processing are localized in the neurosecretory vesicles (Kanmera & Chaiken, 1985); thus, the prohormones are processed into hormones and associated proteins during the axonal transport of the neurosecretory vesicles. The biologically active hormones are stored with neurophysin in the neurosecretory vesicles in the NL until stimulation induces the release of the hormones into capillary plexus within the NL. Upon physiological stimulation, an action potential, initiated in the neuronal somata, propagates down the axon causing calcium to enter the neurosecretory ending, resulting in exocytosis of the
neurosecretory granules. Fusion of the granular membrane with the neurosecretory membrane results in oxytocin or vasopressin entering the perivascular space of the NL, through which the hormones diffuse into the fenestrated capillary vessels surrounding the NL (Hatton, 1990). While oxytocin and vasopressin may both be released following the same stimuli (Miyata & Hatton, 2002), each peptide is generally released preferentially in response to specific physiological stimuli.

Oxytocin is released into the general circulation during parturition, lactation, and osmotic challenge in the rat (for review see; Leng, Caquineau, & Sabatier, 2005; Poulain & Wakerley, 1982). While oxytocin is important for parturition, it is essential for the milk ejection reflex that stimulates the release of milk during lactation (Young et al., 1996). In response to suckling of nursing pups, neuronal stimuli trigger the production and release of oxytocin into the general circulation surrounding the NL, after which, oxytocin acts on the myoepithelial cells of the mammary gland to cause contraction of the smooth muscle which allows the milk to be delivered to the suckling pups. In the rat, pups suckle continuously but are only rewarded with milk every 5-15 minutes (Leng, et al., 2005). This is a result of the oxytocinergic neurons firing in bursts, resulting in oxytocin being released in pulses which leads to effective milk let down (Leng, et al., 2005). The result is a large dose of oxytocin released at one time, which is necessary for mammary gland function because the mammary gland best responds to high concentrations of oxytocin and is rapidly desensitized in response to continued exposure (Leng, et al., 2005). Although bolus injection of
vasopressin can elicit milk ejections, it is much less potent than oxytocin (Bisset et al., 1967), and vasopressinergic neurons do not show synchronized burst firing, and few otherwise respond during suckling (Lincoln & Wakerley, 1974). For this reason, vasopressin cannot substitute for oxytocin during lactation.

The primary physiological role of vasopressin is to conserve body water and maintain plasma osmolality (Verney, 1947). Consequently, in response to osmotic stress such as dehydration or denial of water, vasopressin is produced and released into the general circulation to help conserve water (for review see; Baylis, 1989). In addition, vasopressin in high concentrations raises blood pressure by inducing moderate vasoconstriction (Dunn, Brennan, Nelson, & Robertson, 1973); however, compared to increases in plasma osmolality, it is a less sensitive signal of vasopressin secretion. When the osmolality of the plasma increases, cells involved in sensing changes in body fluid osmolality, called osmoreceptors, have been shown to excite magnocellular neurons leading to the secretion of vasopressin from the NL (Bourque, Oliet, & Richard, 1994). Centrally, osmoreceptors are located in the subfornical organ (SFO) and organum vasculosum lamina terminalis (OVLT) (Bourque, et al., 1994), which are areas that lack the blood-brain-barrier (BBB) and can monitor and respond to changes in plasma osmolality. Tracer studies have demonstrated that the SON receives afferent connections from both the SFO and OVLT (W. A. Anderson, Bruni, & Kaufmann, 1990) and that electrical stimulation within each of these structures has been shown to modify the excitability of the magnocellular neurons (Ferguson, Day, & Renaud, 1984; Renaud, Cunningham, Nissen, &
Yang, 1993). Once vasopressin is in the body's general circulation it will travel to
the kidney where it will bind to the vasopressin V₂ receptors on the principal cell
of the distal tubule and collecting duct. This results in vesicles containing
aquaporin-2 water channels to be incorporated in the luminal membrane of the
principal cell, which increases the water permeability of the distal tubules and
collecting ducts to conserve body water (Fenton, Brond, Nielsen, & Praetorius,
2007).

Anatomy of the Supraoptic Nucleus

The SON are paired hypothalamic nuclei that are positioned immediately
lateral to the optic chiasm and dorsal to the ventral pial surface of the brain. A
homogeneous population of neurons, magnocellular neurons, comprise the
somatic region of the SON and the magnocellular neurons produce the
neuropeptides oxytocin and vasopressin (Silverman & Zimmerman, 1983). The
magnocellular neurons are densely packed throughout the SON (Sofroniew &
Glasmann, 1981) and are spatially organized so that generally oxytocinergic
magnocellular neurons tend to occupy the more dorsal and rostral parts of the
somatic zone while the more ventral and caudal regions contain mostly
vasopressinergic magnocellular neurons (Swaab, Pool, & Nijveldt, 1975;
Vandesande & Dierickx, 1975); however, there is a lot of mixing in the central
regions of the SON (Hou-Yu, Lamme, Zimmerman, & Silverman, 1986).

Immunohistochemical analysis demonstrated that the somata of
magnocellular neurons are round or oval in shape and have a diameter of
20-35 μm (Sofroniew & Glasmann, 1981), hence the term magnocellular.
Magnocellular neurons are classified as multipolar neurons (Sofroniew & Glasmann, 1981) and have two or three dendrites and a single non-myelinated axon arising from the primary dendrite (Glenn Hatton, personal communication) that projects dorsomedial to course over the dorsal extent of the optic chiasm (Silverman & Zimmerman, 1983). In the rat, the dendrites of the magnocellular neurons in the SON project ventrally from the somatic zone into the dendritic zone, a region that also contains numerous astrocytic processes. Similar to the magnocellular neuron axons, neurosecretory granules packaged with oxytocin or vasopressin are found in magnocellular neuron dendrites, and in response to physiological stimuli oxytocin and vasopressin may be released by exocytosis from these dendrites into the extracellular space of the SON (Ludwig, 1998). The dendritic release of oxytocin and vasopressin promotes the magnocellular neurons to stay active during increased neurosecretory activity because V₁ vasopressin receptors are located on vasopressinergic magnocellular neurons (Berlove & Piekut, 1990; Hurbin, Orcel, Alonso, Moos, & Rabie, 2002) and oxytocin receptors are located on oxytocinergic magnocellular neurons in the SON (Freund-Mercier, Stoeckel, & Klein, 1994), which is of major importance for the fine-tuning of neurosecretory activity in the NL (Gouzenes, Desarmenien, Hussy, Richard, & Moos, 1998).

The predominant glia cells in the SON are astrocytes, with ramified microglia (Mander & Morris, 1994) and oligodendrocytes, which myelinate afferent axons, seldom seen in the unstimulated SON (Miyata & Hatton, 2002). Although, an increase in activated microglia in the SON is observed following
chronic osmotic stimulation (Ayoub & Salm, 2003). Within the SON, the majority of the astrocytic cell bodies are located in the ventral glial limitans of the SON (SON-VGL), which is ventral to the dendritic zone and dorsal to the underlying pia mater. These astrocytes have a morphology reminiscent of radial glia in the developing CNS and are immunoreactive for the intermediate filaments glial fibrillary acidic protein (GFAP) and vimentin, with the latter prominently present in developing glia (Bonfanti, Poulain, & Theodosis, 1993). Under normal conditions the astroglial processes project dorsally throughout the parenchyma of the SON and occupy the spaces in between the densely packed magnocellular neuron somata, forming physical barriers by interposing between adjacent SON dendrites and somata (W. E. Armstrong, Scholer, & McNeill, 1982; Bonfanti, et al., 1993). Within the parenchyma of the SON there is a second phenotype of astrocytes. These astrocytes have a stellate morphology and while they are immunoreactive for GFAP they do not exhibit vimentin-immunoreactivity (Bonfanti, et al., 1993; Miyata & Hatton, 2002). Altogether, the astrocyte processes of the SON contribute to an extensive ensheathment of the magnocellular neurons that are necessary for the maintenance of the SON.

Anatomy of the Paraventricular Nucleus

While the SON is a homogenous mixture of magnocellular neurons, the PVN is a heterogeneous mixture of two neuronal cell types, the large magnocellular neurons and the smaller parvocellular neurons (Gurdjian, 1927; Krieg, 1932). Eight distinct subdivisions, three of which are magnocellular, are found in standard Nissl preparations (Swanson & Kuypers, 1980). The
magnocellular divisions can be divided into anterior, medial, and posterior subdivisions. The posterior is the largest magnocellular subdivision and corresponds to the magnocellular division of the PVN referred to by most authors (Swanson & Sawchenko, 1983). The posterior subdivision forms a compact cell mass at the lateral margin of the PVN and can be further divided into medial and lateral halves (Hatton, Hutton, Hoblitzell, & Armstrong, 1976). The parvocellular division of the PVN consists of five distinct subdivisions; anterior, dorsal, lateral, medial, and periventricular. Cell counts estimate that the magnocellular division of the PVN contains approximately 1300-2000 neurons (Bandaranayake, 1971; Bodian & Maren, 1951; Olivecrona, 1957), while the parvocellular division contains around 7000 neurons (Sawchenko & Swanson, 1981b). The bulk of the parvocellular neurons are immunoreactive for biologically active substances other than oxytocin or vasopressin, including but not limited to; somatostatin (Sawchenko & Swanson, 1982), dopamine (Swanson et al., 1981), and corticotropin releasing factor (CRF) (Sawchenko, Swanson, & Joseph, 1982). The majority of oxytocinergic and vasopressinergic neurons in the PVN are magnocellular, with only 20-30% classified as parvocellular (Sawchenko & Swanson, 1982).

The magnocellular neurons of the PVN are similar to those in the SON. They are multipolar neurons with dendritic processes that remain within the confines of the nucleus, although the more caudal groups have some dendrites projecting away from the nucleus (W. E. Armstrong, Warach, Hatton, & McNeill, 1980). Collectively, all of the axons of the PVN neurons leave the nucleus and
contribute to the hypothalamo-neurohypophysial tract by projecting ventrolaterally, coursing above or below the fornix, and the arching posteroverventromedially toward the median eminence (Krieg, 1932). Within the median eminence, all of the PVN axons remain ipsilateral and the parvocellular axons terminate in the external zone of the median eminence and the infundibular stalk, while the magnocellular axons terminate in the NL. Interestingly, direct soma-soma interactions are observed quite frequently between magnocellular neurons in the ipsilateral PVN and SON (Tweedle & Hatton, 1976, 1977). Furthermore, certain magnocellular subdivisions of the PVN have an afferent input to the contralateral PVN (Silverman, Hoffman, & Zimmerman, 1981). These observations indicate that the internuclear connections may facilitate neurosecretion during states of increased neurosecretory activity.

Similar to the SON, the predominant glia cell in the PVN are astrocytes. However, unlike the SON that contains two distinct phenotypes of astrocytes, the astrocytes in the PVN are primarily stellate. In the unstimulated rat, the stellate astrocytes of the PVN express GFAP but do not express vimentin (Yuan, Scott, So, & Wu, 2007). However, in response to hypophysectomy, which will only lesion the PVN magnocellular and not the parvocellular axons, the stellate astrocytes increase their expression of the immature intermediate filaments, vimentin and nestin, in the magnocellular divisions of the PVN (Yuan, et al., 2007). These observations indicate that astrocytes in magnocellular subdivisions of the PVN can be activated by injury.
Anatomy of the Neural Lobe

The NL is primarily occupied by thousands of neurosecretory axons and their terminals from the SON, PVN, and the accessory magnocellular cell groups, which include the anterior and posterior fornical nuclei, the nucleus circularis, and the nucleus of the medial forebrain bundle (Silverman & Zimmerman, 1983). In addition, the NL contains an extensive network of fenestrated capillaries and several intrinsic populations of glia cells including, pituicytes and microglia (Vazquez-Lopez, 1942). Pituicytes, which are astrocytic in nature, are immunoreactive for GFAP and are the only cell bodies of neural origin in the NL (Lederis, 1965; Salm, Hatton, & Nilaver, 1982). Of the three nucleated cell types found in the NL, the majority are pituicytes, which account for 53% of all the nucleated cells followed by endothelia at 34%, and microglia at 13% (Pow, Perry, Morris, & Gordon, 1989). Of the microglia cells, approximately 75% are located within the perivascular spaces and the other 25% are found in the parenchyma of the NL (Mander & Morris, 1994).

The vasculature of the NL is characterized by the lack of a BBB. The vascular compartment of the NL consists of fenestrated endothelia surrounded by a perivascular basal lamina followed by a perivascular space, which in turn is surrounded by a parenchymal basal lamina. The perivascular space not only surrounds vessels but extends in multiple directions to penetrate widely between rows of parenchymal cells. The parenchymal basement membrane is characterized by discontinuities which allow for the penetration of neurosecretory axons into the perivascular space. The perivascular space also includes
macrophages, fibroblasts and collagen fibrils, and the occasional mast cell (Seyama, Pearl, & Takei, 1980).

**Activity-Dependent Plasticity of the Magnocellular Neurosecretory System**

A unique feature of the magnocellular neurosecretory system is that in response to increased neurosecretory activity, such as lactation or dehydration, magnocellular neurons and glia will undergo reversible morphological changes that facilitate neurosecretion (Hatton, 2004). For example, osmotic stimulation increased the amount of soma-somatic contact in the SON (Tweedle & Hatton, 1977) and PVN (Theodosis & Poulain, 1989), and increased the formation of phenotype-specific double synapses (single boutons contacting more than one post-synaptic element) between magnocellular neurons, which are not frequently seen in the unstimulated rat SON (Hatton & Tweedle, 1982; Theodosis, Poulain, & Vincent, 1981). These actions, along with an increase in intercellular communication between magnocellular neurons following osmotic stimulation (Hatton, Yang, & Cobbett, 1987), help to synchronize the activity of the magnocellular neurons during states of heightened neurosecretory activity.

As previously mentioned, osmotic stimulation increased the amount of activated microglia present within the SON (Ayoub & Salm, 2003). Moreover, the predominant glia cells in the SON, astrocytes, undergo several reversible morphological changes. In response to osmotic stimulation, the astrocytic processes, which normally project vertically (dorsally) in between magnocellular neuron somata and dendrites, withdraw and reorient themselves in a horizontal manner (Bobak & Salm, 1996). The reorientation of the astrocyte processes
leads to a thinning of the SON-VGL (Bobak & Salm, 1996) and a 30% increase in the mediolateral width of the SON-VGL (Salm & Hawrylak, 2004). These changes were not due to changes in total astrocyte numbers, increases in the volume of the SON-VGL or dendritic zone, or changes in the length or thickness of the astrocyte processes (Bobak & Salm, 1996; Salm & Hawrylak, 2004). Altogether, these morphological changes are thought to facilitate the activity of the magnocellular neurons during states of heightened neurosecretory activity.

Similar to the SON and PVN, the NL undergoes morphological changes that facilitate neurosecretion. During periods of low secretory activity, pituicytes completely surround and engulf neurosecretory axons and their terminals in the NL (Tweedle & Hatton, 1980, 1982). Also, under these conditions, pituicytes tend to occupy a relatively large portion of the parenchymal basement membrane, interposing themselves between it and the neurosecretory terminals (Tweedle & Hatton, 1987; Wittkowski & Brinkmann, 1974). In response to osmotic stimulation, pituicytes retract from the parenchymal basement membrane resulting in an increase in neuronal-basement membrane contact area thereby facilitating neuropeptide release (Tweedle & Hatton, 1980, 1982, 1987). This response is presumably due to release of the axons by the pituicytes (Hatton, 1988).

To allow for the complex anatomical changes that occur in the SON during states of increased neurosecretory activity, the cells of the SON express proteins that are normally present only in the developing brain and help the SON retain the plastic nature that is similar to the embryonic brain. Cell adhesion molecules
(CAMs) are cell surface glycoproteins that allow for cell to cell interactions and are present in various isoforms throughout the body. Neural cell adhesion molecule (NCAM) is found at the earliest stages of neural tube formation and in most developing neuronal structures; however, NCAM is less abundant in the adult brain (Grumet & Edelman, 1988). The ability of NCAM to promote cell-to-cell adhesion is attenuated by the addition of an unusually high amount of α-2,8-linked polysialic acid (PSA) on the extracellular domain of the molecule (Finne, Finne, Deagostini-Bazin, & Goridis, 1983). Therefore, it has been suggested that the PSA molecule may serve as an overall regulator of contact-dependent cell-to-cell interactions (Sadoul, Hirn, Deagostini-Bazin, Rougon, & Goridis, 1983). Interestingly, PSA-NCAM-immunoreactivity has been observed in the adult rat SON, PVN, and NL (Theodosis, Rougon, & Poulain, 1991). Within the adult rat magnocellular neurosecretory system, PSA-NCAM-immunoreactivity was localized to dendrites, axons, and terminals and in associated astrocytes, but not in neuronal somata (Theodosis, et al., 1991). Removal of PSA from N-CAM abolishes lactation-induced structural plasticity in the SON (Theodosis, Bonhomme, Vitiello, Rougon, & Poulain, 1999). These observations suggest that the retention of the PSA-NCAM allows for the anatomical changes that occur in the SON following activation.

The Magnocellular Neurosecretory System as a Model System for Investigating Axonal Reorganization

Unlike most systems of the central nervous system (CNS), the magnocellular neurosecretory system has the unique ability to structurally reorganize and functionally recover following axotomy of magnocellular neuron
axons (Moll, 1957; Raisman, 1973; Silverman & Zimmerman, 1982; Watt et al., 1999; Watt & Paden, 1991). This phenomenon has been demonstrated across numerous species and by different axotomy approaches including hypophysectomy (Kiernan, 1971), infundibular stalk compression (Dohanics, Hoffman, Smith, & Verbalis, 1992) and transection (Dellmann & Carithers, 1992), and unilateral lesion of the hypothalamo-neurohypophysial tract (Watt & Paden, 1991). These surgical interventions result in degeneration of oxytocinergic and vasopressinergic magnocellular neurons; however, vasopressinergic magnocellular neurons have been demonstrated to be more vulnerable to injury (Dohanics, Hoffman, & Verbalis, 1996). It has been well-documented that in the absence of any external intervention strategies, the neurons surviving these injuries will undergo axonal reorganization to form an ectopic NL or reinnervate the denervated NL (Dellmann & Carithers, 1992; Dohanics, et al., 1992; Kiernan, 1971; Watt, et al., 1999; Watt & Paden, 1991). Thus, the well established regenerative capacity of the magnocellular neurosecretory system has established it as a model system for studying plasticity of the injured CNS.

The previous reports led us to investigate the ability of magnocellular neurosecretory axons to undergo collateral sprouting following partial denervation of the NL (Watt, et al., 1999; Watt & Paden, 1991). We utilized unilateral lesion of the hypothalamo-neurohypophysial tract in which the neurosecretory axons in the animal’s right hemisphere PVN and SON are severed while the contralateral nuclei are spared (Watt & Paden, 1991). The lesion results in the loss of 42% of the neurosecretory axons in the NL followed
by a return to control levels by four weeks post-lesion (Watt & Paden, 1991). The axonal recovery results from a collateral sprouting response arising from the non-injured, contralateral magnocellular neurons with a concomitant increase in the magnocellular neuron somatic and nuclear area, oxytocin and vasopressin mRNA expression (Watt & Paden, 1991), and alpha-I and beta-II tubulin mRNA expression (Paden et al., 1995a). Daily measures of urine osmolality revealed a chronic hyperosmolality with a concomitant decrease in daily water intake and urine excretion volume, which persists throughout the post-surgical period. Together these results indicate that the sprouting event is not a compensatory response as it occurs in the absence of a functional deficit. Hence, the mechanism underlying the axonal sprouting remains the focus of our investigations.

Collateral sprouting has been shown to occur in a variety of neuronal populations; however, the factor or factors responsible for mediating the sprouting response are still largely ill defined. There is evidence suggesting that ciliary neurotrophic factor (CNTF) may play an important role. CNTF has been implicated in hypothalamic magnocellular neuron sprouting in vitro (Vutskits, Bartanusz, Schulz, & Kiss, 1998) and has been demonstrated to promote motor neuron sprouting (Gurney, Yamamoto, & Kwon, 1992; Guthrie, Woods, Nguyen, & Gall, 1997; Kwon & Gurney, 1994; Oyesiku & Wigston, 1996; Siegel, Patton, & English, 2000; Simon, Jablonka, Ruiz, Tabares, & Sendtner, 2010; Ulenkate, Kaal, Gispen, & Jennekens, 1994; Wright, Cho, & Son, 2007; J. J. Xu, Chen, Lu, & He, 2009) and process outgrowth of retinal ganglion cells (Leibinger et al.,
2009; A. Muller, Hauk, & Fischer, 2007; A. Muller, Hauk, Leibinger, Marienfeld, & Fischer, 2009). Previously, we demonstrated a significant increase in CNTF-immunoreactivity within the non-injured, contralateral, sprouting SON between 3 and 10 days post unilateral lesion (Watt, Bone, Pressler, Cranston, & Paden, 2006) in conjunction with an increase in the mRNA for the CNTF-specific receptor, CNTF receptor alpha (CNTFRα), at 7 days post unilateral lesion (Watt, Lo, Cranston, & Paden, 2009). Taken together, these studies suggest that CNTF may be involved in modulating axonal sprouting in the injured magnocellular neurosecretory system.

Ciliary Neurotrophic Factor

Properties of Ciliary Neurotrophic Factor

CNTF was originally described for its effects on the survival of cultured parasympathetic neurons of the chick ciliary ganglia (Adler, Landa, Manthorpe, & Varon, 1979; Barbin, Manthorpe, & Varon, 1984; Manthorpe, Skaper, Adler, Landa, & Varon, 1980). Purification and sequencing of CNTF revealed that it is a 200 amino acid peptide of approximately 23 kDa (Manthorpe, Skaper, Williams, & Varon, 1986; Stockli et al., 1989) and is included in the structurally related family of the interleukin-6 (IL-6) cytokines which include leukemia inhibitory factor (LIF), interleukin-11 (IL-11), oncostatin M (OSM), cardiotrophin 1 (CT-1), and cardiotrophin-like cytokine (CLC) (Barbin, et al., 1984). Moreover, the IL-6 cytokine family share the receptor subunit glycoprotein 130 (gp130) (Hirano, Matsuda, & Nakajima, 1994). This feature is not unique to the IL-6 cytokine family but rather a general feature of the cytokine receptor system (Hirano,
Nakajima, & Hibi, 1997). CNTF is now well established as a survival, sprouting, and differentiation factor for various neuronal and glial populations in vivo and in vitro.

CNTF protein and mRNA are expressed in astrocytes of the CNS and Schwann cells of the peripheral nervous system (Dobrea, Unnerstall, & Rao, 1992; Friedman et al., 1992; Sendtner, Stockli, & Thoenen, 1992; Stockli et al., 1991; Stockli, et al., 1989; Watt, et al., 2006). While CNTF expression is strongly enhanced in reactive astrocytes adjacent to lesions (Choi et al., 2004; M. Y. Lee, Deller, Kirsch, Frotscher, & Hofmann, 1997; T. N. Lin, Wang, Chi, & Kuo, 1998), it was thought that CNTF was not endogenously expressed in the non-injured CNS. However, our lab demonstrated that within the magnocellular neurosecretory system CNTF is endogenously expressed in astrocytes of the SON (Watt, et al., 2006), and consistent with what others have reported, injury increases CNTF expression in the NL and SON (Lo, SunRhodes, & Watt, 2008; Watt, et al., 2006). Therefore, while the neuroprotective functions of CNTF have been extensively demonstrated throughout the nervous system, the high levels of endogenous CNTF in the highly plastic SON provide an excellent model system for studying the physiological role of CNTF in the CNS.

There are conditions other than injury that have been demonstrated to regulate CNTF synthesis; however, these mechanisms remain ill defined. Studies have demonstrated that CNTF protein and mRNA were decreased in primary astrocyte cultures following the addition of forskolin and isoproterenol, which stimulate the production of intracellular cyclic AMP (Nagao, Matsuoka, &
Kurihara, 1995; Rudge, Morrissey, Lindsay, & Pasnikowski, 1994). Similarly, CNTF was down-regulated in cultured astrocytes following addition of adrenergic agonists that increase intracellular cyclic AMP such as; adrenaline, noradrenaline, dopamine, and adenosine (Carroll, Sendtner, Meyer, & Thoenen, 1993; Rudge, et al., 1994). One study demonstrated that phorbol 12-myristate 13-acetate (PMA), an activator of PKC, resulted in an initial decrease in CNTF mRNA expression, which was followed by a persistent increase in CNTF mRNA levels (Nagao, Matsuoka, & Kurihara, 1996). In regard to CNTF up-regulation, Cagnon & Braissant (2009) demonstrated that hyperammonemia increased CNTF protein levels in cultured astrocytes. Altogether, these studies demonstrate that CNTF synthesis may be modulated by factors other than injury.

CNTF lacks the secretory signal sequence which is necessary for protein secretion from the cell via the classic protein secretion pathway through the endoplasmic reticulum (ER) and Golgi apparatus (Vergara & Ramirez, 2004). The secretory signal sequence allows the protein to be packaged in a secretory vesicle at the ER and transported to the cell surface where fusion of the vesicle with the cell's membrane results in the protein released into the extracellular environment. Yet, there are proteins that lack the secretory signal sequence that can be secreted via non-classical pathways such as interleukin 1-beta (IL-1β) and basic fibroblast growth factor (bFGF) (Mignatti, Morimoto, & Rifkin, 1992; Rubartelli & Sitia, 1991). Thus far it has not been conclusively demonstrated if, or how, rat CNTF is secreted, however, chick CNTF has been demonstrated to be secreted through a non-classical secretory pathway (Reiness et al., 2001) and
reports have demonstrated that CNTF may be secreted in the absence of injury from cultured rat astrocytes (Kamiguchi et al., 1995) and cultured bovine corneal endothelium (Koh, 2002). Moreover, there are reports of detectable levels of serum CNTF in healthy individuals and patients with amyotrophic lateral sclerosis (ALS) indicating that CNTF may be released in both the presence and absence of a pathological condition.

**CNTF Receptor Complex**

To have an effect on the target cell’s transcriptional activities, CNTF binds to a three part receptor complex consisting of the ligand-specific binding subunit CNTFRα that is attached to the membrane via a glycosylphosphatidylinositol (GPI) linkage, and the intracellular signal transducing subunits gp130 and leukemia inhibitory factor receptor beta (LIFRβ) (Davis, Aldrich, Stahl, et al., 1993; Ip et al., 1993; Ip et al., 1992; Schuster et al., 2003). Our lab previously demonstrated that CNTFRα is localized to the magnocellular neurons and astrocytes of the SON (Watt, et al., 2009), however, the localization of the gp130 and LIFRβ receptor components in the magnocellular neurosecretory system has not been reported. Knockout studies performed in mice have confirmed the neuroprotective role of CNTF and have demonstrated the importance of the CNTF receptor components. While knockouts of CNTF resulted in fairly healthy offspring with relatively minor motor deficiencies, individual knockouts of gp130, LIFRβ, or CNTFRα led to either embryonic or perinatal death (Heinrich, Behrmann, Muller-Newen, Schaper, & Graeve, 1998). Specifically, CNTFRα gene disruption is devastating, with deletions in motor nuclei causing perinatal
death from the inability to suckle (DeChiara et al., 1995). In contrast to the relatively minor effects seen because of a loss of CNTF, deletion of CLF-1, the co-factor for CLC signaling, resulted in a phenotype similar to that seen in CNTFRα knockouts (Alexander et al., 1999), suggesting that CLC may be the more critical ligand for CNTFRα during development.

In order to modulate intracellular signaling, CNTF first binds to CNTFRα, then recruits gp130 and finally complexes with LIFRβ (Stahl & Yancopoulos, 1994). It has been demonstrated that CNTFRα, but not gp130 or LIFRβ, is located within lipid rafts on the plasma membrane; however, CNTF binding to CNTFRα induces a rapid translocation of gp130 and LIFRβ into the lipid rafts (Port, Gibson, & Nathanson, 2007). Interestingly, LIF stimulation does not induce receptor translocation of gp130 or LIFRβ, suggesting that CNTF induces different patterns of signaling and/or receptor trafficking due to the membrane localization of CNTFRα (Port, et al., 2007). The differential signaling patterns of cell surface receptors in lipid rafts are possible because lipid rafts are microdomains of the plasma membrane that are enriched in cholesterol, sphingomyelin, and glycosphingolipids, and lipid rafts contain distinct subsets of proteins and lipids and can thus create distinct environments for cell signaling (Simons & Ikonen, 1997; Simons & Toomre, 2000). Localization of CNTFRα within the CNS has been largely considered to be neuronal (M. Y. Lee, Hofmann, & Kirsch, 1997; MacLennan et al., 1996). However, within the SON, CNTFRα is localized to magnocellular neurons and astrocytes (Watt, et al., 2009). To date, few reports have demonstrated gp130 and LIFRβ localization within the CNS; however,
differential localization of the three receptor components would not be surprising since other members of the IL-6 cytokine family utilize the gp130 and LIFRβ receptor components in various combinations.

Following CNTF binding to CNTFRα and the subsequent recruitment of the gp130 and LIFRβ signal transducing subunits, CNTF has been demonstrated to activate multiple intracellular signal transduction pathways (Figure 1) including: The Jak-STAT (janus kinase-signal transducer and activator of transcription) pathway (Bonni, Frank, Schindler, & Greenberg, 1993; Dolcet et al., 2001; Lutticken et al., 1994; A. Muller, et al., 2009; K. Park, Luo, Hisheh, Harvey, & Cui, 2004; Peterson, Wang, Tzekova, & Wiegand, 2000; Rhee, Goureaux, Chen, & Yang, 2004; Sango, Yanagisawa, Komuta, Si, & Kawano, 2008; Symes et al., 1994), the PI3K-AKT (phosphoinositide 3-kinase-AKT) pathway (Dolcet, et al., 2001; A. Muller, et al., 2009; K. Park, et al., 2004; Sango, et al., 2008), the MAPK-ERK (mitogen-activated extracellular signal-regulated kinases, ERK½ isoforms or p44/42) pathway (Kassen et al., 2009; A. Muller, et al., 2009; K. Park, et al., 2004; Sango, et al., 2008; Trimarchi, Pachuau, Shepherd, Dey, & Martin-Caraballo, 2009), the p38-MAPK (p38 α, β, γ, and δ isoforms) pathway (Loy et al., 2011), the JNK-MAPK (c-Jun N-terminal kinase, JNK-1, -2, -3, isoforms, also named Stress-Activated Protein Kinase or SAPK) pathway (Cagnon & Braissant, 2009), as well as the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) (Gallagher et al., 2007; Nishimune et al., 2000).
CNTF-Mediated Signal Transduction

The Jak-STAT Pathway

The Jak-STAT intracellular signal transduction pathway (Figure 1) is considered the canonical signaling pathway utilized by CNTF (Bonni, et al., 1993; Bonni et al., 1997; Darnell, Kerr, & Stark, 1994). After ligand binding and receptor dimerization the of LIFRβ and gp130 receptor components, their constitutively bound Janus kinase (Jak) molecules, which includes the ubiquitously expressed Jak1, Jak2, and Tyk2, are tyrosine phosphorylated (Stahl & Yancopoulos, 1994). The resultant phosphotyrosines on LIFRβ and gp130 serve as specific Src homology 2 (SH2) domain-docking sites for other cytoplasmic substrates that include members of the STAT family (Segal & Greenberg, 1996; Stahl et al., 1994; Stahl & Yancopoulos, 1994). Subsequently, STAT molecules, preferentially STAT3, are recruited to the β-components of the receptor complex (gp130 and LIFRβ), are tyrosine phosphorylated by Jaks, dimerize and translocate to the nucleus, and bind DNA in a sequence specific fashion, thus activating gene transcription (Bonni, et al., 1993; Boulton et al., 1995; Lutticken, et al., 1994; Stahl et al., 1995). Other transcription factors have been demonstrated to associate with STAT molecules, including; c-Jun, CREB (cAMP response element binding), and NF-κB (Brown, Ades, & Nordan, 1995; Kojima, Nakajima, & Hirano, 1996). Several genes have been linked to Jak-STAT activation, including; vasoactive intestinal peptide (VIP) (Symes, Gearan, Eby, & Fink, 1997), Bcl-XL (Fujio, Kunisada, Hirota, Yamauchi-Takahara, & Kishimoto, 1997),
gp130 (O'Brien & Manolagas, 1997), and suppressors of cytokine signaling (SOCS) (Bjorbaek et al., 1999).

When signaling through Jak, CNTF preferentially phosphorylates STAT3 at tyrosine 705 (Tyr705) (Bonni, et al., 1997; Darnell, et al., 1994; Wegenka, Buschmann, Lutticken, Heinrich, & Horn, 1993). However, STAT molecules can become activated by members of the MAPK pathway. Unlike Jak molecules, which are tyrosine kinases, members of the MAPK pathway are serine/threonine (Ser/Thr) kinases. Thus, MAPK-dependent activation of STAT3 occurs at serine 727 (Ser727) as opposed to the more commonly Jak-dependent phosphorylated Tyr705 (Decker & Kovarik, 2000). Interestingly, Chung et al. (1997) demonstrated that serine phosphorylation of STAT3 via MAPK pathways negatively modulates the tyrosine phosphorylation of STAT3, suggesting a possible feedback mechanism to regulate STAT3 activity. However, others have reported that serine phosphorylation of STAT3 is required for maximal activation of STAT3 (Yokogami, Wakisaka, Avruch, & Reeves, 2000). A more commonly appreciated negative feedback mechanism for STAT activity is the SOCS proteins. There are seven known SOCS proteins, and they have been implicated in the regulation of many cytokines that signal through the Jak-STAT pathway (Croker, Kiu, & Nicholson, 2008). There are four major ways by which SOCS can inhibit cytokine signaling; blocking STAT recruitment to the receptor, targeting the receptor for degradation by the proteosome, binding to Jaks and inhibiting their kinase activity, or targeting Jaks for degradation by the proteosome (Palmer & Restifo, 2009). Moreover, others have demonstrated that SOCS3 mRNA is activated by
CNTF within the hypothalamus (Bjorbaek, et al., 1999), indicating that CNTF can negatively regulate its signaling response.

The MAPK Pathways

Along with the Jak-STAT pathway, CNTF can activate the MAPK pathways via other SH2 domain-containing proteins, such as the phosphatases SHP2 and Shc, which are bound to the activated receptors (Giordano et al., 1997; Stahl, et al., 1995). Four conventional families of MAPKs have been described: MAPK-ERK\(^{\frac{1}{2}}\), MAPK-ERK5, p38-MAPK, and JNK-MAPK (Cargnello & Roux, 2011).

Each group of conventional MAPKs is comprised of a set of three conserved, consecutively acting kinases; a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). The MAPKKKs are activated through phosphorylation and/or as a result of their interaction with a small GTP-binding protein of the Ras/Rho family in response to extracellular stimuli (Cargnello & Roux, 2011). MAPKKK activation leads to phosphorylation and activation of a MAPKK, on serine and threonine residues, which then stimulates MAPK activity through phosphorylation of serine, threonine, and tyrosine residues (Cargnello & Roux, 2011). Phosphorylation of these residues is essential for enzymatic activities (Robbins et al., 1993). The wide range of functions regulated by the MAPKs is mediated through phosphorylation of several substrates, including member of a family of protein kinases termed MAPK-activated protein kinases (MAPKAPKs) (Gaestel, 2008). Within this family are the p90 ribosomal S6 kinases (RSKs) (Carriere, Ray, Blenis, & Roux, 2008), mitogen- and stress-
activated kinases (MSKs) (Arthur, 2008), and MAPK-activated protein kinases 2/3 (MK2/3) and 5 (MK5) (Perander, Keyse, & Seternes, 2008; Ronkina, Kotlyarov, & Gaestel, 2008).

**MAPK-ERK½ pathway.** The MAPK-ERK½ pathway (Figure 1) is primarily activated by cell surface receptors, especially receptors that undergo Tyr phosphorylation (Cargnello & Roux, 2011), which include the CNTF receptor components. These phosphorylated residues serve as specific binding sites for proteins that contain SH2 domains, such as Grb2 (growth factor receptor-bound protein 2) (Cargnello & Roux, 2011). A guanine nucleotide exchange factor (GEF) called SOS (son of sevenless) is recruited from the cytosol to the plasma membrane as a result of its interaction with Grb2. SOS stimulates the exchange of GDP bound to Ras (rat sarcoma), which is a small GTPase, to GTP, which is required for Ras activation (Campbell, Khosravi-Far, Rossman, Clark, & Der, 1998). This nucleotide exchange allows Ras to interact directly with its target effectors, one of which is Raf, the initiating kinase of the ERK½ pathway (Campbell, et al., 1998). Activated Raf binds to and phosphorylates the dual-specificity kinases MEK½ (MAPKK1/2), which in turn phosphorylates ERK1 at Thr²⁰²/Tyr²⁰⁴ and ERK2 at Thr¹⁸⁵/Tyr¹⁸⁷ (Ferrell & Bhatt, 1997; Roux & Blenis, 2004). Significant ERK activation requires phosphorylation at both sites, with Tyr phosphorylation preceding that of Thr (Ferrell & Bhatt, 1997). While in quiescent cells ERK½ is localized to the cytoplasm; however, upon extracellular stimulation, a significant proportion of ERK½ accumulates in the nucleus (R. H. Chen, Sarnecki, & Blenis, 1992; Lenormand et al., 1993). In addition to
activation of the cytosolic RSKs, transcription factors such as CREB are activated by ERK½. Interestingly, ERK½ activated RSKs have been demonstrated to inhibit the pro-apoptotic molecule BAD, while ERK½-induced CREB activity directs transcription of the anti-apoptotic proteins Bcl-2 and Bcl-Xₐ (Nagata, 1999). These proteins provide evidence of one of the functions of the MAPK-ERK½ pathway; induction of cellular survival.

MAPK-ERK5 pathway. The ERK5 pathway is a separate MAPK pathway from the MAPK-ERK½ pathway (Figure 1). ERK5 has 51% homology to ERK2 (Cargnello & Roux, 2011) and there are three spliced forms of ERK5: ERK5a, ERK5b, and ERK5c (C. Yan, Luo, Lee, Abe, & Berk, 2001). ERK5 is ubiquitously expressed in all tissues, with particularly high levels in the brain, thymus, and spleen (L. Yan et al., 2003). Genetic deletion of ERK5 demonstrated that it is essential for early embryonic development (Regan et al., 2002; L. Yan, et al., 2003) and ERK5 is also required for normal development of the vascular system as well as cell survival (Sohn, Sarvis, Cado, & Winoto, 2002).

MEK5 was identified as the MAPKK that phosphorylates ERK5, while other MEKs do not appear to influence ERK5 activity (English, Vanderbilt, Xu, Marcus, & Cobb, 1995; G. Zhou, Bao, & Dixon, 1995). MEK5 is phosphorylated by both MEKK2 and MEKK3 in a stimulus- and cell type-dependent manner (Wang et al., 2006); however, the signaling cascade at this level is not entirely specific to ERK5 since MEKK2 and MEKK3 can also activate the JNK and p38 pathways. Similar to ERK½, ERK5 localizes to the cytoplasm in resting cells and upon activation ERK5 is found in the nucleus (Wang, et al., 2006). There are a
number of molecules that have been identified as ERK5 substrates, including; c-Myc and connexin 43 (Hayashi & Lee, 2004; Wang, et al., 2006). ERK5 activity is increased by growth factors and oxidative stress and was found to regulate cell survival and proliferation (Wang, et al., 2006).

*p38-MAPK pathway.* Generally more responsive to stimuli such as environmental stressors, the p38 family (Figure 1) contains the p38 α, β, γ, and δ isoforms (Han, Lee, Bibbs, & Ulevitch, 1994; J. C. Lee et al., 1994; Rouse et al., 1994). p38α and p38β are more ubiquitously expressed, while p38γ and p38δ have more restricted expression patterns and may have specialized functions (Y. Jiang et al., 1996). Since p38α is generally more highly expressed than p38β, most of the published reports on p38-MAPKs refer to p38α.

In mammalian cells, the four p38 isoforms are strongly activated by various environmental stresses and inflammatory cytokines (Cuadrado & Nebreda, 2010). MKK3 (MAPKK3) and MKK6 (MAPKK6) are thought to be the major kinases responsible for p38 activation (Derijard et al., 1995; Han et al., 1996; Stein, Brady, Yang, Young, & Barbosa, 1996), but MKK4 (MAPKK4) has also been shown to possess some activity toward p38 (Meier, Rouse, Cuenda, Nebreda, & Cohen, 1996). Most stimuli that activate p38-MAPKs also stimulate JNK isoforms, and many MAPKKKs, including MEKK2/3, in the p38 pathway are shared by the ERK5 and JNK pathways (Cargnello & Roux, 2011). Upon activation, p38 has been demonstrated within the nucleus (Ben-Levy, Hooper, Wilson, Paterson, & Marshall, 1998). Moreover, p38 signaling has been demonstrated to utilize, among others, the NF-κB transcription factor (Karin,
2006), and many transcriptional products are produced by the p38 pathway, including the pro-apoptotic molecule, Bax (Cuadrado & Nebreda, 2010). While some studies have reported pro-survival functions for the p38 pathway, many more have associated p38 activity with the induction of apoptosis (Cuenda & Rousseau, 2007).

**JNK-MAPK pathway.** Similar to the p38-MAPK pathway, the JNK-MAPK pathway (Figure 1) is largely responsive to cellular stresses, cytokines, and some growth factors. There are three JNK isoforms, JNK1-3 (also known as SAPKγ (p46), SAPKα (p54), and SAPKβ, respectively) (Derijard et al., 1994; Kyriakis et al., 1994). JNK1 and JNK2 are ubiquitously expressed, while JNK3 seems to be localized primarily to neuronal tissues, testis, and cardiac myocytes (Bode & Dong, 2007). Furthermore, it has been demonstrated that JNK2 and JNK3 are expressed within the SON and localized to magnocellular neurons *in vivo* and *in vitro* (Meeker & Fernandes, 2000), while JNK1 localization within the SON has not been reported.

The MAPKKs involved in the JNK-MAPK pathway are MKK4 and MKK7, which appear to work in conjunction in the phosphorylation of the JNKs (Lawler, Fleming, Goedert, & Cohen, 1998). MKK4 and MKK7 are activated by several MAPKKKs, many of which are involved in ERK5 and p38 signaling, including MEKK1-4, MLK1-3, and Tpl-2 (Kyriakis & Avruch, 2001; Wagner & Nebreda, 2009). Thus, due to the overlap of signaling components between the MAPK pathways, multiple pathways may be activated by similar stimuli. Conversely, the activation of a specific pathway would be dependent on a combination of the
following; the stimuli, the cellular phenotype activated, and the research paradigm. Similar to the other MAPK pathways, activated JNKs have been shown to translocate into the nucleus following stimulation (Mizukami, Yoshioka, Morimoto, & Yoshida, 1997). The transcription factor cJun is a well-described substrate for JNKs. cJun is a component of the activator protein-1 (AP-1) transcription factor complex (Tibbles & Woodgett, 1999), which also include members of the cFos and cJun families. Interestingly, one of the primary targets of AP-1 is the cJun gene itself (Tibbles & Woodgett, 1999), indicating a positive feedback loop regulating cJun activity. Additional transcription factors have been shown to be phosphorylated by JNK, including; STAT3, c-Myc, and JunB (Bogoyevitch, Ngoei, Zhao, Yeap, & Ng, 2010; Raman, Chen, & Cobb, 2007). Analogous to the p38-MAPK pathway, the JNK-MAPK pathway has been primarily implicated in the induction of neuronal apoptosis (Dhanasekaran & Reddy, 2008).

The PI3K-AKT Pathway

CNTF may also activate the PI3K-AKT pathway (Figure 1) in some cell types, possibly via a Jak-dependent signaling mechanism (Dolcet, et al., 2001; Gold, Duronio, Saxena, Schrader, & Aebersold, 1994; Oh et al., 1998). Activation results in the recruitment of PI3K to the plasma membrane where the catalytic subunit of PI3K phosphorylates the membrane phospholipid phosphatidylinositol (4, 5)-diphosphate (PIP2), generating phosphatidylinositol (3, 4, 5)-triphosphate (PIP3). The accumulation of PIP3 promotes the translocation of AKT to the plasma membrane, where AKT binds to PIP3 via its PH domain, allowing
phosphorylation of Thr^{308} on AKT by phosphoinositide-dependent kinase 1 (PDK1) (Alessi et al., 1996; K. E. Anderson, Coadwell, Stephens, & Hawkins, 1998). Maximal AKT activity requires the additional phosphorylation of Ser^{473}, and reports have demonstrated that mammalian target of rapamycin (mTOR), in a rapamycin-insensitive complex with Sin1 and rictor (mTORC2), functions as the ‘PDK2’ and provides the kinase activity at Ser^{473} (Hresko & Mueckler, 2005; Sarbassov, Guertin, Ali, & Sabatini, 2005).

AKT can directly regulate apoptotic machinery by phosphorylating BAD, which inhibits the pro-apoptotic functions of BAD (Datta et al., 1997). In addition, there are many transcriptional targets of AKT, including the transcription factors CREB (Du & Montminy, 1998), NF-κB (Kane, Shapiro, Stokoe, & Weiss, 1999; Romashkova & Makarov, 1999), and mTOR, in a rapamycin-sensitive complex with raptor, DEPTOR, and GβL (mTORC1) (Asnaghi et al., 2004; Nave, Ouwens, Withers, Alessi, & Shepherd, 1999). There are multiple downstream targets of mTORC1; however, the best characterized are p70 S6 kinase (S6K1) and eukaryotic initiation factor 4E (eIF4E) (H. Zhou & Huang, 2010). After p70S6K1 and eIF4E are phosphorylated, mTOR has been reported to be involved in mechanisms inducing process outgrowth, including axonal regeneration and dendritic arborization (Jaworski & Sheng, 2006; K. K. Park, Liu, Hu, Kanter, & He, 2010).

In general, the PI3K-AKT pathway has been implicated in promoting cell survival, differentiation, and several aspects of neurite outgrowth, including elongation, branching, and neurite caliber (Read & Gorman, 2009). Interestingly,
while the Jak-STAT pathway was important to mediate neuronal survival, the PI3K-AKT pathway was necessary to promote CNTF-induced process outgrowth of primary neuronal cultures (Ozog, Modha, Church, Reilly, & Naus, 2008). These reports indicate that CNTF-mediated activation of intracellular signal transduction pathways is phenotype specific and individual pathways may mediate different responses dependent on the cellular phenotypes.

The NF-κB Transcription Factor

CNTF has been demonstrated to signal through NF-κB in various experimental conditions (Gallagher, et al., 2007; Nishimune, et al., 2000). Interestingly, NF-κB was implicated in mediating CNTF-induced process outgrowth in developing sensory neurons (Gallagher, et al., 2007). However, as indicated above, multiple signaling pathways are capable of utilizing NF-κB; thus, the CNTF-induced immediate signaling responses that activate NF-κB are not clear.

NF-κB is a ubiquitously expressed transcription factor system that consists of homodimers or heterodimers of five structurally related proteins: p65, RelB, c-Rel, p50, and p52, of which the p50/p65 heterodimer is the most abundant and widely expressed (Hayden & Ghosh, 2004). NF-κB is held in an inactive form in the cytosol by interaction with a member of the IκB family of inhibitory proteins, which include; IκBα, IκBβ, IκBγ, IκBε, p100, p105, of which IκBα is the predominantly expressed inhibitor. In the NF-κB signaling pathway, NF-κB is activated by phosphorylation on serine residues 32 and 36 by an IκB kinase complex (Hayden & Ghosh, 2004). This leads to ubiquitination and proteosome-
mediated degradation of IκBα and translocation of the liberated NF-κB to the nucleus where it binds to responsive genes (Hayden & Ghosh, 2004).

**Biological Actions of CNTF**

While it has already been mentioned that CNTF acts as a survival factor for cultured parasympathetic neurons, CNTF has also been demonstrated to promote the survival of retinal ganglion cells (Lehwalder, Jeffrey, & Unsicker, 1989), cortical (Magal, Burnham, & Varon, 1991), hippocampal (Ip et al., 1991), motor (Albrecht, Dahl, Stoltzfus, Levenson, & Levison, 2002; Arakawa, Sendtner, & Thoenen, 1990), Purkinje (Larkfors, Lindsay, & Alderson, 1994), and sensory neurons (Barbin, et al., 1984) in vitro. Within the magnocellular neurosecretory system, organotypic cultures of the SON and PVN cultured with CNTF rich media have increased survival of hypothalamic magnocellular neurons over control cultures (House, Li, Yue, & Gainer, 2009; Rusnak, House, Arima, & Gainer, 2002; Rusnak, House, & Gainer, 2003; Shahar, House, & Gainer, 2004; Vutskits, et al., 1998; Vutskits, Gascon, & Kiss, 2003). In vivo, CNTF has been shown to prevent neuronal death in the facial motor nucleus following axotomy by application of 5 μg CNTF to the stump of the lesioned facial nerve (Sendtner, Kreutzberg, & Thoenen, 1990). CNTF has also been shown to protect retinal ganglion cells from apoptosis following optic nerve transection (van Adel, Arnold, Phipps, Doering, & Ball, 2005), and rescue axotomized anterior thalamic neurons from retrograde cell death following infusion of 500 μg/mL of rat recombinant CNTF (rrCNTF) (Clatterbuck, Price, & Koliatsos, 1993). Furthermore, over-expression of CNTF using a retroviral vector rescued 30% of ciliary ganglia
neurons that would normally undergo cell death during development (Finn, Kim, & Nishi, 1998).

Along with being a survival factor, CNTF has also been reported as being a sprouting factor for injured neurons. Following transection of fibers innervating the gastrocnemius muscle, CNTF null mice did not demonstrate motoneuron sprouting; however, when exogenous CNTF was administered to mice lacking CNTF the typical sprouting response was observed (Siegel, et al., 2000). When organotypic cultures of rat PVN are cultured in the presence of the NL, there is an observed process outgrowth from the severed magnocellular neurons of the PVN towards the NL (Vutskits, et al., 1998), which contains a cellular source of CNTF (Lo, et al., 2008; Watt, et al., 2006) as well as CNTF mRNA (Vutskits, et al., 1998), suggesting that CNTF may play a role in the sprouting of neurosecretory axons following partial denervation of the NL (Watt, et al., 1999; Watt & Paden, 1991).

Given the dramatic and widespread neuroprotective effects of CNTF, several phase II-III clinical trials were undertaken with recombinant human CNTF (rhCNTF) in patients with ALS (R. G. Miller, Bryan, et al., 1996; R. G. Miller, Petajan, et al., 1996). Ultimately, these studies reported that systemically administered CNTF had no beneficial effect, as well as unacceptable side-effects including cachexia, fever, and a marked immune response, all of which have limited CNTF’s clinical application. The limited efficacy and undesired side-effects were attributed to ineffective CNTF administration, because doses greater than 5μg/kg rhCNTF caused unacceptable toxicity (R. G. Miller, Bryan, et al.,
Consequently, several phase I-II clinical studies have been subsequently performed using encapsulated cells genetically engineered to secrete CNTF for the treatment of ALS, Huntington’s disease, and retinal degeneration (Bloch et al., 2004; Sieving et al., 2006; Zurn et al., 2000). Collectively, these studies have demonstrated the safety and feasibility of using the CNTF gene therapy procedure, but subsequent phase II-III clinical trials have not yet been reported. Moreover, because of the weight loss side effects from the early clinical trials (R. G. Miller, Bryan, et al., 1996; R. G. Miller, Petajan, et al., 1996), CNTF is now being actively pursued as an anorexic therapy (Foster-Schubert & Cummings, 2006).

Research Goal

While it is known that CNTF promotes the survival of a myriad of neuronal phenotypes, little is understood on the specific CNTF-induced neuroprotective mechanisms in the CNS. Elucidation of these mechanisms may provide insight into the development of future therapeutic strategies due to the deleterious side-effects of systemic CNTF administration. Because of the well-established plasticity and high endogenous expression of CNTF and CNTFRα, we believe that the magnocellular neurosecretory system, and specifically the SON, provides an excellent model system for investigating the neuroprotective role of CNTF. Therefore, the goal of my research is to determine the mechanisms underlying the neuroprotective effects of CNTF within the rat magnocellular neurosecretory system.
Hypothesis

The central hypothesis of my research is that CNTF acts as both a neuronal survival and sprouting factor for hypothalamic magnocellular neurons in vitro and in vivo. The following Specific Aims testing this hypothesis were pursued.

Specific Aims

Specific Aim 1: Determine the Effect that Injury, Neuronal Activity, and Age Have on the Protein Levels of CNTF and the CNTF Receptor Components in the SON and NL

Previous studies have demonstrated that following unilateral lesion of the hypothalamic-neurohypophysial tract there is a collateral sprouting response that reinnervates the partially denervated NL (Watt, et al., 1999; Watt & Paden, 1991). Moreover, the observed sprouting response does not occur as a result of a functional deficit. Conversely, the magnocellular neurons undergo an increase in neurosecretory activity (Watt, et al., 1999). Therefore, the factor or factors responsible for the sprouting remained to be determined. Following reports demonstrating that CNTF promotes magnocellular neuron survival and possibly potentiates process outgrowth (Rusnak, et al., 2002; Vutskits, et al., 1998), we demonstrated an increase in CNTF-immunoreactivity and CNTFRα mRNA levels in the sprouting SON (Watt, et al., 2006; Watt, et al., 2009). However, it remained be determined if the observed changes in CNTF and CNTFRα were due to the sprouting or the increase in neurosecretory activity. Thus, I designed experiments to determine the protein levels of CNTF and the CNTF receptor components following chronic physiological activation in the male and female rat.
Furthermore, these studies will extend our previous studies to demonstrate the protein levels of CNTF and the CNTF receptor components in the SON and NL following unilateral lesion of the hypothalamo-neurohypophysial tract. In addition, the localization of gp130 and LIFRIβ within the SON and NL had not been reported, and these data would provide valuable insight into possible mechanisms of action by CNTF in the magnocellular neurosecretory system.

While the collateral sprouting response occurs in 35 day old rats following unilateral hypothalamic lesion, there is an absence of a sprouting response following unilateral hypothalamic lesion in 125 day old rats (J.A. Watt, unpublished observation). We have hypothesized that this is due to a deficit in the CNTF signaling process within the magnocellular neurosecretory system. Thus, I compared the protein levels of the CNTF signaling components, including STAT3, in the SON and NL of 35 day old rats to 125 day old rats. In addition, I determined the protein levels of CNTF and the CNTF receptor components in the SON and NL following unilateral lesion of the hypothalamo-neurohypophysial tract in 125 day old rats. The results of these studies may lead to future experiments demonstrating the role of CNTF in the collateral sprouting response in the magnocellular neurosecretory system.

**Specific Aim 2: Determine the Functional Role of the Jak-STAT Pathway in CNTF Signal Transduction in Hypothalamic Organotypic Cultures**

The Jak-STAT pathway is considered the canonical pathway for CNTF signal transduction (Bonni, et al., 1993; Bonni, et al., 1997; Darnell, et al., 1994).

In this regard, our lab has demonstrated that exogenous rrCNTF injection in to
the SON in vivo activates STAT3 in astrocytes (Askvig et al., 2012b). Thus, I will extend this observation and determine the temporal activation of the Jak-STAT pathway following exogenous rrCNTF injection into the SON in vivo.

Others have demonstrated the neuroprotective effects that CNTF has on hypothalamic magnocellular neurons in organotypic cultures (House, et al., 2009; Rusnak, et al., 2002; Rusnak, et al., 2003; Shahar, et al., 2004; Vutskits, et al., 1998; Vutskits, et al., 2003). However, the mechanisms of these neuroprotective effects have not been reported. Therefore, I designed experiments to determine the functional role(s) of the contribution of the Jak-STAT pathway in CNTF-induced neuroprotection in hypothalamic organotypic cultures. 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, Thomas, Kusano, & Gainer, 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.

Specific Aim 3: Determine the Functional Role of the MAPK and PI3K-AKT Pathways in CNTF Signal Transduction in Hypothalamic Organotypic Cultures

While the Jak-STAT pathway is considered the canonical CNTF signal transduction pathway, CNTF has been demonstrated to activate multiple intracellular signaling pathways (Bonni, et al., 1993; Cagnon & Braissant, 2009;
Dolcet, et al., 2001; Gallagher, et al., 2007; Kassen, et al., 2009; Loy, et al., 2011; Luticken, et al., 1994; A. Muller, et al., 2009; K. Park, et al., 2004; Peterson, et al., 2000; Rhee, et al., 2004; Sango, et al., 2008; Symes, et al., 1994; Trimarchi, et al., 2009). This occurs because there are multiple points of convergence between the signaling components of the different pathways. Moreover, it appears that the activation of a specific pathway is dependent on a combination of various factors, such as; the stimulus, the cellular phenotype activated, and the research paradigm. Therefore, I sought to determine if exogenous rrCNTF injection into the SON in vivo results in the activation of other major signaling pathways. Additionally, I assessed the functional role(s) of these pathways in mediating the CNTF-induced neuroprotective mechanisms that are observed in hypothalamic organotypic cultures. The results and a discussion of the implications of our findings are presented herein.
CHAPTER II
MATERIALS AND METHODS

To test the previously stated hypothesis-driven questions regarding CNTF acting as both a neuronal survival factor and sprouting factor for hypothalamic magnocellular neurons in vitro and in vivo, various experimental techniques were employed. Extensive animal work was performed including stereotaxic surgeries and organotypic explant culture preparation. In addition to the animal preparations, Western blot and immunohistochemical analyses were performed.

Animals

Male and female 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, unless otherwise noted. 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 number of animals used in this study and their suffering.
Rats in the *in vivo* analyses were assigned randomly to the following experimental groups: 35 day old male control (75-100g), 35 day old male lesion (75-100g), male salt-loaded control (200-250g), male salt-loaded (200-250g), male acute CNTF-injected (250-400g), 125 day old male control (450-600g), 125 day old male lesion (450-600g), female virgin control (250-300g), female lactating (250-300g), and female salt-loaded (250-300g). Rats in the salt-loaded groups were given 2% salt water for 10 days in place of tap water and female lactating rats were taken from their cage following 21 days of nursing their pups and sacrificed within five minutes of their pups suckling.

**Stereotaxic Surgery**

*Unilateral Lesion of the Hypothalamo-Neurohypophysial Tract*

Male rats were either 35-40 (75-100g) or 125 days of age (450-600g) at the time a unilateral hypothalamic knife cut of the hypothalamo-neurohypophysial tract was performed. The animals were secured in a stereotaxic apparatus (Stoelting, Wood Dale, IL) and kept under constant isoflurane anesthesia (2.5%; Abbot Laboratories; Abbott Park, IL) using a tabletop anesthesia machine (Matrx Quantiflex Low Flow V.M.C.; Matrx, Orchard Park, NY) equipped with an isoflurane Vaporizer (Matrx VIP 3000; Matrx). The knife tract extended from the dorsal to the ventral surface of the brain, medial to the ipsilateral SON, but passing through the lateral aspect of the ipsilateral PVN. Stereotaxic lesion coordinates were 0.6 mm (for 35 day old lesion) or 0.8 mm (for 125 day lesion) lateral to the midsagittal suture, and the lesion extended from -4.0 mm to +4.0 mm anterior-posterior from bregma. This results in complete transection of
the ipsilateral hypothalamo-neurohypophysial tract (Watt & Paden, 1991). The animals were sacrificed 10 days post surgery along with age-matched non-injured control animals. A 90% success rate was confirmed using a cresyl violet (Sigma, St. Louis, MO) counterstain to demarcate the lesion tract and only the animals with a complete transection were included in these studies.

Acute rrCNTF Injections

For acute injection of exogenous 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 mOsmo/l) to a final concentration of 100 ng/µl was drawn into a 10 µl syringe (26 gauge; Hamilton, Reno, NV). The syringe was placed in a stereotaxic injector (Quintessential Stereotaxic Injector; Stoelting) mounted onto a stereotaxic apparatus (Stoelting) and 1 µl 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 µl/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. We observed an 80-90% success rate in cannula targeting and only those animals with a confirmed syringe tract were used in these studies. The following groups were prepared for quantitative Western blot analysis following
CNTF injection (n=6 per time point); 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 hour prior to rrCNTF injection. These rats were sacrificed at 1 hour post-CNTF injection (AG490 + 1 hr CNTF-injected SON). Moreover, a qualitative Western blot analysis (n=1 per time point) was performed at 15, 30, 45 minutes, 6, 12, 24, and 48 hours post CNTF-injection.

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 per time point) 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). Following other experimental paradigms (i.e. unilateral lesion, salt-loading, lactation), SON and NL samples were pooled in RIPA from 6 rats (30 total rats, n=5 per group). The tissue 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
either 10 µg (for tubulin experiment), 15 µg (for signal transduction analysis), or
50 µg (for other experiments) of protein and separated by a 12% SDS-PAGE gel
(Precise Protein Gels; Pierce) at 90 V for approximately 1.25 hours and then
electrophoretically transferred to a PVDF membrane (0.2 µm; 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 4x15 minute intervals between
incubations. Membranes were then incubated in blocking solution (5% BSA or
dehydrated milk in PBS-Tween; Sigma) for 1 hour at room temperature. Next, the
membranes were incubated in a primary antibody (Table 1) overnight at 4°C.
Following PBS-Tween washes for 2 hours, the membranes were incubated in the
appropriate species-specific horseradish peroxidase secondary antibody
(1:150,000; Santa Cruz). Subsequently, the bands were visualized, following
PBS washes for 2 hours, using the West Femto chemiluminescent detection kit
(Pierce) with high performance chemiluminescence film (Amersham Hyperfilm
ECL; VWR; West Grove, PA) developed using an AGFA CP1000 film processor.
Afterward 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 with subsequent antibodies
including the loading control, anti-β-actin.

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. Moreover, to assess specificity of the anti-CNTF and -CNTFRα antibodies to the antigen of interest, the Western blot was incubated with the antibody plus a 10 molar excess of the appropriate purified rat recombinant protein overnight at 4°C (pre-adsorption control).

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. The ROD of all bands was normalized to the respective ROD of β-actin bands to obtain ratios. However, in order to determine the amount of total protein that is phosphorylated in the signal transduction analyses, 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.

Immunohistochemistry

Peroxidase Immunohistochemistry

Following experimental periods rats utilized in immunohistochemical analyses were perfused intracardially with 100 mL of saline under deep isoflurane anesthesia followed by perfusion of 450 mL of periodate-lysine-
paraformaldehyde fixative (PLP; 3.2% paraformaldehyde, 2.2% lysine, 0.33% sodium-(meta) periodate; Sigma) prepared immediately before use (McLean & Nakane, 1974). The brains and NLs were removed intact and then the brains were blocked with a razor blade to contain the magnocellular neurosecretory system nuclei, post-fixed in PLP overnight and then cryoprotected in 20% sucrose (Sigma) in phosphate buffered saline (PBS) for three to five days. Next, the tissue was embedded in OCT (Ted Pella; Redding, CA) and frozen in isopentane (Sigma) chilled to -80°C for three to five minutes. Once the tissue was frozen, serial cryosections were collected coronally throughout the brain (16 μm) or NL (10 μm) using a cryostat (Microm HM550; Fisher Scientific; Pittsburgh, PA) and thaw mounted on gelatin coated slides.

For all immunohistochemical staining runs sections were first rehydrated in PBS followed by a treatment with 0.3% hydrogen peroxide in PBS for 30 minutes to remove endogenous peroxidase activity. All incubations were followed by a minimum of 3x10 minute washes with PBS containing 0.1% triton (PBS-T; Sigma). Next, non-specific staining was alleviated by treatment with 4% normal serum from the secondary antibody species in PBS-T (blocking buffer) for 1 hour at room temperature followed by an overnight incubation at 4°C in the primary antibody prepared in blocking buffer (Table 1). The tissue sections were then incubated in a biotinylated immunoglobulin G (IgG; 1:500 in blocking buffer; Vector; Burlingame, CA), specific for the species of the primary antibody, for 1 hour, followed by an incubation in avidin-biotin complex (ABC; ABC Elite kit; Vector) in PBS for 1 hour. Binding of ABC reagent was visualized with a
14.5 minute reaction in 0.05% diaminobenzidine (DAB; 200 mg glucose, 40 mg ammonium chloride, 50 mg DAB per 100 mL PBS; Sigma) with the enzyme glucose oxidase (300 μL per 100 mL DAB; Sigma) used to generate the hydrogen peroxide needed for the reaction (Itoh et al., 1979). Following PBS washes, sections labeled with anti-oxytocin and -vasopressin were incubated in the nuclear counterstain hematoxylin (Vector) for 30 seconds before proceeding through the alcohol dehydration steps. All sections were dehydrated in increasing concentrations of alcohol followed by xylene washes and coverslipped with Permount (Fisher).

**Fluorescence Immunohistochemistry**

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 goat anti-CNTFRα, rabbit anti-LIFRβ, or rabbit anti-gp130 with either mouse anti-GFAP, mouse anti-s100β, guinea pig anti-vasopressin, or guinea pig anti-oxytocin (Table 1) in blocking buffer overnight at 4°C followed by species-specific secondary IgG and fluorescently conjugated Texas Red or FITC (1:1000; Jackson ImmunoResearch, West Grove, PA). Fluorescent sections were coverslipped with Vectashield mounting medium containing DAPI (Vector). All sections were viewed and images were captured using either an Olympus BX-51 light microscope with attached DP-71 color camera and dedicated software or an Olympus fluoview confocal microscope. Images were prepared for reproduction using Adobe Photoshop CS3.
As a control for the dual label fluorescence immunohistochemistry, incubations with either goat anti-CNTFRα, rabbit anti-LIFRβ, or rabbit anti-gp130 antibodies on separate sections of tissue were followed by an incubation with the species-specific fluorescent-conjugated secondary antibodies for anti-GFAP, -oxytocin, -s100β, and -vasopressin. Similarly, after separate sections of tissue were incubated with the anti-GFAP, -oxytocin, -s100β, or -vasopressin antibodies the tissue was exposed to the fluorescent-conjugated secondary antibodies for the opposing primary antibodies. These controls demonstrated an absence of immunoreactivity in the rat SON (see Results), 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.

Stationary Hypothalamic Organotypic Cultures

Organotypic cultures were prepared using a modification of techniques described previously (House, et al., 2009; House, Rusnak, Liu, Youle, & Gainer, 2006; House, et al., 1998; Rusnak, et al., 2002; Rusnak, et al., 2003; Vutskits, et al., 1998). 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 μm 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 0.4μm, 30mm diameter; Millipore, Bedford, MA) and each filter insert was then placed in a 35x10 mm Petri dish containing 1.1-1.2 ml 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%, #26050-088 lot #1034545; 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. Incubation of the cultures was stationary in 5% CO₂-enriched air at 35°C for the entire experimental period.

In the current studies, hypothalamic slices were cultured in the presence or absence of rrCNTF (#C3835, lot #080M1730 and #091M1403, Sigma) for 7 or 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 the small molecule pharmacological inhibitor (Table 2) 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. Briefly, inhibition of the Jak-STAT pathway was achieved by utilizing AG490 and cucurbitacin I (Figure 2), various components of the MAPK pathway were inhibited with U0126, PD98059, PD184352, SP600125, and SB203580 (Figure 3), the PI3K-AKT pathway was inhibited with LY294002 and wortmannin (Figure 4), mTOR signaling was inhibited utilizing rapamycin and torin-1 (Figure 4), and the NF-κB transcription factor was inhibited by bay 11-7082 and sc-514 (Figure 5). In order to be thorough, multiple inhibitors were used to inhibit the pathways at various points. The concentrations used for each inhibitor was determined from literature searches of inhibitor concentrations used in primary cell cultures, or when available, in organotypic cultures.

As a control for the rrCNTF 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 SSAFRLDHIS RVTWQSLEQL 51V KLGWLKKEF 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 3x10 minute intervals before and after all incubations. For single-label peroxidase immunocytochemistry, 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 oxytocin-neurophysin or vasopressin-neurophysin (Table 1). These antibodies were characterized by Ben-Barak et al. (1985) and have been consistently utilized to label oxytocinergic and vasopressinergic 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). All images were captured using an Olympus BX-51 light microscope with attached DP-71 color camera and dedicated software. Montage images were prepared for reproduction using the 'photomerge' option in Adobe Photoshop CS3.

Data Analysis

In Vivo Oxytocin and Vasopressin Magnocellular Neuron Counts

To ensure that the same magnocellular neuron was not counted twice, adjacent sections were stained for oxytocinergic and vasopressinergic magnocellular neurons and a minimum of five sections (80 μm) were skipped before the next section was processed. Following immunocytochemical labeling of oxytocinergic and vasopressinergic magnocellular neurons, the sections were randomly coded by a third-party blind to experimental conditions. Immunopositive neurons containing a counterstained nucleolus were counted using a drawing tube attached to an Olympus BX51 microscope. In order to account for the rostral-caudal size difference of the SON, we determined the numbers of cells per unit area of SON by obtaining the total area of each SON using Image J. Data are expressed as the percentage of the age-matched controls.

Organotypic Culture 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, SON, and accessory nuclei (ACC), 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.

Organotypic Culture Process Outgrowth Analysis

In order to quantify the neuronal process outgrowth in the SON of organotypic cultures, we utilized a previously described organotypic culture process stereological analysis technique modified for our culture system (Bilsland, Rigby, Young, & Harper, 1999). First, slides were coded by a third party blind to the experimental conditions and the SON was viewed at 10x magnification on an Olympus BX51 microscope. In order to ensure consistency across groups, the SON was placed in the lower center frame of the picture (1360x1024 image size). A grain counting function in MCID image analysis software (Cambridge, England) was used in order to quantify the area occupied by the neuronal processes. Grain counting is an automatic target detection and measurement feature that quantifies the area in pixels occupied by a target according to the defined target criteria (Bilsland, et al., 1999). The target criteria are set combining the optical density and spatial characteristics of the target. In order to establish this criterion, the image was opened in MCID and 'Grain Count' was selected, which was under 'Settings' – 'Study' tab. In the 'Scan Area' box, the density for the target was adjusted to fill the neuronal processes and cell bodies. Once the density was set, any pixel falling within this range in the image was automatically counted. A full image scan of the micrograph was performed
which gave the proportional area of the entire micrograph that was occupied by the neuronal processes and cell bodies (total proportional area). Next, the density was determined as described above, however, just for the neuronal cell bodies, which gave the proportional area of the picture that was occupied by the neuronal cell bodies (somata proportional area). The somata proportional area was subtracted from the total proportional area to determine the proportional area that was occupied by the neuronal processes (process proportional area), which was the value that was utilized in the statistical analysis. As each organotypic culture differs in terms of intensity of immunoreactivity and background, the density was set for each image before quantification was undertaken. Since each SON varies in the amount of magnocellular neurons, we corrected for the number of neurons in the SON by standardizing the process proportional area to the total number of neurons in the SON. Thus, the values utilized for statistical analyses, as described below, represent the ratio of process proportional area to total number of neurons in the SON.

Statistical Analysis

Distribution normality of each group of data was tested using the Kolmogorov-Smirnov test (GraphPad InStat) and all groups were normally distributed. Student's *t* test, one-way ANOVA with post *hoc* Tukey's tests, or 2-way ANOVA with Bonferroni post *hoc* test to compare replicate means by row (GraphPad InStat, version 3.06 for Windows, San Diego California) were used, where appropriate, to compare groups with *p*<0.05 considered statistically significant. Results are expressed as the group means ± SD.
CHAPTER III

RESULTS

Specific Aim I: Determine the Effect that Injury, Heightened Neurosecretory Activity, and Age Have on the Protein Levels of CNTF and the CNTF Receptor Components in the SON and NL

We demonstrated previously that unilateral lesion of the hypothalamo-neurohypophysial tract results in an increase in CNTF mRNA expression in the axotomized SON (Watt, et al., 2006). This occurs concomitant with an increase in CNTF-immunoreactivity (Watt, et al., 2006) and CNTFRα mRNA expression (Watt, et al., 2009) in the contralateral, sprouting SON. However, the protein levels of CNTF and the CNTF receptor components following unilateral lesion of the hypothalamo-neurohypophysial tract remained to be determined. Therefore, in the current study we quantified the protein levels of CNTF and components of the tripartite receptor complex, CNTFRα, gp130, and LIFRβ, in the contralateral intact and axotomized SON and NL and sought to determine if the previously observed responses in the contralateral SON are due to increased neurosecretory activity. Moreover, previous studies have demonstrated the complete absence of sprouting in the mature, 125 day old rat following unilateral hypothalamic lesion (J.A. Watt, unpublished observations). Therefore, we will determine the protein levels of CNTF and the CNTF receptor components in the SON and NL of the 125 day old rat in the absence and presence of unilateral hypothalamic lesion.
Protein Levels of CNTF and the Receptor Components Following Unilateral Lesion of Hypothalamo-Neurohypophysial Tract in the 35 Day Old Rat

Confirmation of the lesion tract was performed using a cresyl violet counterstain, which clearly demarcates the lesion tract as well as the surviving magnocellular neurons (Figure 6A). Only those animals with a complete rostral-caudal transection of the hypothalamo-neurohypophysial tract were included in the following analyses. At 10 days post-lesion (10 dpi), it is apparent that there was a dramatic reduction in the number of magnocellular neurons in the SON following unilateral lesion in the 35 day old rat (Figure 6A). Moreover, Figure 6B illustrates the reduction in oxytocinergic axonal profiles arising from the right SON within the median eminence. These observations were quantified by performing oxytocinergic and vasopressinergic magnocellular neuron counts that was corrected for the size of the SON. Our analysis demonstrated that at 10 dpi there was an 85% and 90% loss of oxytocinergic and vasopressinergic magnocellular neurons, respectively, in the axotomized SON of the 35 day old rat (one-way ANOVA, F=119.0, p<0.0001; Figure 7). Furthermore, there was no significant difference in the number of oxytocinergic or vasopressinergic magnocellular neurons in the SON contralateral to the injury (one-way ANOVA, F=119.0, p=0.6533, p=0.5327; Figure 7), which is the SON from which the sprouting response arises (Watt, et al., 1999; Watt & Paden, 1991). These data indicate that the axotomy only results in neuronal loss ipsilateral to the lesion tract.

As a control to ensure specificity of the antibody to the antigen of interest, a pre-adsorption control for the anti-CNTF and -CNTFRα antibodies utilized in
the Western blot analyses was performed. When staining for anti-CNTF, Western blot analysis revealed multiple bands for CNTF in the SON (Figure 8A). In addition to the expected band for CNTF at 23 kDa, there were two prominent bands observed between 36 and 55 kDa that have been previously reported in the literature (Henderson, Seniuk, & Roder, 1994; Narhi et al., 1997). Reports have demonstrated that CNTF exists primarily as monomers; however, as a means of physiological storage of CNTF, dimer formation occurs as the protein concentration increases (McDonald, Panayotatos, & Hendrickson, 1995; Narhi, et al., 1997). Together, these reports suggest that the observed higher molecular weight bands for CNTF are dimeric CNTF. Following the pre-adsorption of the anti-CNTF antibody with a 10 molar excess of exogenous rrCNTF, there was a dramatic reduction in band intensity (Figure 8B), indicating that the anti-CNTF antibody is specific for rat CNTF. When staining for anti-CNTFRα, Western blot analysis revealed two bands at approximately 40 and 60 kDa corresponding to the non-glycosylated and glycosylated forms of CNTFRα, respectively (Figure 8C). Following the pre-adsorption of the anti-CNTFRα antibody with 10 molar excess of exogenous rrCNTFRα, there was a complete loss of the bands (Figure 8D). Furthermore, when 50 ng of rrCNTF or rrCNTFRα were run on a Western blot, the anti-CNTF and anti-CNTFRα antibodies detected prominent bands at 23 (Figure 9A) and 40 kDa (Figure 9B), respectively. Therefore, all Western blot analyses for CNTF and CNTFRα were performed on the bands at their reported molecular weights, 23 and 40 kDa, respectively, as opposed to the higher molecular weight bands that were also observed.
Our lab has previously demonstrated an increase in CNTF-immunoreactivity in the SON and NL (Lo, et al., 2008; Watt, et al., 2006), and CNTFRα mRNA levels in the SON following unilateral lesion (Watt, et al., 2009). We sought to confirm and extend these observations by determining the protein levels of CNTF and the CNTF receptor components in the SON and NL following unilateral hypothalamic lesion. By 10 dpi Western blot analysis demonstrated that CNTF protein levels were significantly elevated by 79% in the SON contralateral to the injury (one-way ANOVA, F=91.94, \( p=0.0003 \); Figure 9A) and by 169% in the axotomized SON compared to age-matched control SON (one-way ANOVA, F=91.94, \( p<0.0001 \); Figure 9A). Protein levels of CNTFRα undergo similar changes in the SON following unilateral lesion. As shown in Figure 9B, in the non-injured control SON, low endogenous CNTFRα protein levels were detectable by Western blot analysis with a small and faint band. However, at 10 dpi we observed a 140% increase in CNTFRα protein levels in the SON contralateral to the injury and a 233% increase in the axotomized SON compared to age-matched control SON (one-way ANOVA, F=81.24, \( p<0.0001 \); Figure 9B). While CNTFRα provides the specificity for CNTF signaling, it is the gp130 and LIFRIβ components that directly mediate intracellular signaling in response to CNTF (Davis, Aldrich, Stahl, et al., 1993). Therefore, we next examined whether the changes in CNTFRα were accompanied by changes in gp130 and LIFRIβ protein levels. As shown in Figure 9C, Western blot analysis demonstrated a prominent band at approximately 190 kDa corresponding to LIFRIβ in the non-injured control SON. LIFRIβ protein levels in the SON contralateral to the injury
and the axotomized SON were significantly elevated by 34% (one-way ANOVA, 
F=65.28, p=0.0013; Figure 9C) and 98% (one-way ANOVA, F=65.28, p<0.0001; Figure 9C), respectively, from the age-matched control levels at 10 dpi. As shown in Figure 9D, Western blot analysis demonstrated a faint band at approximately 130 kDa corresponding to gp130 in the non-injured control SON. By 10 dpi protein levels of gp130 increased by 176% in the SON contralateral to the injury (one-way ANOVA, F=22.67, p=0.0004; Figure 9D) and 258% in the axotomized SON (one-way ANOVA, F=22.67, p=0.0002; Figure 9D) compared to age-matched control SON.

In addition to CNTF being endogenously expressed in the SON, our lab previously demonstrated that CNTF is endogenously expressed in perivascular cells of the NL (Lo, et al., 2008). Furthermore, similar to the response observed in the SON, unilateral hypothalamic lesion results in the increase in the number of CNTF-immunoreactive cells in the NL (Lo, et al., 2008). However, the protein levels of CNTF the CNTF receptor components in the NL following unilateral lesion of the hypothalamo-neurohypophysial tract remained to be determined. Therefore, we quantified the change in the protein levels of CNTF and the CNTF receptor components in the NL following unilateral lesion.

At 10 dpl Western blot analysis demonstrated that CNTF protein levels were significantly elevated by 72% in the axotomized NL compared to age-matched control NL (Student’s t test, p=0.0024; Figure 10A). Similarly, protein levels of CNTFRα were significantly increased in the axotomized NL above age-matched control NL by 160% at 10 dpl (Student’s t test, p<0.0001; Figure 10B).
Moreover, Western blot analysis demonstrated a significant increase in LIFRβ and gp130 protein levels by 69% (Student's t test, \( p=0.0003 \); Figure 10C) and 108% (Student's t test, \( p=0.0183 \); Figure 10D), respectively, at 10 dpi in the axotomized NL compared to age-matched control NL.

Altogether, these data demonstrate that the injury associated with unilateral hypothalamic lesion increased the protein levels of CNTF and its receptor components in the axotomized SON in spite of an almost complete loss of magnocellular neurons. In addition, protein levels of CNTF and the CNTF receptor components were elevated in the terminal field following unilateral hypothalamic lesion. Furthermore, protein levels of CNTF and its receptor components are significantly elevated in the sprouting, metabolically active SON, in the absence of neuronal loss, suggesting that either the sprouting response per se or the increase in neurosecretory activity resulted in the increase in protein levels of CNTF and its receptor components.

*Tubulin Protein Levels Following Unilateral Lesion of Hypothalano-Neurohypophysial Tract in 35 Day Old Rat*

The regulation of axonal growth following axotomy is believed to be under the influence of the levels of α- and β-tubulin subunits since their products form microtubules which are the major structural components that enable neurite extension (Paden, et al., 1995a). Others have demonstrated that axonal injury that results in robust axonal growth is accompanied by elevations in several tubulin mRNAs (Hoffman & Cleveland, 1988; K. J. Jones & Oblinger, 1994; Wong & Oblinger, 1990). Of interest to our lab, it was demonstrated that unilateral hypothalamic lesion results in an increase in αI- and βII-tubulin mRNAs in the
sprouting SON at 10 dpl (Paden, et al., 1995a). Thus, we sought to determine if the mRNA increases translate to changes in the protein levels of α- and βII-tubulin in the magnocellular neurosecretory system following unilateral hypothalamic lesion.

Since a reliable antibody for the αI-tubulin subtype was not commercially available, we utilized a general α-tubulin antibody that recognizes all α-tubulin subtypes and a specific βII-tubulin antibody. Moreover, due to the high protein levels of α- and βII-tubulin in the magnocellular neurosecretory system, 10 μg of total protein was run for the tubulin experiments. Unlike the increases observed in the αI- and βII-tubulin mRNAs at 10 dpl, our results demonstrated that there was no significant difference in the α-tubulin (one-way ANOVA, F=1.307, p=0.1812; Figure 11A) or βII-tubulin (one-way ANOVA, F=0.6302, p=0.3327; Figure 11B) protein levels in the sprouting SON. Similarly, we did not observe a significant difference in the α-tubulin (one-way ANOVA, F=1.307, p=0.2297; Figure 11A) or βII-tubulin (one-way ANOVA, F=0.6302, p=0.3352; Figure 11B) protein levels in the axotomized SON. We extended the observations to determine the tubulin protein levels in the NL following unilateral hypothalamic lesion. Similar to the SON, our results demonstrated that there was no significant difference in the α-tubulin (Student’s t test, p=0.2068; Figure 11C) or βII-tubulin (Student’s t test, p=0.2242; Figure 11D) protein levels in the NL at 10 dpl. These data demonstrate that the previously demonstrated increases in tubulin message levels did not translate in to increases in the tubulin protein levels.
Protein Levels of CNTF and the Receptor Components Following Heightened Neurosecretory Activity

Male Rat

In order to determine if the increase in neurosecretory activity resulted in the increase in the protein levels of CNTF and its receptor components following unilateral hypothalamic lesion, we utilized a chronic hypernatremia paradigm in which non-injured magnocellular neurons are physiologically activated for 10 days by substituting tap water with a 2% salt-water solution. Previous reports have demonstrated that physiological activation of the MNS via salt loading results in heightened neurosecretory output and synthetic activity which is reflected in increased size of magnocellular somata and nuclei in the SON (Hatton & Walters, 1973; S. H. Lin, Miyata, Kawarabayashi, Nakashima, & Kiyohara, 1996; Theodosis & Poulain, 1984; Watt, et al., 1999). Consistent with those reports, we demonstrated a significant hypertrophy of 25% and 32% in oxytocinergic and vasopressinergic magnocellular neuron nuclei area, respectively, in the salt-loaded SON (Askvig, Leiphon, & Watt, 2012a). These data demonstrate that 10 days of chronic salt loading successfully induces physiological activation of the magnocellular neurons of the SON. Quantitative Western blot analysis was then performed to determine CNTF, CNTFRα, LIFRβ, and gp130 protein levels in the SON and NL following physiological activation.

In contrast to our observations in the axotomized and contralateral sprouting SON, our Western blot analysis revealed a significant decrease of 60% in CNTF protein levels in the salt-loaded SON compared to the age-matched control SON from animals maintained on tap water (Student’s t test, \( p<0.001 \)).
Figure 12A). Western blot analysis demonstrated no significant difference between CNTFRα (Student’s *t* test, *p*=0.5784; Figure 12B), LIFRβ (Student’s *t* test, *p*=0.5279; Figure 12C), or gp130 (Student’s *t* test, *p*=0.6128; Figure 12D) protein levels in the salt-loaded SON and the age-matched control SON. When extending these observations to the effect that chronic salt-loading has on CNTF and the CNTF receptor component protein levels in the NL, we found that there was no change in the protein levels of CNTF (Student’s *t* test, *p*=0.5763; Figure 13A), CNTFRα (Student’s *t* test, *p*=0.2182; Figure 13B), LIFRβ (Student’s *t* test, *p*=0.9670; Figure 13C), or gp130 (Student’s *t* test, *p*=0.7721; Figure 13D) compared to control NL from animals maintained on tap water.

Thus, these data demonstrate that an increase in neurosecretory activity in the absence of injury does not result in increased CNTF or CNTF receptor component protein levels in the SON as was observed in the sprouting SON contralateral to unilateral hypothalamic lesion. In addition, we extended these observations and determined that following chronic salt-loading there was no change in the protein levels of CNTF or the CNTF receptor components in the NL. However, we did observe the first evidence of an activity-dependent change, in the absence of injury, in CNTF protein levels in the CNS.

**Female Rat**

While the unilateral hypothalamic lesion paradigm has been exclusively performed in male rats, the magnocellular neurosecretory system of female rats can be activated to sustain chronic periods of increased neurosecretory activity by lactation and chronic salt loading. Thus, we sought to determine if the
observed response in male rats following increased neurosecretory activity occurs within the female magnocellular neurosecretory system. Female rats were physiologically activated either via 10 days of chronic salt-loading or 21 days of sustained lactation. In addition to the hypertrophy of magnocellular neuron nuclei following chronic salt loading, we demonstrated that 21 days of sustained lactation induced a significant hypertrophy of 17% and 8% in oxytocinergic and vasopressinergic magnocellular neuron nuclei area, respectively (Askvig, 2008). These data indicate that similar to 10 days of chronic salt loading, 21 days of sustained lactation are stimuli capable of inducing physiological activation of the magnocellular neurons of the SON.

Quantitative Western blot analysis was performed to determine the protein levels of CNTF and the CNTF receptor components in the SON and NL following physiological activation. Unlike the male SON, there was no significant difference observed between CNTF protein levels in the female physiologically activated SON and the female control SON (one-way ANOVA, F=1.028, \( p=0.3870 \); Figure 14A). However, the CNTFR\( \alpha \) bands present in the female physiologically activated groups were more intense than the female control CNTFR\( \alpha \) band. Quantitative analysis of the CNTFR\( \alpha \) band normalized to the ROD of the \( \beta \)-actin loading control revealed a significant increase of 73% in CNTFR\( \alpha \) protein levels in the female lactating SON (one-way ANOVA, F=16.98, \( p=0.0020 \); Figure 14B) and by 116% in the female salt-loaded SON (one-way ANOVA, F=16.98, \( p=0.0010 \); Figure 14B) compared to age-matched control SON. Conversely, neither of the signal transducing receptor subunits, LIFR\( \beta \) (one-way ANOVA,
F=0.6958, p=0.5177; Figure 14C) or gp130 (one-way ANOVA, F=1.111, p=0.3607; Figure 14D), demonstrated a change in protein levels following sustained neurosecretory activity in the female SON.

We extended these observations to determine the effect that chronic salt-loading has on CNTF and the CNTF receptor component protein levels in the female NL. Western blot analysis demonstrated that there was no change in the protein levels of CNTF (one-way ANOVA, F=0.3583, p=0.7061; Figure 15A), CNTFRα (one-way ANOVA, F=0.004481, p=0.9955; Figure 15B), or gp130 (one-way ANOVA, F=0.8948, p=0.4343; Figure 15D) in the female activated NL compared to control NL from animals maintained on tap water. Surprisingly, we observed a significant decrease in LIFRβ protein levels following 21 days of sustained lactation compared to control NL (one-way ANOVA, F=38.72, p=0.0002; Figure 15C) and female salt loaded NL (one-way ANOVA, F=38.72, p<0.0001; Figure 15C). Altogether, these data indicate a gender-specific response of CNTF and the CNTF receptor protein levels following an increase in neurosecretory activity in the SON.

Protein Levels of CNTF and CNTF Receptor Components in the Maturing Magnocellular Neurosecretory System

While the collateral sprouting response occurs in 35 day old rats following unilateral hypothalamic lesion, there is an absence of a sprouting response following unilateral hypothalamic lesion in 125 day old rats (J.A. Watt, unpublished observation). We have hypothesized that this is due to a deficit in the CNTF signaling process within the magnocellular neurosecretory system. Thus, I compared the protein levels of the CNTF signaling components, including
total STAT3 (tSTAT3), in the non-injured control SON and NL of 35 day old rats versus 125 day old rats.

Western blot analysis demonstrated that within the control SON (one-way ANOVA, F=3.331, \( p=0.0707 \); Figure 16A) and NL (one-way ANOVA, F=2.063, \( p=0.1698 \); Figure 16B) there was not a significant difference in the CNTF protein levels between 35, 60, or 125 day old rats. However, CNTFR\(\alpha\) protein levels in the maturing SON were significantly decreased by 38% in the 125 day old rat compared to the 35 day old rat (one-way ANOVA, F=9.290, \( p=0.0029 \); Figure 17A) and by 31% compared to the 60 day old rat (one-way ANOVA, F=9.290, \( p=0.0087 \); Figure 17A). The observed decrease in CNTFR\(\alpha\) protein levels only occurred within the SON. Western blot analysis demonstrated that there was no significant difference in the CNTFR\(\alpha\) protein levels in the maturing NL (one-way ANOVA, F=1.342, \( p=0.2978 \); Figure 17B). We did not observe a statistical difference in the protein levels of LIFR\(\beta\) in the maturing SON (one-way ANOVA, F=2.034, \( p=0.1049 \); Figure 18A) or NL (one-way ANOVA, F=1.469, \( p=0.2687 \); Figure 18B), of gp130 in the maturing SON (one-way ANOVA, F=1.398, \( p=0.2846 \); Figure 19A) or NL (one-way ANOVA, F=2.192, \( p=0.1543 \); Figure 19B), or tSTAT3 in the maturing SON (one-way ANOVA, F=0.3135, \( p=0.7367 \); Figure 20A) or NL (one-way ANOVA, F=0.5549, \( p=0.5882 \); Figure 20B). Altogether, these data indicate that as the rat matures, there is a reduction in the protein levels of CNTFR\(\alpha\) in the SON.
Protein Levels of CNTF and the Receptor Components Following Unilateral Lesion of Hypothalamo-Neurohypophysial Tract in 125 Day Old Rat

In addition to determining the effect that age has on the protein levels for CNTF signal transduction, we also wanted to determine if unilateral hypothalamic lesion in the 125 day old rat elicits a similar response in the protein levels of CNTF and the CNTF receptor components as seen in the 35 day old rat following unilateral lesion. Thus, unilateral hypothalamic lesions were performed on male rats at the age of 125 days and were sacrificed 10 dpi for Western blot and neuronal cell count analyses.

Unlike the 35 day contralateral SON, Western blot analysis demonstrated that CNTF protein levels were not significantly different in the SON contralateral to the injury of the 125 day rat at 10 dpi compared to age-matched control SON (one-way ANOVA, \( F=4.222, \ p=0.2315 \); Figure 21A). However, the lesion did result in a significant increase of 81% in CNTF protein levels in the axotomized SON compared to age-matched control SON (one-way ANOVA, \( F=4.222, \ p=0.0139 \); Figure 21A). Protein levels of CNTFRα undergo similar changes in the SON following unilateral lesion in the 125 day old rat. As shown in Figure 21B, we did not observe a significant difference in CNTFRα protein levels in the SON contralateral to the injury (one-way ANOVA, \( F=14.76, \ p=0.0572 \); Figure 21B); however, we did observe a 311% increase in the axotomized SON compared to age-matched control SON (one-way ANOVA, \( F=14.76, \ p=0.0013 \); Figure 21B). Western blot analysis demonstrated that LIFRβ protein levels in the 125 day rat SON contralateral to the injury were not significantly different from age-matched control SON (one-way ANOVA, \( F=10.66, \ p=0.0696 \); Figure 21C) and were
significantly elevated by 33% in the axotomized SON (one-way ANOVA, F=10.66, p=0.0408; Figure 21C) compared to age-matched control levels. Similarly, there was no significant difference in the protein levels of gp130 in the SON contralateral to the injury (one-way ANOVA, F=97.55, p=0.3568; Figure 21D) but we did observe a significant increase of 251% in the axotomized SON of the 125 day old rat (one-way ANOVA, F=97.55, p<0.0001; Figure 21D) compared to age-matched control SON.

Next, we quantified the change in the protein levels of CNTF and the CNTF receptor components in the NL following unilateral hypothalamic lesion in the 125 day old rat. Unlike the response that occurs in the 35 day old rat following unilateral hypothalamic lesion, there was no significant difference in CNTF protein levels the axotomized 125 day rat NL (Student's t test, p=0.6760; Figure 22A) compared to age-matched control NL. Similarly, protein levels of CNTFRα (Student's t test, p=0.2415; Figure 22B), LIFRβ (Student's t test, p=0.4521; Figure 22C), and gp130 (Student's t test, p=0.9890; Figure 22D) were not significantly different in the axotomized NL compared to age-matched control NL at 10 dpi in the 125 day old rat.

While unilateral hypothalamic lesion did not elicit a similar response in CNTF and its receptor components in the 125 day old rat, the effect of the injury on neuronal numbers was similar to the 35 day old rat. At 10 dpi there was a loss of 88% and 93% of the oxytocinergic and vasopressinergic magnocellular neurons, respectively, in the axotomized SON of the 125 day old rat (one-way ANOVA, F=184.7, p<0.0001; Figure 23). Moreover, there was no significant
difference in the number of oxytocinergic (one-way ANOVA, F=184.7, p=0.6941; Figure 23) or vasopressinergic (one-way ANOVA, F=184.7, p=0.9851; Figure 23) magnocellular neurons in the SON contralateral to the injury.

Altogether, these data indicate that the injury in the 125 day old rat results in similar responses to CNTF and CNTF receptor component protein levels and number of neurons surviving in the axotomized SON. However, while the 35 day old rat elicits a dramatic increase in CNTF and the CNTF receptor components in the sprouting SON and NL, which unlike the 125 day old rat also happens to undergo a collateral sprouting response following the injury (Watt, et al., 1999; Watt & Paden, 1991), there was no statistically significant increase within the 125 day old rat contralateral SON or NL.

*Tubulin Protein Levels Following Unilateral Lesion of Hypothalamo-Neurohypophysial Tract in 125 Day Old Rat*

Similar to the results observed in the α- and βII-tubulin protein levels in the 35 day old rat at 10 dpi, our results demonstrated that there was no significant difference in the α-tubulin (one-way ANOVA, F=0.2695, p=0.6605; Figure 24A) or βII-tubulin (one-way ANOVA, F=3.604, p=0.4600; Figure 24B) protein levels in the 125 day old contralateral SON. Similarly, we did not observe a significant difference in the α-tubulin (one-way ANOVA, F=0.2695, p=0.5128; Figure 24A) or βII-tubulin (one-way ANOVA, F=3.604, p=0.1467; Figure 24B) protein levels in the axotomized SON of the 125 day old rat. We extended the observations to determine the tubulin protein levels in the 125 day NL following unilateral hypothalamic lesion. Similar to the SON, our results demonstrated that there was no significant difference in the α-tubulin (Student's t test, p=0.7459; Figure 24C)
or βII-tubulin (Student’s t test, p=0.4106; Figure 24D) protein levels in the NL at 10 dpi. These data demonstrate that there was no change in the tubulin protein levels in the magnocellular neurosecretory system of the 125 day old rat following unilateral hypothalamic lesion.

**Localization of CNTF Receptor Components in the Magnocellular Neurosecretory System**

Our lab has previously demonstrated that CNTFRα is localized to magnocellular neurons and astrocytes within the SON (Watt, et al., 2009). However, there are no reports of gp130 and LIFRβ localization within the SON. Therefore, we utilized 35 day, 10 dpi contralateral SON tissue sections and performed immunohistochemical analysis of gp130 and LIFRβ localization to identify cell phenotypes that express these receptors within the SON. We observed robust immunoreactivity for gp130 on neuronal somata distributed throughout the SON (Figure 25A-F), suggesting that virtually all magnocellular neurons in the SON express the protein. Dual-label immunohistochemistry confirmed the presence of gp130-immunoreactivity associated with both vasopressinergic (Figure 25A-C) and oxytocinergic (not shown) magnocellular neurons. We also observed gp130-immunoreactivity localized to GFAP-immunoreactive astrocytes in the VGL of the SON (Figure 25D-F).

In contrast to gp130-immunoreactivity, we did not observe any LIFRβ-immunoreactive profiles that were consistent with neuronal somata (Figure 25G-I). However, we did observe a strong band of LIFRβ-immunoreactivity in the VGL of the SON (Figure 25G-L). Dual-label immunohistochemistry demonstrated that the astrocytes of the SON (Figure 25J-L) contain the LIFRβ protein and that
vasopressinergic (Figure 25G-I) and oxytocinergic (not shown) magnocellular neurons do not contain the LIFRβ protein.

As a control for the dual label fluorescence immunohistochemistry, incubations with rabbit anti-LIFRβ or rabbit anti-gp130 antibodies on separate sections of tissue were followed by incubation with the species-specific fluorescent-conjugated secondary antibodies for anti-GFAP and -vasopressin. Similarly, after separate sections of tissue were incubated with the anti-GFAP or -vasopressin antibodies the tissue was exposed to the fluorescent-conjugated secondary antibodies for the opposing primary antibodies. These controls demonstrated an absence of immunoreactivity in the rat SON (Figure 25M-N), 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.

While our lab has demonstrated previously that CNTF is localized to perivascular cells in the NL (Lo, et al., 2008), there are no reports demonstrating the localization of the CNTF receptor components within the NL. Thus, we utilized 35 day 10 dpi lesioned NL tissue sections and performed immunohistochemical analysis of the receptors to identify cell phenotypes that express these receptors within the NL. Figure 26 demonstrates that each of the CNTF receptor components have similar immunoreactive profiles. Dual-label immunohistochemistry demonstrated that CNTFRα- (Figure 26A-C), LIFRβ- (Figure 26I-K), and gp130- (Figure 26Q-S) immunoreactive profiles are localized to oxytocinergic and vasopressinergic (not shown) axons. However, it is apparent
that there are immunoreactive profiles that do not co-localize with the axonal profiles. We further determined that CNTFRα- (Figure 26E-G), LIFRβ- (Figure 26M-O), and gp130- (Figure 26U-W) immunoreactive profiles also are localized to s100β-immunoreactive pituicytes, which are the astrocyte population of the NL. In addition, pre-adsorption controls for anti-CNTFRα with Texas red (Figure 26D) and FITC (Figure 26H) demonstrated a strong decrease in immunoreactivity, indicating that the anti-CNTFRα antibody is specific for CNTFRα. Moreover, negative controls for LIFRβ (Figure 26L and P), and gp130 (Figure 26T and X) demonstrated a lack of immunoreactivity, indicating that the secondary antibody was specific for species of the primary antibody. Together, these data indicate that all of the CNTF receptor components are localized to astrocyte populations in the SON and NL, and axons within the NL.

Specific Aim 2: Determine the Functional Role of the Jak-STAT Pathway in CNTF Signal Transduction in Hypothalamic Organotypic Cultures

Others have demonstrated the neuroprotective effects that CNTF has on hypothalamic magnocellular neurons in organotypic cultures (House, et al., 2009; Rusnak, et al., 2002; Rusnak, et al., 2003; Shahar, et al., 2004; Vutskits, et al., 1998; Vutskits, et al., 2003). However, the mechanisms of these neuroprotective effects have not been reported. The Jak-STAT pathway is considered the canonical pathway for CNTF signal transduction (Bonni, et al., 1993; Bonni, et al., 1997; Darnell, et al., 1994). To this point, our lab has demonstrated that exogenous rrCNTF injection in to the SON in vivo activates STAT3 in astrocytes (Askvig et al., 2012b). Thus, I extended this observation and determine the
temporal activation of the Jak-STAT pathway following exogenous rrCNTF injection in to the SON in vivo. Furthermore, I utilized hypothalamic organotypic cultures to determine the functional role(s) of the contribution of the Jak-STAT pathway in CNTF-induced neuroprotection. 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). In addition, 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.

Assessment of the Jak-STAT Pathway Activation Following Exogenous rrCNTF Injection in the SON in Vivo

Quantitative Western blot analysis demonstrated that pressure injection of exogenous rrCNTF (100 ng/μl) into the SON in vivo induced a statistically significant increase in pSTAT3 (Tyr705) levels at 1 hr post injection (one-way ANOVA, F=44.77, p<0.0001; Figure 27A) compared to the control SON (vehicle-injected and contralateral, non-injected SON). At 3 hrs post injection, pSTAT3 (Tyr705) levels remained significantly elevated over control values (one-way ANOVA, F=44.77, p<0.0001; Figure 27A); but had decreased significantly from the peak level observed at 1 hour post injection (one-way ANOVA, F=44.77, p=0.0285; Figure 27A). In contrast, pSTAT3 (Ser727), which is primarily activated by the MAPK pathway in a mechanism different from pSTAT3 (Tyr705), was not
activated following pressure injection of rrCNTF compared to control levels (one-way ANOVA, F=0.3898, p=0.7616; Figure 27B).

While CNTF preferentially signals through STAT3, there have been reports of CNTF-induced STAT1 activation (Bonni, et al., 1997; Darnell, et al., 1994; Wegenka, et al., 1993). Thus, we also screened for rrCNTF-induced activation of other members of the Jak-STAT pathway, including STAT1, Jak2, and Tyk2. Quantitative Western blot analysis demonstrated that following pressure injection of rrCNTF into the SON there was no significant difference in pSTAT1 activation at the Tyr701 (one-way ANOVA, F=0.2985, p=0.8261; Figure 28A) or the Ser727 (one-way ANOVA, F=0.03798, p=0.9898; Figure 28B) residues compared to the control SON. Similarly, at 1 hour and 3 hours post rrCNTF-injection we did not observe a change in pJak2 (one-way ANOVA, F=12.38, p=0.0668; Figure 28C) or pTyk2 (one-way ANOVA, F=0.1789, p=0.9095; Figure 28D) levels compared to control SON. In addition to Jak2 and Tyk2, Jak1 is also a member of the Jak-STAT signaling pathway. However, after trying several sources of commercial antibodies for tJak1 and pJak1, we were unable to detect bands for either tJak1 or pJak1 in our samples.

Intraperitoneal (IP) administration of 10 mg/kg AG490, which is a protein tyrosine kinase inhibitor of Jak2 (Ozog et al., 2004; K. Park, et al., 2004), was administered 1 hour prior to pressure injection of rrCNTF in order to confirm the activation of the Jak-STAT pathway in the SON. Western blot analysis demonstrated that administration of 10 mg/kg AG490 1 hour before rrCNTF injection significantly suppressed the phosphorylation of STAT3 (Tyr705) (one-way ANOVA, F=0.0171, p=0.927; Figure 28E).
ANOVA, F=44.77, p<0.0001; Figure 27A) and Jak2 (one-way ANOVA, F=12.38, p<0.0001; Figure 28C) compared to the levels at 1 hr post-CNTF injection without affecting protein levels of tSTAT3 or tJak2. These data demonstrate that even though we did not observe Jak2 activation at 1 or 3 hours post rrCNTF pressure injection, STAT3 activation in the SON may be through Jak2.

Since we did not observe activation of a Jak component, but did observe activation of a STAT component at 1 and 3 hours post rrCNTF injection, we performed a survey of the Jak-STAT signaling components at various time points. However, these data are a qualitative observation because they only have an n=1. Similar to what was observed at 1 and 3 hours post rrCNTF injection, we did not see activation of pSTAT3 (Ser727) at 15, 30, 45 minutes, 6, 12, 24, or 48 hours post rrCNTF injection (Figure 29). The bands present in the pSTAT3 (Tyr705) survey extend the quantitative observations observed at 1 and 3 hours post rrCNTF injection. In Figure 29, it appears that pSTAT3 (Tyr705) activation is apparent by 30 minutes post rrCNTF injection, where it reaches a peak at 1 hour post rrCNTF injection (Figure 27A) and begins to decline at 3 hours post rrCNTF injection (Figure 27A). Visible bands of pSTAT3 (Tyr705) are still apparent at 6 and 12 hours post rrCNTF injection (Figure 29); however, by 24 and 48 hours post rrCNTF injection we no longer see pSTAT3 (Tyr705) bands in the SON (Figure 29). Consistent with the levels of pSTAT1 (Ser727) and (Tyr701), pJak2, and pTyk2 at 1 and 3 hours post rrCNTF injection, we did not see an apparent increase in activation of these band sizes at any of the time points surveyed (Figure 29). Altogether, we did not observe a visual change in band size of
pSTAT3 (Ser\(^{727}\)), pSTAT1 (Ser\(^{727}\)) and (Tyr\(^{701}\)), pJak2, or pTyk2 in our survey of additional time points, but we were able to extend our observations of the temporal pSTAT3 (Tyr\(^{705}\)) activation following rrCNTF injection into the SON.

**Characterization of Hypothalamic Organotypic 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), which are defined as the vasopressinergic or oxytocinergic neurons that were not confined within the PVN or SON. 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). *In vivo*, magnocellular neurons are multipolar neurons (Sofroniew & Glasmann, 1981) that have two or three dendrites and a single non-myelinated axon arising from the primary dendrite (Glenn Hatton, personal communication). As seen in Figure 30A, the cytoarchitecture of the magnocellular neuron is maintained *in vitro*. The oxytocinergic magnocellular neuron contains two dendrites (thick arrows, Figure 30A) with one axon (thin arrows, Figure 30A) branching from one of the dendrites. Moreover, the axon contains punctate staining that is consistent with neurosecretory vesicles. In addition to maintenance of individual magnocellular neurons, Figure 30B demonstrates the preservation of the specific magnocellular nuclei after being in culture for 14 days. The organization of the oxytocinergic 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. Furthermore, analysis of control organotypic culture media demonstrated that the osmolality and pH did not change during the entire experimental period, with levels maintained around 310 mmol/kg and 8.1, respectively (Figure 30C), which was consistent with previous reports (House, et al., 2009; Rusnak, et al., 2003). These observations were critical since all media was made and used throughout the same 14 day experimental period.

**Determination of Exogenous rrCNTF Concentration Necessary for Neuroprotection**

We first determined the number of oxytocinergic and vasopressinergic neurons that were in the nuclei at the time of sacrifice (P6; Table 3). It is apparent how severe the injury is when comparing the number of neurons in the P6 group to the groups receiving control media for 14 days (Table 3), and that this injury results in the loss of greater than 90% of the neurons. Moreover, even though exogenous rrCNTF administration promotes survival of oxytocinergic neurons, it still does not result in the recovery to the P6 number of neurons (Table 3). We also established the optimum concentration of exogenous rrCNTF that significantly increased neuronal survival (Table 3). Unlike previous reports (House, et al., 2009; Rusnak, et al., 2002; Rusnak, et al., 2003; Vutskits, et al., 1998), our results demonstrated that addition of 10 ng/ml rrCNTF to control media for 14 days did not increase the survival of oxytocinergic neurons over control values in either the SON (one-way ANOVA, F=24.84, p=0.2302; Figure 31A), PVN (one-way ANOVA, F=23.16, p=0.1837; Figure 31C), or ACC (one-way ANOVA, F=22.34, p=0.1730; Figure 31D).
ANOVA, F=12.76, p=0.0768; Figure 31E). However, in our hands, addition of 25 ng/ml rrCNTF for 14 days significantly increased the number of oxytocinergic neurons in the SON by 683% (one-way ANOVA, F=24.84, p<0.0001; Figure 31A), in the PVN by 73% (one-way ANOVA, F=23.16, p=0.0064; Figure 31C), and in the ACC by 1835% (one-way ANOVA, F=12.76, p<0.0001; Figure 31E) over control values. Treatment with 50 ng/ml rrCNTF for 14 days did not promote oxytocinergic magnocellular neuron survival above that observed in the 25 ng/ml rrCNTF treatment group in the SON (one-way ANOVA, F=24.84, p=0.6565; Figure 31A), PVN (one-way ANOVA, F=23.16, p=0.0858; Figure 31C), or ACC (one-way ANOVA, F=12.76, p=0.9862; Figure 31E).

In contrast to published reports (House, et al., 2009; Rusnak, et al., 2002; Rusnak, et al., 2003; Vutskits, et al., 1998), our analysis of vasopressinergic neuronal survival demonstrated that administration of 10 or 25 ng/ml rrCNTF for 14 days did not promote the survival of vasopressinergic neurons in the SON (one-way ANOVA, F=2.953, p=0.0793, p=0.0588; Figure 31B), the PVN (one-way ANOVA, F=5.491, p=0.6141, p=0.0207; Figure 31D), or the ACC (one-way ANOVA, F=1.816, p=0.4011, p=0.5496; Figure 31F) nuclei of hypothalamic organotypic explant cultures. However, administration of 50 ng/ml rrCNTF for 14 days resulted in a small, but statistically significant, increase in vasopressinergic magnocellular neurons in the SON (one-way ANOVA, F=2.953, p=0.0026; Figure 31B). While there was no change in the amount of vasopressinergic neurons in the ACC nuclei following administration of 50 ng/ml rrCNTF (one-way ANOVA, F=1.816, p=0.1739; Figure 31F), there was a
significant reduction in the amount of vasopressinergic neurons seen in the PVN following exogenous rrCNTF (25 and 50 ng/ml) treatment (one-way ANOVA, \( F=5.491, \ p=0.0207, \ p=0.0004 \); Figure 31D). Therefore, due to the more robust survival of oxytocinergic neurons following 25 ng/ml rrCNTF administration, all future experiments utilized a concentration of 25 ng/ml rrCNTF and focused primarily on oxytocinergic neuron survival.

**Determine if Soluble CNTFRα plus CNTF Enhanced Neuroprotection**

CNTFRα imparts its functionality either as a membrane bound receptor, which lacks a transmembrane sequence and is attached via a GPI-linkage or as a soluble receptor that results from the cleavage of the GPI-linked membrane-bound receptor in a phosphatidylinositol-specific phospholipase C (PI-PLC)-dependent mechanism (Davis, Aldrich, Ip, et al., 1993; Davis et al., 1991). The enzymatic cleavage of membrane-bound CNTFRα releasing the soluble CNTFRα into the extracellular space is thought to occur in response to cellular injury, as demonstrated by its release from skeletal muscle following peripheral nerve injury (Davis, Aldrich, Ip, et al., 1993). While most examples of soluble receptors are thought to interfere with the binding of their ligands to membrane-bound receptors, soluble CNTFRα increases the biological activity of CNTF 30-fold (Ip, et al., 1993; Saggio, Gloaguen, Poiana, & Laufer, 1995). Interestingly, a complex of the soluble CNTFRα bound to CNTF (hereafter referred to as ‘complex’) further enhanced the survival and nerve regeneration of cortical neurons compared to CNTF administration (Ozog, et al., 2008). Thus, we wanted to determine if the complex had an enhanced effect on the neuroprotective
functions of CNTF-induced neuronal survival in hypothalamic organotypic cultures.

Unlike the results previously reported in a primary neuronal culture paradigm, (Ozog, et al., 2008), complex administration did not enhance the survival (Figure 32) or process outgrowth (compare Figures 33C and 33D) of oxytocinergic magnocellular neurons in hypothalamic organotypic cultures compared to exogenous rrCNTF administration. Quantitative neuronal cell counts demonstrated no significant difference between the number of oxytocinergic neurons between CNTF and complex in the SON (one-way ANOVA, F=29.87, p=0.7716; Figure 32A), PVN (one-way ANOVA, F=12.11, p=0.1416; Figure 32C), or ACC (one-way ANOVA, F=16.02, p=0.7115; Figure 32E). Similarly, we did not observe a significant difference in the number of surviving oxytocinergic neurons between control and soluble CNTFRα in the SON (one-way ANOVA, F=29.87, p=0.2473; Figure 32A), PVN (one-way ANOVA, F=12.11, p=0.1916; Figure 32C), or ACC (one-way ANOVA, F=16.02, p=0.0542; Figure 32E).

The results of vasopressinergic neuron survival following complex administration were similar to what was observed with rrCNTF administration. We did not observe a significant increase between the number of vasopressinergic neurons between CNTF and complex in the SON (one-way ANOVA, F=1.075, p=0.9034; Figure 32B), or ACC (one-way ANOVA, F=1.146, p=0.3299; Figure 32F); although, we did observe a significant increase in the number of surviving vasopressinergic neurons in the PVN following complex administration compared to exogenous rrCNTF administration (one-way ANOVA,
However, there was a significant decrease in the number of surviving vasopressinergic neurons in the PVN following exogenous rrCNTF administration (one-way ANOVA, $F=6.898, p=0.0004$; Figure 32D) and complex administration recovered the amount of vasopressinergic neurons in the PVN to control levels (one-way ANOVA, $F=6.898, p=0.4936$; Figure 32D). Comparable to oxytocinergic neuronal counts, we did not observe a significant difference in the number of surviving vasopressinergic neurons between control and soluble CNTFRα in the SON (one-way ANOVA, $F=1.075, p=0.2430$; Figure 32B), PVN (one-way ANOVA, $F=6.898, p=0.3058$; Figure 32D), or ACC (one-way ANOVA, $F=1.146, p=0.3874$; Figure 32F).

Figure 33A is a micrograph montage of oxytocinergic 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 oxytocinergic 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 33A also demonstrates the hypothalamo-neurohypophysial tract (arrows), which contains the magnocellular neuron axons that project to the NL. Figure 33B illustrates oxytocinergic magnocellular neurons from a representative explant slice that received only control media for 14 days. These hypothalamic organotypic explant cultures still exhibit easily distinguishable oxytocinergic magnocellular neuron cell bodies and processes located within the PVN, SON,
and ACC nuclei; however, due to the inherent neuronal death associated with the axotomy, there are significantly fewer oxytocinergic magnocellular neurons present when compared to Figure 33A. Figure 33C illustrates a representative explant slice that received 25 ng/ml rrCNTF for 14 days. When compared to Figure 33B, the substantial increase in the survival of oxytocinergic magnocellular neurons in the magnocellular neurosecretory system following exogenous rrCNTF administration is apparent, as well as a prominent increase in oxytocinergic magnocellular neuron process density throughout the magnocellular neurosecretory system nuclei, particularly processes extending between the nuclei (arrows, Figure 33C), indicating that exogenous rrCNTF may be promoting process outgrowth from oxytocinergic magnocellular neurons. Figure 33D received complex (25 ng/ml rrCNTF plus 250 ng/ml rrCNTFRα) for 14 days. Quantitative neuronal cell counts demonstrated that the amount of surviving oxytocinergic neurons was no different between CNTF and complex treated groups (Figure 32A, C, E), and when comparing their representative micrograph montages, it is apparent that the exogenous rrCNTF (Figure 33C) and complex (Figure 33D) organotypic groups are visually indistinguishable. Moreover, similar to the culture receiving only exogenous rrCNTF, the culture receiving complex for 14 days also displayed a prominent increase in oxytocinergic magnocellular neuron process density throughout the magnocellular neurosecretory system nuclei, particularly processes extending between the nuclei (arrows, Figure 33D).
Figure 34 depicts representative micrograph montages of vasopressinergic neurons in the magnocellular neurosecretory system. While there are an observable number of vasopressinergic neurons in the control PVN, the majority of the neurons that are present appear to be parvocellular neurons (Figure 34A), as opposed to the larger, more prominent, magnocellular neurons that are visually apparent in the PVN of organotypic cultures immunohistochemically stained for oxytocinergic neurons (Figure 33B). Moreover, it is apparent that there was not a significant increase in the amount of vasopressinergic neurons following exogenous rrCNTF treatment (Figure 34B).

**Determine the Effect of Exposure Duration of Exogenous rrCNTF**

In order to further characterize the hypothalamic organotypic cultures in our hands, we sought to determine the number of neurons that survive exogenous rrCNTF administration for a shorter experimental period and whether an acute dose of rrCNTF is adequate to promote neuronal survival.

*Temporal response of exogenous rrCNTF.* For the first experiment, we compared the number of surviving neurons in the organotypic cultures in the absence and presence of exogenous rrCNTF for experimental periods of 7 and the previously studied 14 days in vitro (DIV). Within the SON, our analysis demonstrated that the number of surviving oxytocinergic magnocellular neurons were not significantly different between control at 7 and 14 DIV (2-way ANOVA, F=0.0005084, p=0.5065; Figure 35A), or between exogenous rrCNTF treatment for 7 or 14 DIV (2-way ANOVA, F=0.0005084, p=0.8098; Figure 35A). However, there was a significant increase in the number of surviving oxytocinergic
magnocellular neurons between control and rrCNTF treatment after 7 DIV (2-way ANOVA, F=195.2, \( p<0.0001 \); Figure 35A) and 14 DIV (2-way ANOVA, F=195.2, \( p<0.0001 \); Figure 35A). For vasopressinergic magnocellular neurons in the SON, there was no significant difference between control at 7 and 14 DIV (2-way ANOVA, F=17.64, \( p=0.5858 \); Figure 35B). Conversely, there was a significant reduction in the number of surviving vasopressinergic magnocellular neurons at 14 DIV with exogenous rrCNTF treatment compared to 7 DIV with exogenous rrCNTF treatment (2-way ANOVA, F=17.64, \( p<0.0001 \); Figure 35B). When comparing the treatment categories, we observed a significant increase of 479% in the number of surviving vasopressinergic magnocellular neurons after exogenous rrCNTF treatment for 7 DIV (2-way ANOVA, F=37.21, \( p<0.0001 \); Figure 35B); although, there was still a very low number of surviving vasopressinergic neurons present in the SON (mean=32). Furthermore, as previously demonstrated in Figure 31B and 32B, we did not see a significant difference in the number of vasopressinergic magnocellular neurons in the SON following 14 days of exogenous rrCNTF treatment (2-way ANOVA, F=37.21, \( p=0.0969 \); Figure 35B).

Within the PVN, from which we did not distinguish magnocellular or parvocellular neurons from each other, we did not see the same effects on oxytocinergic neuron survival that we saw within the SON. Our analysis demonstrated a significant decrease in the number of oxytocinergic neurons in the PVN between control (2-way ANOVA, F=32.28, \( p<0.0001 \); Figure 35C) and exogenous rrCNTF treatment (2-way ANOVA, F=32.28, \( p=0.0002 \); Figure 35C).
from 7 to 14 DIV. However, we observed a significant increase in the number of surviving oxytocinergic neurons in the PVN following exogenous rCNTF treatment for 7 (2-way ANOVA, F=39.48, p<0.0001; Figure 35C) and 14 DIV (2-way ANOVA, F=39.48, p<0.0001; Figure 35C). For vasopressinergic neurons in the PVN, there was no significant difference between control at 7 and 14 DIV (2-way ANOVA, F=16.78, p=0.1507; Figure 35D). Similar to the SON, there was a significant reduction in the number of surviving vasopressinergic neurons in the PVN at 14 DIV with exogenous rCNTF treatment compared to 7 DIV with exogenous rCNTF treatment (2-way ANOVA, F=16.78, p<0.0001; Figure 35D).

When comparing the treatment categories, we observed no significant difference in the number of surviving vasopressinergic neurons after exogenous rCNTF treatment for 7 DIV (2-way ANOVA, F=1.068, p=0.5844; Figure 35D).

Furthermore, as previously demonstrated in Figure 31D and 32D, we observed a significant decrease in the number of vasopressinergic magnocellular neurons in the PVN following 14 days of exogenous rCNTF treatment (2-way ANOVA, F=1.068, p=0.0041; Figure 35D).

For the ACC nuclei, which are characterized as being neurons not located within the confines of the SON and PVN, we observed responses similar to the PVN. Our analysis demonstrated a significant decrease in the number of oxytocinergic neurons in the ACC between control (2-way ANOVA, F=55.86, p=0.0123; Figure 35E) and exogenous rCNTF treatment (2-way ANOVA, F=55.86, p<0.0001; Figure 35E) from 7 to 14 DIV. Moreover, we observed a significant increase in the number of surviving oxytocinergic neurons in the ACC.
following exogenous rrCNTF treatment for 7 (2-way ANOVA, F=89.45, p<0.0001; Figure 35E) and 14 DIV (2-way ANOVA, F=89.45, p<0.0001; Figure 35E). For vasopressinergic neurons in the ACC, there was no significant difference between control at 7 and 14 DIV (2-way ANOVA, F=19.68, p=0.0871; Figure 35F). Similar to the SON and PVN, there was a significant reduction in the number of surviving vasopressinergic neurons in the ACC at 14 DIV with exogenous rrCNTF treatment compared to 7 DIV with exogenous rrCNTF treatment (2-way ANOVA, F=19.68, p=0.0015; Figure 35F). When comparing the treatment categories, we observed no significant difference in the number of surviving vasopressinergic neurons after exogenous rrCNTF treatment for 7 DIV (2-way ANOVA, F=10.26, p=0.0156; Figure 35D). Furthermore, as previously demonstrated in Figure 31D and 32D, we observed no significant difference in the number of vasopressinergic magnocellular neurons in the ACC following 14 days of exogenous rrCNTF treatment (2-way ANOVA, F=10.26, p=0.2938; Figure 35D). In summary, there were less oxytocinergic neurons present after 14 days of exogenous rrCNTF treatment in the PVN and ACC nuclei compared to 7 days; however, there were still enough neurons present in these nuclei after 14 DIV to allow for reproducible analyses. Thus, for our pharmacological inhibition experiments, we maintained consistency with other reports (House, et al., 2009; Rusnak, et al., 2002; Rusnak, et al., 2003; Vutskits, et al., 1998) and utilized the experimental period of 14 DIV.

**Acute response of rrCNTF.** It was recently demonstrated that pretreatment with CNTF promoted neuronal survival when an insult occurred two days later,
suggesting that CNTF reprograms the cell to be neuroprotective (Bechstein et al., 2012). We hypothesized that an acute dose of exogenous rrCNTF would promote the survival of oxytocinergic neurons for the entire 14 day experimental period. Thus, we exposed the organotypic cultures for either 24, 48, or 72 hours and after which the cultures received control media for the duration of the 14 days. Our results demonstrated that 24, 48, or 72 hours of acute exposure to exogenous rrCNTF did not result in a significant increase in the number of oxytocinergic neurons in the SON (one-way ANOVA, F=11.91, p=0.1697, p=0.1133, p=0.6593; Figure 36A), PVN (one-way ANOVA, F=22.56, p=0.9057, p=0.3402, p=0.2616; Figure 36B), or ACC nuclei (one-way ANOVA, F=56.94, p=0.8357, p=0.1772, p=0.1416; Figure 36C) compared to control. These data indicate that an acute exposure period of less than 72 hours to exogenous rrCNTF is not adequate to promote the survival of oxytocinergic neurons over a 14 day experimental period in hypothalamic organotypic explant cultures.

Determine the Functional Role(s) of the Contribution of the Jak-STAT Pathway in CNTF-Induced Neuroprotection

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; K. 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, indicating that CNTF utilizes the Jak-STAT pathway in the SON. We previously demonstrated that activation of the Jak-STAT pathway was specific to
astrocytes in the SON (Askvig et al., 2012a). Therefore, we sought to determine the cellular phenotype(s) that undergo activation of the Jak-STAT pathway in vitro. In addition, we determined the contribution of the Jak-STAT pathway to rrCNTF-induced oxytocinergic neuron survival in the magnocellular neurosecretory system.

**STAT3 Activation In Vitro**

We previously demonstrated that astrocytes, and not the magnocellular neurons, are the only cellular phenotype within the SON that exhibit activation of the Jak-STAT pathway following exogenous rrCNTF injection (Askvig et al., 2012a). While hypothalamic organotypic cultures contain all of the cells that are present in vivo, it is still an in vitro environment maintained in culture media. Thus, we wanted to determine which cells in the SON undergo activation of the Jak-STAT pathway in vitro.

Our results have shown that there is no difference in the amount of oxytocinergic magnocellular neurons in the SON between 7 and 14 DIV (see Figure 35). Therefore, we maintained four cultures in control media for 7 days to allow the for the natural cell death process to occur. Next, we placed two filter inserts into 25 ng/ml rrCNTF and the other two filter inserts were placed into fresh control media. After 1 hour, the cultures were fixed with paraformaldehyde and fluorescently stained for anti-pSTAT3 and -GFAP or -oxytocin. Figures 37B and 37E demonstrated that even following 7 days of control media there were pSTAT3-immunoreactive profiles present. These data suggest an injury-dependent activation of pSTAT3. However, it is interesting to note, that even in
the control treated cultures, pSTAT3 activation in the SON occurs exclusively in GFAP-immunoreactive astrocytes (arrows, Figure 37A-C) and not oxytocinergic magnocellular neurons of the SON (asterisks, Figure 37D-F). Similarly, after a 1 hour exogenous rrCNTF treatment, pSTAT3 activation only occurred in GFAP-immunoreactive astrocytes (arrows, Figure 37G-I) and not in oxytocinergic magnocellular neurons of the SON (asterisks, Figure 37J-L). Thus, even though we did not observe a CNTF-specific activation of pSTAT3, pSTAT3 activation was only observed in astrocytes in vitro.

Pharmacological Inhibition of the Jak-STAT Pathway

First, 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 oxytocinergic neuron numbers in the SON (one-way ANOVA, F=42.72, p=0.2175; Figure 38A), PVN (one-way ANOVA, F=11.8, p=0.8791; Figure 38B), and ACC (one-way ANOVA, F=20.87, p=0.0871; Figure 38C) 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 oxytocinergic neurons in the SON, PVN, and ACC.
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; K. 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 μM AG490 there was a small, but statistically significant reduction of oxytocinergic magnocellular neurons by 39% in the SON (one-way ANOVA, F=42.72, p=0.0002; Figure 38A) compared to the 25 ng/ml rrCNTF treated group. Within the PVN (one-way ANOVA, F=11.8, p>0.05; Figure 38B) and ACC (one-way ANOVA, F=20.87, p=0.8542; Figure 38C), the amount of oxytocinergic neurons in the 25 ng/ml rrCNTF plus 50 μM AG490 group was not significantly different from the 25 ng/ml rrCNTF treated group. Although, subsequent statistical analysis directly comparing the two groups in the PVN with a Student’s t-test demonstrated a significant decrease (p=0.0230; Figure 38B). However, incubation with 25 ng/ml rrCNTF plus 100 μM AG490 resulted in a statistically significant decrease in oxytocinergic neurons in the SON (one-way ANOVA, F=42.72, p<0.0001; Figure 38A), PVN (one-way ANOVA, F=11.8, p=0.0054; Figure 38B), and ACC (one-way ANOVA, F=20.87, p=0.0145; Figure 38C) to control levels. Similarly, inhibition of STAT3 activation via 10 μM cucurbitacin I in the presence of 25 ng/ml rrCNTF resulted in a statistically significant decrease in oxytocinergic neurons in the SON (one-way ANOVA, F=42.72, p<0.0001; Figure 38A), PVN (one-way ANOVA, F=11.8, p<0.0001; Figure 38B), and ACC (one-way ANOVA, F=20.87, p<0.0001;
Figure 38C) to control levels. When the hypothalamic organotypic explant cultures were treated with inhibitors alone there was not a statistical difference in the number of oxytocinergic neurons in the SON (one-way ANOVA, F=42.72, p>0.05; Figure 38A), PVN (one-way ANOVA, F=11.8, p>0.05; Figure 38B), or ACC (one-way ANOVA, F=20.87, p>0.05; Figure 38C) compared to the control media-treated group, demonstrating that in the absence of rrCNTF, the concentrations of the inhibitors used did not adversely affect oxytocinergic neuron survival. Altogether, these data demonstrate that pharmacological inhibition of the Jak-STAT pathway prevented CNTF-induced survival of oxytocinergic neurons in the SON, PVN, and ACC.

Representative low magnification montages and higher magnification 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 (Figures 39B and 40D) compared to control media treated cultures (Figures 39A and 40A), suggesting that exogenous CNTF may have a role in mediating process outgrowth of oxytocinergic magnocellular neurons. Moreover, no visual difference was observed in magnocellular neuron process density following inhibition of the Jak-STAT pathway with AG490 (Figures 39C and 40B) or cucurbitacin I (Figures 39D and 40C) compared to control SON (Figures 39A and 40A). Similar results were observed when analyzing individual PVN following pharmacological inhibition of the Jak-STAT pathway (see Figure 39).
Specific Aim 3: Determine the Functional Role of the MAPK and PI3K-AKT Pathways in CNTF Signal Transduction in Hypothalamic Organotypic Cultures

While the Jak-STAT pathway is considered the canonical CNTF signal transduction pathway, CNTF has been demonstrated to activate multiple intracellular signaling pathways, including the PI3K-AKT and MAPK pathways (Bonni, et al., 1993; Cagnon & Braissant, 2009; Dolcet, et al., 2001; Gallagher, et al., 2007; Kassen, et al., 2009; Loy, et al., 2011; Lutticken, et al., 1994; A. Muller, et al., 2009; K. Park, et al., 2004; Peterson, et al., 2000; Rhee, et al., 2004; Sango, et al., 2008; Symes, et al., 1994; Trimarchi, et al., 2009). This occurs because there are multiple points of convergence between the signaling components of the different pathways. Moreover, it appears that the activation of a specific pathway is dependent on a combination of various factors, such as; the stimulus, the cellular phenotype activated, and the research paradigm. Therefore, I determined the temporal activation of other major signaling pathways following exogenous rrCNTF injection on to the SON in vivo and I assessed the functional role(s) of these pathways in mediating the CNTF-induced neuroprotective mechanisms that are observed in the hypothalamic organotypic cultures as described below.

_Determine if Exogenous rrCNTF Injection Activates Components of the MAPK and PI3K-AKT Pathways_

In order to determine if exogenous rrCNTF injection activated signaling components of pathways other than the Jak-STAT pathway, we performed quantitative Western blot analysis of SON samples post-injection for phosphorylated signaling proteins. Our analysis demonstrated that there was no
significant difference in the protein levels of phosphorylated components of the MAPK-ERK½ pathway. Figure 41A demonstrated a band at 44 kDa for ERK1 and 42 kDa for ERK2. Quantitative Western blot analysis demonstrated no change in the protein levels of pERK1 (one-way ANOVA, F=1.354, p=0.2853; Figure 41A) or pERK2 (one-way ANOVA, F=0.1676, p=0.9170; Figure 41A) in the SON at 1 or 3 hours post rrCNTF injection. Similarly, the protein levels of pMEK½, which was observed at 45 kDa, at 1 or 3 hours post rrCNTF injection were not significantly different than control levels in the SON (one-way ANOVA, F=3.236, p=0.0540; Figure 41B). Moreover, when we analyzed signaling components of the p38- and JNK-MAPK pathways we did not see a significant difference in the levels of the phosphorylated proteins following rrCNTF injection. Figure 42A demonstrated a band at approximately 38 kDa for p-p38 and t-p38. Quantitative Western blot analysis demonstrated that there was no change in the protein levels of p-p38 at 1 or 3 hours post rrCNTF injection (one-way ANOVA, F=3.388, p=0.1345, p=0.1562; Figure 42A) compared to control levels. We observed two bands for JNK, at 46 and 54 kDa, which correspond to the JNK1 and JNK2 isoforms, respectively. Quantitative Western blot analysis demonstrated that there was no change in the protein levels of pJNK1 (p46; one-way ANOVA, F=1.489, p=0.2478; Figure 42B) or pJNK2 (p54; one-way ANOVA, F=2.889, p=0.0609; Figure 42B) in the SON at 1 or 3 hours post rrCNTF injection compared to control levels. As purely a qualitative observation, we performed a survey of the MAPK signaling components at other time points. Similar to what was observed at 1 and 3 hours post rrCNTF injection in the SON, we did not see
activation of pERK\(^{1/2}\), pMEK\(^{1/2}\), p-p38, or pJNK at 15, 30, 45 minutes, 6, 12, 24, or 48 hours post rrCNTF injection (Figure 43).

AKT, which has been demonstrated to be activated by CNTF in a Jak-dependent manner (Dolcet, et al., 2001; Gold, et al., 1994; Oh, et al., 1998), has two phosphorylation sites. The initial phosphorylation occurs at the Thr\(^{308}\) residue and is phosphorylated by PDK1, (Alessi, et al., 1996; K. E. Anderson, et al., 1998), with a secondary phosphorylation site located at the Ser\(^{473}\) residue, which is believed to be phosphorylated by mTORC2 in order to achieve maximal activation of AKT (Hresko & Mueckler, 2005; Sarbassov, et al., 2005). Figure 44A demonstrates a band for AKT at the expected 60 kDa. Quantitative Western blot analysis demonstrated that following pressure injection of rrCNTF into the SON there was no change in the protein levels of pAKT (Thr\(^{308}\)) (one-way ANOVA, F=1.262, \(p=0.3143\); Figure 44A) or pAKT (Ser\(^{473}\)) (one-way ANOVA, F=0.4443, \(p=0.7239\); Figure 44B) compared to control levels.

Next, we analyzed our samples for activation of transcription factors that CNTF has been demonstrated to activate, CREB and cJun. CREB was seen around 43 kDa and Western blot analysis demonstrated that there was no activation of CREB following rrCNTF injection into the SON (one-way ANOVA, F=1.029, \(p=0.4008\); Figure 45A). Similarly, cJun was found at approximately 50 kDa and quantitative Western blot analysis demonstrated that there was no significant difference in the amount of p-cJun in the SON following exogenous rrCNTF injection compared to control (one-way ANOVA, F=3.879, \(p=0.1206\); Figure 45B). Next, as a qualitative observation, we performed a survey of AKT,
CREB, and cJun at other time points. Similar to what was observed at 1 and
3 hours post rrCNTF injection in the SON, we did not see activation of pAKT
(Thr^{308}), pAKT (Ser^{473}), pCREB, or p-cJun at 15, 30, 45 minutes, 6, 12, 24, or
48 hours post rrCNTF injection (Figure 46).

Pharmacological Inhibition of the MAPK-ERK Pathway

We utilized three pharmacological inhibitors of the MAPK-ERK pathway,
U0126, PD98059, and PD184352. All of these inhibitors inhibit at the MAPKK
(MEK) level, which will prevent activation of the immediate downstream target,
ERK. Two of the inhibitors are structurally related, U0126 and PD98059, and
they have both been demonstrated to inhibit ERK½ and ERK5 (Ballif & Blenis,
2001; Su, Underwood, Rybalchenko, & Singh, 2011). However, the PD184352
has been recommended by others to be used to inhibit MEK½ because it is
specific for ERK½ (Bain et al., 2007). We also treated organotypic cultures with
U0124, which is a control molecule for the pharmacological inhibitor, U0126, and
does not inhibit MEK activity even at concentrations of 100 μM (manufacturer’s
technical sheet). Not surprisingly, when we treated organotypic cultures with
25 ng/ml rrCNTF plus 1 μM U0124, the control molecule, there was no significant
difference in the amount of surviving oxytocinergic neurons in the SON (one-way
ANOVA, F=46.83, p=0.4664; Figure 47A), PVN (one-way ANOVA, F=21.90,
p=0.8261; Figure 47B), and ACC (one-way ANOVA, F=32.72, p=0.7151; Figure
47C) compared to the 25 ng/ml rrCNTF group. Treatment with 25 ng/ml rrCNTF
plus 1 μM U0126, the pharmacological inhibitor, resulted in a statistically
significant decrease in the amount of surviving oxytocinergic neurons by 55% in
the SON (one-way ANOVA, $F=46.83, p<0.0001$; Figure 47A), by 40% in the PVN (one-way ANOVA, $F=21.90, p<0.0001$; Figure 47B), and by 42% in the ACC (one-way ANOVA, $F=32.72, p=0.0166$; Figure 47C) compared to the 25 ng/ml rrCNTF group. Although, only in the PVN did 1 μM U0126 plus 25 ng/ml rrCNTF treatment reduce the amount of surviving oxytocinergic neurons to control levels. Next, we treated organotypic cultures with the structurally related, later generation inhibitor, PD98059 (75 μM) plus 25 ng/ml rrCNTF. Our analysis demonstrated that 75 μM PD98059 in the presence of 25 ng/ml rrCNTF resulted in a statistically significant decrease, to control levels, in the amount of surviving oxytocinergic neurons by 80% in the SON (one-way ANOVA, $F=46.83, p<0.0001$; Figure 47A), by 65% in the PVN (one-way ANOVA, $F=21.90, p<0.0001$; Figure 47B), and by 82% in the ACC (one-way ANOVA, $F=32.72, p<0.0001$; Figure 47C) compared to the 25 ng/ml rrCNTF group. In order to determine if ERK½ or ERK5 is the signaling component necessary for CNTF-induced survival, we utilized the ERK½-specific inhibitor, PD184352. Our analysis demonstrated that 5 μM PD184352 in the presence of 25 ng/ml rrCNTF resulted in a statistically significant decrease, to control levels, in the amount of surviving oxytocinergic neurons by 84% in the SON (one-way ANOVA, $F=46.83, p<0.0001$; Figure 47A), by 53% in the PVN (one-way ANOVA, $F=21.90, p<0.0001$; Figure 47B), and by 99% in the ACC (one-way ANOVA, $F=32.72, p<0.0001$; Figure 47C) compared to the 25 ng/ml rrCNTF group. As controls for the inhibitors, we treated organotypic cultures with the inhibitors alone and there was not a statistical difference in the number of oxytocinergic neurons in the SON (one-way ANOVA, $F=42.72$, 95
$p>0.05$; Figure 47A), PVN (one-way ANOVA, $F=11.8$, $p>0.05$; Figure 47B), or ACC (one-way ANOVA, $F=20.87$, $p>0.05$; Figure 47C) compared to the control media-treated group, demonstrating that in the absence of rrCNTF, the concentrations of the inhibitors used did not adversely affect oxytocinergic neuron survival. Altogether, these data demonstrate that pharmacological inhibition of the MAPK-ERK½ pathway prevented CNTF-induced survival of oxytocinergic neurons in the SON, PVN, and ACC.

Representative low magnification montages of the magnocellular neurosecretory system nuclei and higher magnification micrographs of the SON further illustrate these results. The significant increase in the amount of oxytocinergic neurons between control (Figure 48A) and the rrCNTF plus U0124 control molecule (Figure 48B), which is not different than rrCNTF alone (Figure 49D), is apparent. Moreover, no visual difference was observed in magnocellular neuron process density following inhibition of the MAPK-ERK pathway with U0126 (Figures 48C and 49A), PD98059 (Figures 48D and 49B), or PD184352 (Figures 48E and 49C) compared to control SON (Figure 48A). Similar observations were observed when analyzing individual PVN following pharmacological inhibition of the MAPK-ERK pathway (see Figure 48).

**Pharmacological Inhibition of the p38- and JNK-MAPK Pathways**

For the pharmacological inhibition of the p38-MAPK pathway, we used SB203580, which inhibits the p38α and β isoforms, but not the γ and δ isoforms, and does not inhibit any of the JNK- or ERK-MAPK isoforms (English & Cobb, 2002; Greenwood & Bushell, 2010; J. C. Lee, et al., 1994). Our analysis...
demonstrated that 75 μM SB203580 in the presence of 25 ng/ml rrCNTF did not result in a statistically significant difference in the number of surviving oxytocinergic neurons in the SON (one-way ANOVA, F=50.10, p=0.1358; Figure 50A) and PVN (one-way ANOVA, F=50.49, p=0.1915; Figure 50B) compared to the 25 ng/ml rrCNTF group; but the number of surviving oxytocinergic neurons was still significantly elevated from control. However, following p38 inhibition with SB203580 in the presence of 25 ng/ml rrCNTF, we observed a statistically significant increase in the number of surviving oxytocinergic neurons by 60% in the ACC (one-way ANOVA, F=21.41, p<0.0001; Figure 50C) compared to the 25 ng/ml rrCNTF group. Interestingly, when we treated organotypic cultures with just SB203580 there was a statistically significant increase in the number of oxytocinergic neurons by 184% in the SON (one-way ANOVA, F=50.10, p<0.0001; Figure 50A), by 72% in the PVN (one-way ANOVA, F=50.49, p<0.0001; Figure 50B), and by 732% in the ACC (one-way ANOVA, F=21.41, p<0.0001; Figure 50C) compared to the control media-treated group. These data suggest that the p38-MAPK pathway may be involved in the injury-induced cell death process in hypothalamic organotypic cultures.

In order to inhibit the JNK-MAPK pathway, we utilized the SP600125 (JNK Inhibitor II) inhibitor which has equal potency towards all three of the JNK isoforms (JNK1-3) and has been shown to dose-dependently inhibit cJun (Bennett et al., 2001), and was demonstrated to have a 300 fold greater selectively for JNK compared to ERK or p38 (Ohnishi et al., 2010). Our analysis demonstrated that 50 μM SP600125 in the presence of 25 ng/ml rrCNTF did not
result in a statistically significant difference in the number of surviving oxytocinergic neurons in the SON (one-way ANOVA, F=50.10, p=0.4440; Figure 50A) and ACC (one-way ANOVA, F=21.41, p=0.0893; Figure 50B) compared to the 25 ng/ml rrCNTF group; but the number of surviving oxytocinergic neurons was still significantly elevated from control. However, following JNK inhibition with SP600125 in the presence of 25 ng/ml rrCNTF, we observed a statistically significant increase in the number of surviving oxytocinergic neurons by 66% in the PVN (one-way ANOVA, F=50.49, p<0.0001; Figure 50B) compared to the 25 ng/ml rrCNTF group. Similar to inhibition with SB203580, when we treated organotypic cultures with just SP600125 there was a statistically significant increase in the number of oxytocinergic neurons by 194% in the SON (one-way ANOVA, F=50.10, p<0.0001; Figure 50A), by 125% in the PVN (one-way ANOVA, F=50.49, p<0.0001; Figure 50B), and by 2152% in the ACC (one-way ANOVA, F=21.41, p<0.0001; Figure 50C) compared to the control media-treated group. These data suggest that, in addition to the p38-MAPK pathway, the JNK-MAPK pathway may also be involved in the injury-induced cell death process in hypothalamic organotypic cultures.

Representative low magnification montages of the magnocellular neurosecretory system nuclei and higher magnification micrographs of the SON further illustrate these results. When visibly comparing the amount of oxytocinergic neurons between SB203580 (Figures 51A and 52A) and the rrCNTF plus SB203580 (Figures 51B and 52B) groups, a slight increase in the number of surviving neurons in the rrCNTF plus SB203580 cultures can be
appreciated. Consistent with the quantitative neuronal count data (Figure 50), it is visibly apparent that there is a slight increase in the number of neurons in the SON and PVN of the rrCNTF plus SP600125 (Figures 51D and 52D) group compared to the SP600125 cultures (Figures 51C and 52C). Moreover, there appears to be similar amount of processes present in both the inhibitor and rrCNTF plus inhibitor groups (Figures 51 and 52). Similar observations were observed when analyzing individual PVN following pharmacological inhibition of the p38- and JNK-MAPK pathways (see Figure 51).

Pharmacological Inhibition of PI3K-AKT Pathway

We utilized two pharmacological inhibitors of the PI3K-AKT pathway that act on two separate and distinct signaling components: LY294002, which acts on the ATP binding site of PI3K (Zamin et al., 2006), and wortmannin (KY12420), which blocks the catalytic activity of PI3K (Luo et al., 2007). Moreover, we also utilized a control molecule for LY294002, LY303511, which contains a single atom substitution in the morpholine ring compared to LY294002 and does not inhibit PI3K even at 100 μM (manufacturer’s technical sheet). Our analysis demonstrated that when exposing the cultures to the control molecule, 15 μM LY303511, in the presence of 25 ng/ml rrCNTF we did not observe a statistically significant difference in the number of surviving oxytocinergic neurons in the SON (one-way ANOVA, F=36.29, p=0.6014; Figure 53A), PVN (one-way ANOVA, F=19.73, p=0.4931; Figure 53B), and ACC nuclei (one-way ANOVA, F=38.48, p=0.7503; Figure 53C) compared to the 25 ng/ml rrCNTF group; but the number of surviving oxytocinergic neurons were still significantly elevated from
control. When inhibiting PI3K with 15 μM LY294002 in the presence of 25 ng/ml rrCNTF, we did not observe a statistically significant difference in the number of surviving oxytocinergic neurons in the SON (one-way ANOVA, F=36.29, p=0.8791; Figure 53A) and ACC nuclei (one-way ANOVA, F=38.48, p=0.0738; Figure 53C) compared to the 25 ng/ml rrCNTF group. However, there was a statistically significant increase in the number of surviving oxytocinergic neurons by 35% in the PVN (one-way ANOVA, F=19.73, p=0.0373; Figure 53B) compared to the 25 ng/ml rrCNTF group. When we exposed the organotypic cultures to 1 μM wortmannin plus 25 ng/ml rrCNTF, we did not observe a significant difference in the number of surviving oxytocinergic neurons in the SON (one-way ANOVA, F=36.29, p=0.3364; Figure 53A), PVN (one-way ANOVA, F=19.73, p=0.7280; Figure 53B), or ACC (one-way ANOVA, F=38.48, p=0.3753; Figure 53C) compared to the 25 ng/ml rrCNTF group. As controls for the inhibitors, we treated organotypic cultures with the inhibitors alone and there was not a statistical difference in the number of oxytocinergic neurons in the SON (one-way ANOVA, F=38.48, p>0.05; Figure 53A), PVN (one-way ANOVA, F=19.73, p>0.05; Figure 53B), or ACC (one-way ANOVA, F=38.48, p>0.05; Figure 53C) compared to the control media-treated group, demonstrating that in the absence of rrCNTF, the concentrations of the inhibitors used did not adversely affect oxytocinergic neuron survival. Altogether, these data demonstrate that pharmacological inhibition of the PI3K-AKT pathway does not affect CNTF-induced oxytocinergic neuronal survival in hypothalamic organotypic
explant cultures indicating that PI3K is not involved in the CNTF-induced neuronal survival intracellular signaling cascade.

Representative low magnification montages of the magnocellular neurosecretory system nuclei and higher magnification micrographs of the SON further illustrate these results. When visibly comparing the number of oxytocinergic neurons between rrCNTF (Figures 54B and 55B), the rrCNTF plus LY294002 (Figures 54C and 55C), and the rrCNTF plus wortmannin (Figures 54D and 55D) groups, there are similar numbers of oxytocinergic neurons present, and these groups all have more oxytocinergic neurons than the control group (Figures 54A and 55A). However, what is visually apparent is that there are fewer neuronal processes present in the groups receiving PI3K inhibition compared to the rrCNTF group, even though they have the same number of surviving oxytocinergic neurons (compare 54B to 54C and 55B to 55C). The oxytocinergic neurons in the PI3K inhibited groups (Figures 55C and D) appear to have short stubby processes, if any at all, emanating from their soma, while the rrCNTF (Figure 55B) and control group (Figure 55A) have long, branching, punctate processes radiating from their cell bodies. In addition, there are numerous processes coursing between the nuclei in the rrCNTF group (Figures 54B and 55B), while there is a lack of these processes present in the PI3K inhibited groups.

In order to quantify this observation, we utilized a quantitative optical densitometric stereological analysis using the grain counting function in image analysis software to determine the proportional area of neuronal processes.
Figure 56 shows the various steps involved in generating these data in the SON of hypothalamic organotypic cultures following PI3K inhibition. Figure 56A shows the image of the SON captured such that the entire SON is centered in the bottom of the image. A highlighted SON is shown following setting of the density to highlight the neuronal processes and cell bodies (Figure 56B). Note that the analysis is a conservative estimate of the proportional area of neuronal processes because not all of the neuronal processes are highlighted (arrowhead, Figure 56A, B). A highlighted SON is shown following the setting of the density to highlight just the neuronal somata (Figure 56C). Note that similar to their immunoreactive profiles, the nuclei are not highlighted by the density setting (arrows, Figure 56A, C).

The value utilized in the statistical analysis was the ratio of process proportional area to total number of neurons in the SON. Figure 57 demonstrated that following administration of exogenous rrCNTF there was a significant increase in the proportional area of neuronal processes compared to control (one-way ANOVA, F=24.87, p<0.0001; Figure 57). The LY303511 molecule served as a control molecule for the LY294002 inhibitor and the proportional area of neuronal processes in the LY303511 plus 25 ng/ml rrCNTF group did not differ from the 25 ng/ml rrCNTF group (one-way ANOVA, F=24.87, p=0.6496; Figure 57). However, when the PI3K inhibitors, LY294002 and wortmannin, were administered to the organotypic cultures in the presence of 25 ng/ml rrCNTF, there was a significant reduction in the proportional area of neuronal processes in the SON compared to the 25 ng/ml rrCNTF group (one-way ANOVA, F=24.87,
Figure 57). Moreover, the LY294002 plus 25 ng/ml rrCNTF group was significantly reduced from control values (one-way ANOVA, F=24.87, \(p<0.0001\); Figure 57). These observations indicate that while PI3K signaling does not appear to be involved in CNTF-mediated neuronal survival, it is involved in CNTF-induced process outgrowth from oxytocinergic magnocellular neurons in the SON.

Pharmacological Inhibition of mTOR

mTOR is a signaling molecule that is primarily involved in PI3K-AKT signaling. We utilized two pharmacological inhibitors to inhibit mTOR; rapamycin and torin-1. Rapamycin is a selective and potent inhibitor of mTORC1 (Marwarha, Dasari, Prabhakara, Schommer, & Ghribi, 2010), and in order to understand the role of mTORC2 we also utilized torin-1, which is a selective and potent inhibitor for both mTORC1 and mTORC2 (Thoreen et al., 2012; Thoreen et al., 2009; Thoreen & Sabatini, 2009). Moreover, torin-1 has been demonstrated to provide more complete inhibition of mTORC1 when compared to rapamycin (Thoreen & Sabatini, 2009). Our analysis demonstrated that 10 \(\mu\)M rapamycin in the presence of 25 ng/ml rrCNTF did not result in a statistically significant difference in the number of surviving oxytocinergic neurons in the SON (one-way ANOVA, F=44.16, \(p=0.1113\); Figure 58A), PVN (one-way ANOVA, F=24.50, \(p=0.6633\); Figure 58B), and ACC (one-way ANOVA, F=23.92, \(p=0.0865\); Figure 58C) compared to the 25 ng/ml rrCNTF group; but the number of surviving oxytocinergic neurons were still significantly elevated from control. Similarly, when we treated organotypic cultures with 500 nM torin-1 plus 25 ng/ml
rrCNTF, our results demonstrated that there was no significant difference in the number of oxytocinergic neurons in the SON (one-way ANOVA, $F=44.16, p=0.1487$; Figure 58A), PVN (one-way ANOVA, $F=24.50, p=0.4250$; Figure 58B), and ACC (one-way ANOVA, $F=23.92, p=0.1816$; Figure 58C) compared to the 25 ng/ml rrCNTF group; but the number of surviving oxytocinergic neurons were still significantly elevated from control. When we treated organotypic cultures with rapamycin or torin-1, not in the presence of rrCNTF, there was a statistically significant increase in the number of oxytocinergic neurons in the SON (one-way ANOVA, $F=44.16, p<0.0001$; Figure 58A), PVN (one-way ANOVA, $F=24.50, p<0.0001$; Figure 58B), and ACC (one-way ANOVA, $F=23.92, p<0.0001$; Figure 58C) compared to the control media-treated group. These data suggest that mTOR signaling may be involved in the injury-induced cell death process in hypothalamic organotypic cultures.

Representative low magnification montages of the magnocellular neurosecretory system nuclei and higher magnification micrographs of the SON further illustrate these results. When visibly comparing the amount of oxytocinergic neurons between rapamycin (Figures 59A and 60A) and the rrCNTF plus rapamycin (Figures 59B and 60B) groups, you can appreciate that there was a slight increase in the number of surviving neurons in the SON of the rrCNTF plus rapamycin cultures. Consistent with the quantitative neuronal count data (Figure 58), it is visibly apparent that there is a slight increase in the number of neurons in the SON of the rrCNTF plus torin-1 (Figures 59D and 60D) group compared to the torin-1 cultures (Figures 59C and 60C). Moreover, while there
appears to readily visible processes present in the inhibitor and rrCNTF plus
inhibitor groups, the neuronal processes in the rrCNTF plus inhibitor groups were
more commonly organized in bundles or tracts spanning in between the SON
and ipsilateral PVN (Figure 59B, D).

*Pharmacological Inhibition of NF-κB*

We utilized two pharmacological inhibitors to inhibit NF-κB; bay 11-7082
and sc-514, which both inhibit IκBα phosphorylation and prevent activation of
NF-κB (Phulwani, Esen, Syed, & Kielian, 2008). Our analysis demonstrated that
15 and 30 μM bay 11-7082 in the presence of 25 ng/ml rrCNTF did not result in a
statistically significant difference in the number of surviving oxytocinergic neurons
in the SON (one-way ANOVA, F=46.56, p=0.9228, p=0.0828; Figure 61A), PVN
(one-way ANOVA, F=25.59, p=0.9003, p=0.0667; Figure 61B), and ACC (one-
way ANOVA, F=41.72, p=0.7507, p=0.2082; Figure 61C) compared to the
25 ng/ml rrCNTF group; but the number of surviving oxytocinergic neurons were
still significantly elevated from control. Similarly, when we treated organotypic
cultures with 20 μM sc-514 plus 25 ng/ml rrCNTF, our results demonstrated that
there was no significant difference in the number of oxytocinergic neurons in the
SON (one-way ANOVA, F=46.56, p=0.7561; Figure 61A), PVN (one-way
ANOVA, F=25.59, p=0.3522; Figure 61B), and ACC (one-way ANOVA, F=41.72,
 p=0.4565; Figure 61C) compared to the 25 ng/ml rrCNTF group; but the number
of surviving oxytocinergic neurons were still significantly elevated from control.
When we treated organotypic cultures with bay 11-7082, not in the presence of
rrCNTF, there was no significant difference in the number of oxytocinergic
neurons in the SON (one-way ANOVA, F=46.56, p=0.1130, p=0.9030; Figure 61A), PVN (one-way ANOVA, F=25.59, p=0.1822, p=0.1013; Figure 61B), and ACC (one-way ANOVA, F=41.72, p=0.9098, p=0.1128; Figure 61C) compared to the control media-treated group. However, when we treated the organotypic cultures with sc-514 we observed a statistically significant increase in the number of oxytocinergic neurons by 181% in the SON (one-way ANOVA, F=46.56, p<0.0001; Figure 61A), by 166% in the PVN (one-way ANOVA, F=25.59, p<0.0001; Figure 61B), and by 2058% in the ACC (one-way ANOVA, F=41.72, p<0.0001; Figure 61C) compared to the control media-treated group. Altogether, these data indicate that NF-κB is not involved in CNTF-mediated oxytocinergic neuronal survival.

Representative low magnification montages of the magnocellular neurosecretory system nuclei and higher magnification micrographs of the SON further illustrate these results. The significant increase in the amount of oxytocinergic neurons between control (Figures 62A and 63A) and the rrCNTF (Figures 62B and 63B) is apparent. When comparing the cultures that received NF-κB inhibition (Figures 62C, D and 63C, D), it is also visually apparent that the number of oxytocinergic neurons is similar to rrCNTF (Figures 62B and 63B). Moreover, no visual difference was observed in magnocellular neuron process density following inhibition of NF-κB with bay 11-7082 (Figures 62C and 63C) and sc-514 (Figures 62D and 63D) compared to rrCNTF (Figures 62B and 63B). Similar observations were observed when analyzing individual PVN following pharmacological inhibition of NF-κB (see Figure 61).
**Consistency of Organotypic Cultures**

Within each study, separate control and rrCNTF groups were performed. The previous experiments were performed over the course of three years, and utilized many different vials of exogenous rrCNTF from two separate lots. Lot #080M1730 of exogenous rrCNTF was utilized in the initial organotypic studies, the Jak-STAT study (Figure 38), the MAPK-ERK study (Figure 47), and the PI3K-AKT study (Figure 53), while lot #091M1403 was utilized for the p38- and JNK-MAPK study (Figure 50), the mTOR study (Figure 58), and the NF-κB study (Figure 61). Thus, we compared the control and rrCNTF groups from all of the studies in order to determine if there were changes in the number of oxytocinergic numbers within these groups across studies. Figure 64 demonstrated that throughout all of the experiments, there was no statistical difference in the number of oxytocinergic neurons in control SON (one-way ANOVA, $F=1.437, p=0.1752$; Figure 64A), PVN (one-way ANOVA, $F=2.072, p=0.0762$; Figure 64B), and ACC (one-way ANOVA, $F=1.153, p=0.3283$; Figure 64C). Similarly, when we compared the rrCNTF groups across the studies, our analysis demonstrated that there was no significant difference in the number of oxytocinergic neurons in the SON (one-way ANOVA, $F=1.325, p=0.2256$; Figure 65A), PVN (one-way ANOVA, $F=0.2128, p=0.9924$; Figure 65B), and ACC (one-way ANOVA, $F=1.458, p=0.1659$; Figure 65C). These analyses demonstrated that the organotypic cultures maintained consistency throughout the entirety of the studies.
CHAPTER IV
DISCUSSION
Specific Aim I

Previous studies have demonstrated that following unilateral lesion of the hypothalamo-neurohypophysial tract there is a collateral sprouting response that reinnervates the partially denervated NL (Watt, et al., 1999; Watt & Paden, 1991). The observed sprouting response does not occur as a result of a functional deficit; conversely, the magnocellular neurons undergo an increase in neurosecretory activity (Watt, et al., 1999). Therefore, the factor or factors responsible for the sprouting remain to be determined. Following reports demonstrating that CNTF promotes magnocellular neuron survival and possibly potentiates process outgrowth (Rusnak, et al., 2002; Vutskits, et al., 1998), we demonstrated an increase in the protein levels of CNTF and the CNTF receptor complex in the sprouting and axotomized SON and NL. However, it remained be determined if the observed changes in CNTF and the receptor complex were due to the sprouting response or the increase in neurosecretory activity. The results reported herein demonstrated that increased neurosecretory activity resulted in a decrease in CNTF protein levels, indicating that the post-injury sprouting response may be responsible for the observed increases in CNTF and the receptor complex. In addition, we determined that astrocytes in the SON and pituicytes and axons in the NL are the cellular phenotypes that express the entire
CNTF receptor complex, suggesting that CNTF-mediated neuroprotective actions in the SON occur in a paracrine mechanism.

While the collateral sprouting response occurs in 35 day old rats following unilateral hypothalamic lesion (Watt & Paden, 1991), the same post-injury sprouting response does not occur in 125 day old rats (J.A. Watt, unpublished observation). We have hypothesized that this is due to a deficit in the CNTF signaling process within the magnocellular neurosecretory system. To this point, we demonstrated a reduction in CNTFRα protein levels in the 125 day old rat SON. Moreover, following unilateral hypothalamic lesion in the 125 day old rat, the protein levels of CNTF and the CNTF receptor complex were not significantly heightened in the contralateral SON or axotomized NL. These data are suggestive that CNTF is involved in the post-injury sprouting response in the magnocellular neurosecretory system and a deficit in that response may underlie the reduced sprouting observed in older animals.

Unilateral Lesion of Hypothalamo-Neurohypophysial Tract in 35 Day Old Rat

The present observations confirm and extend our previous reports demonstrating an increase in CNTF-immunoreactivity and CNTFRα mRNA expression in the contralateral sprouting and axotomized SON (Watt, et al., 2006; Watt, et al., 2009). It has been widely reported that injury results in increased levels of CNTF in the proximity of the injury (M. Y. Lee, Deller, et al., 1997; X. G. Xia, Hofmann, Deller, & Kirsch, 2002). Not surprisingly, within the axotomized SON, protein levels of CNTF and the CNTF receptor complex are significantly elevated. In the axotomized SON, however, these increases did not prevent the
death of the injured magnocellular neurons. This may be due to the fact that CNTF has not conclusively been demonstrated to be secreted in vivo and it has been proposed that it is only released following loss of membrane integrity of the astrocytes (Richardson, 1994). CNTF lacks the secretory signal sequence which is necessary for protein secretion from the cell via the classic protein secretion pathway through the ER and Golgi apparatus (Vergara & Ramirez, 2004). While it has not been conclusively demonstrated if, or how, rat CNTF is secreted, chick CNTF is secreted through a non-classical secretory pathway (Reiness, et al., 2001) and reports have demonstrated that CNTF may be secreted in the absence of injury from cultured rat astrocytes (Kamiguchi, et al., 1995) and cultured bovine corneal endothelium (Koh, 2002). However, a more likely explanation for the extensive loss of the magnocellular neurons following the unilateral hypothalamic lesion is the proximity of the injury. The lesion tract is typically within 500 µm of the SON, and even if CNTF was being secreted, the degree of injury might be too severe for the magnocellular neurons to overcome. It was demonstrated that injury to the hypothalamo-neurohypophysial tract within the median eminence via nerve compression, which is much more distal to the magnocellular neurons than unilateral hypothalamic lesion, results in a significant loss of 31% oxytocinergic and 64% vasopressinergic neurons in the SON (Dolcet, et al., 2001; Shahar, et al., 2004). Magnocellular neuron loss following unilateral hypothalamic lesion reaches 85-93%; further demonstrating that the more proximal the injury is to the SON, the more severe the loss of magnocellular neurons.
Within the contralateral, sprouting SON, it was previously demonstrated that there is an increase in the message levels of α- and βII-tubulin (Paden et al., 1995b), which are subunits that form the major structural units, microtubules, that enable neurite extension. However, we did not observe this response in α- or βII-tubulin protein levels in the 35 day sprouting SON at 10 dpi. The observed changes in the message levels of α- and βII-tubulin were analyzed at 10 and 30 dpi; thus, the translational activity producing the α- and βII-tubulin proteins may not be initiated at 10 dpi since the post-lesion sprouting response occurs over the 1 to 4 week post-lesion period (Watt, et al., 1999; Watt & Paden, 1991). Therefore, studies assessing the α- or βII-tubulin protein levels in the SON during the entire 1 to 4 week post-lesion period would provide insight into the temporal pattern of the collateral sprouting response.

In addition to the increases in CNTF and the CNTF receptor complex in the axotomized SON, protein levels of CNTF and its receptor complex increased in the contralateral SON during the period of robust collateral sprouting of intact neurosecretory axons. The observed increases in CNTF and the CNTF receptor complex occurred at 10 dpi, which is during the collateral sprouting response that repopulates the axonal numbers to control levels between 1 and 4 weeks post lesion (Watt, et al., 1999; Watt & Paden, 1991). CNTF is of particular interest with regard to axonal sprouting due to its demonstrated effects on sprouting in the central and peripheral nervous systems. Numerous reports have demonstrated that CNTF promotes motorneuron sprouting (Gurney, et al., 1992; Guthrie, et al., 1997; Kwon & Gurney, 1994; Oyesiku & Wigston, 1996; Siegel, et al., 2000;
Simon, et al., 2010; Ulenkate, et al., 1994; Wright, et al., 2007; J. J. Xu, et al., 2009) and process outgrowth of retinal ganglion cells (Leibinger, et al., 2009; A. Muller, et al., 2007; A. Muller, et al., 2009). In addition, CNTF was previously implicated in hypothalamic magnocellular neuron process outgrowth *in vitro* (Vutskits, et al., 1998). Consistent with our results, others have demonstrated an increase in astrocytic CNTF and CNTFRα expression during the period of entorhinal cortex (M. Y. Lee, Deller, et al., 1997) and hippocampal sprouting (Guthrie, et al., 1997), providing further evidence for a direct involvement of CNTF in promoting post-injury axonal sprouting. The expression of CNTF and the CNTF receptor complex by SON astrocytes indicates that astrocyte-derived CNTF acts through an autocrine signaling mechanism to promote indirectly magnocellular survival and axonal sprouting. This is further evidenced by our data showing the absence of LIFRβ-immunoreactivity on magnocellular neurons, which would preclude receptor-mediated signaling in response to CNTF. These data are consistent with the observations of Rusnak, et al., who indicated that addition of CNTF to dissociated cultures of magnocellular neurons did not result in increased neuronal survival (Rusnak, et al., 2003). However, when CNTF is applied to stationary organotypic cultures of the SON and PVN, survival of axotomized magnocellular neurons is increased significantly (House, et al., 2009; Rusnak, et al., 2002; Rusnak, et al., 2003; Vutskits, et al., 1998; Vutskits, et al., 2003), indicating that CNTF is acting through astrocytes or other glial elements. Thus, CNTF may influence axonal sprouting by stimulating production of other factors known to influence process outgrowth including fibroblast growth factor-2
(FGF-2) and nerve growth factor (NGF) (Albrecht, et al., 2002; F. Jiang, Levison, & Wood, 1999; Semkova & Krieglstein, 1999; Wang et al., 2008). Consequently, the precise mechanism(s) by which CNTF influences axonal sprouting remains unclear.

Our results also demonstrated that following unilateral hypothalamic lesion, the protein levels of CNTF and the CNTF receptor complex were elevated in the NL, which is the terminal sprouting field. Consistent with our results, others have shown that CNTF expression is elevated in the terminal sprouting field following axotomy in the hippocampus (Guthrie, et al., 1997). There is evidence indicating that CNTF-immunoreactive perivascular cells of the NL are neuronophagic during the axonal degeneration that follows partial denervation of the 35 day old rat NL (Lo et al., in preparation), providing a possible explanation for the up-regulation of CNTF. Alternatively, another hypothesis for the observed increases in CNTF and the CNTF receptor components in the NL following axotomy is that CNTF in the NL may promote the collateral sprouting response; although, magnocellular neuron sprouting still occurs following hypophysectomy (Kiernan, 1971), which is an injury paradigm that completely removes the NL. However, unilateral hypothalamic lesion is an injury paradigm that differs from hypophysectomy in that it leaves a population of magnocellular neurons intact following the injury, suggesting that the post-lesion responses following these injuries may also be different. Therefore, while it is unlikely that the observed collateral sprouting response is due directly to a CNTF signaling response in the NL, there are reports indicating the CNTF/CNTFRα complex are retrogradely
transported in sensory neuron axons following injury (Curtis et al., 1993), and within the NL we demonstrated that oxytocinergic and vasopressinergic axons, in addition to pituicytes, are the cellular source of the CNTF receptor components. Therefore, these data indicate that a CNTF signaling response within the axotomized NL may play a role in the collateral sprouting response, but a more likely hypothesis may be that the collateral sprouting response is a combination of coordinated signaling responses between the intact SON and axotomized NL.

An alternative hypothesis to the role of CNTF in the NL is that CNTF may assist in neurosecretion of the neuropeptides from the neurosecretory axons. This hypothesis is based on the localization of the CNTF receptor complex to the neurosecretory axons and pituicytes within the NL. Early investigations of the NL led to the hypothesis that pituicytes synthesized and released neuropeptides; however, ultrastructural analyses later revealed that the neurosecretory granules were inside the neurosecretory axons that were surrounded or engulfed by pituicyte cytoplasm (Dreifuss, Sandri, Akert, & Moor, 1975). Although, numerous reports have suggested that pituicytes influence the control of neuropeptide release. For example, increased neurosecretory activity results in morphological changes in the pituicytes that assist in neurosecretion (Hatton, 1988; Tweedle & Hatton, 1980, 1982, 1987). In addition, CNTF has been demonstrated to induce the morphological alterations of astrocytes in vivo (Lisovoski et al., 1997). Thus, during states of increased neurosecretory activity, functioning through the CNTF receptor complex located on the pituicytes, CNTF may promote the morphological changes in the pituicytes to allow for the neuropeptide release.
from the neurosecretory axons. Future experiments involving the administration of exogenous rrCNTF to *ex vivo* NL cultures and analyzing oxytocin and vasopressin release would aid in the understanding of the possible role of CNTF in the NL.

*Increased Neurosecretory Activity in the Male Rat*

In our previous studies we demonstrated that the cross sectional area of oxytocinergic and vasopressinergic somata and nuclei are significantly increased in the sprouting SON by 10 and 30 dpi, respectively (Watt, et al., 1999). This hypertrophy is accompanied by increased neurosecretory activity as evidenced by a persistent increase in urine osmolality accompanied by decreased urine volume and water intake beginning 3 dpi and maintained throughout the sprouting event (Watt & Paden, 1991). These data lead us to hypothesize that collateral sprouting by intact magnocellular neurons occurs as a direct response to increased activity rather than as a consequence of partial denervation in the NL. This possibility is supported by studies demonstrating that the survival and collateral axonal sprouting of oxytocinergic and vasopressinergic neurons after axotomy is attenuated by decreased neuronal activity (Dohanics, et al., 1996; Herman, Marciano, Wiegand, & Gash, 1987; Shahar, et al., 2004; Watt, et al., 1999) and by evidence of increased magnocellular survival in response to potassium chloride stimulation (Shahar, et al., 2004) and chronic intermittent salt loading (Huang & Dellmann, 1996). Key to understanding the interaction between increased activity and collateral sprouting is to determine the responses of various growth factors to these events. The hypothesis that increased neuronal
activity may be sufficient in itself to induce changes in expression levels of CNTF and the CNTF receptor complex is supported by evidence for activity-induced increases in brain-derived neurotrophic factor (BDNF) mRNA expression in neurons in the PVN (Castren, Thoenen, & Lindholm, 1995) and SON (Aliaga, Arancibia, Givalois, & Tapia-Arancibia, 2002) following osmotic stimulation. Indeed, our data show that heightened metabolic activity in the absence of the post-denervation sprouting response resulted in a significant decrease in CNTF protein levels within the SON, but with no measureable change in CNTFRα, LIFRβ, or gp130 protein levels. However, it remains to be determined whether the decrease in CNTF protein levels resulted from a down-regulation in CNTF message levels or increased secretion as these two events would have predictably different effects on neurosecretory behavior.

Heightened neuronal activity has been demonstrated to modulate neurotrophin expression throughout the brain, such as increases in hippocampal BDNF during seizure (Katoh-Semba, Takeuchi, Inaguma, Ito, & Kato, 1999) and physical activity (Neeper, Gomez-Pinilla, Choi, & Cotman, 1996). Likewise, increased expression of BDNF and its secretion from dendrites of magnocellular neurons in the SON has been reported in salt-loaded rats (Aliaga, et al., 2002; Arancibia, Lecomte, Silhol, Aliaga, & Tapia-Arancibia, 2007). While the precise mechanisms of activity-dependent regulation of CNTF remain unclear, CNTF has been demonstrated to increase the expression of GFAP (Kahn, Ellison, Chang, Speight, & de Vellis, 1997) and morphological alterations in astrocytes in vivo (Lisovoski, et al., 1997). SON astrocytes are highly plastic and modulate
neurosecretory activity in part by altering their process morphology to facilitate increased neuron to neuron contact (Bobak & Salm, 1996; Salm & Hawrylak, 2004). Thus, the activity-dependant decrease in CNTF levels observed herein may influence magnocellular neuron behavior indirectly through decreased astrocyte GFAP synthesis and process retraction.

Increased excitatory afferent input may also modulate CNTF expression in the SON. The SON is the main target for the noradrenergic system arising from the brainstem (Cunningham & Sawchenko, 1988; Sawchenko & Swanson, 1981a). During states of heightened neurosecretory activity, noradrenaline, via the $\alpha_1$-adrenoreceptor, plays a critical excitatory role in the release of oxytocin and vasopressin from magnocellular neurons (W. E. Armstrong, Gallagher, & Sladek, 1986; Leibowitz, Eidelman, Suh, Diaz, & Sladek, 1990; Willoughby, Jervois, Menadue, & Blessing, 1987). Within the SON, the majority of adrenergic varicosities are found ventral to the magnocellular cell bodies in the dendritic zone (McNeill & Sladek, 1980; Swanson, et al., 1981). The dendritic zone is the location of the magnocellular neuron dendrites and astrocyte processes (W. E. Armstrong, et al., 1982; Dyball & Kemplay, 1982; Yulis, Peruzzo, & Rodriguez, 1984) and adrenergic receptors are expressed on astrocytes of the SON (Lafarga, Berciano, Del Olmo, Andres, & Pazos, 1992). In addition, reports have demonstrated that noradrenaline decreases CNTF mRNA expression and protein levels when applied to cultured astrocytes (Carroll, et al., 1993; Rudge, et al., 1994). Collectively, these reports suggest that during states of heightened neurosecretory activity in the SON, increased noradrenergic stimulation results in
down-regulation of CNTF protein levels in the SON. Future experiments will test this hypothesis by analyzing CNTF protein levels following chronic infusion of adrenergic receptor agonists and antagonists into the SON of salt-loaded and control rats in vivo.

During states of heightened neurosecretory activity, the NL also undergoes reversible morphological changes that help facilitate neurosecretion. During periods of low secretory activity, pituicytes completely surround and engulf neurosecretory axons and their terminals in the NL (Tweedle & Hatton, 1980, 1982). However, in response to osmotic stimulation, pituicytes retract from the parenchymal basement membrane resulting in an increase in neuronal-basement membrane contact area thereby facilitating neuropeptide release (Tweedle & Hatton, 1980, 1982, 1987). During chronic neurosecretory activity, we did not observe any changes in the protein levels of CNTF or the CNTF receptor complex in the male rat NL. Thus, in the male rat, only the protein levels of CNTF were decreased in the SON following increased neurosecretory activity.

*Increased Neurosecretory Activity in the Female Rat*

While the unilateral hypothalamic lesion paradigm has been exclusively performed in male rats, the magnocellular neurosecretory system of female rats can be activated to sustain chronic periods of increased neurosecretory activity by lactation and chronic salt-loading. Thus, we sought to determine if the observed response in male rats following increased neurosecretory activity occurs within the female magnocellular neurosecretory system. Following physiological activation in female rats via chronic salt-loading and sustained
lactation there was an up-regulation in CNTFRα protein levels in the SON. Within
the SON, CNTFRα is localized to oxytocinergic and vasopressinergic
magnocellular neurons and astrocytes (Watt, et al., 2009). Previous results have
demonstrated that within the SON increased neuronal activity promotes neuron
survival of injured vasopressinergic magnocellular neurons (Huang & Dellmann,
1996; Shahar, et al., 2004), while decreased neuronal activity reduces the
survival of injured vasopressinergic magnocellular neurons in the SON
(Dohanics, et al., 1996). Thus, the increase in CNTFRα protein levels in the
female SON that occurred as a result of increased neuronal activity may make
the magnocellular neurons more responsive to trophic support (i.e. CNTF) and
contribute to the neuroprotective role of increased neuronal activity. However, the
same response was not observed in the male SON, and the previous
experiments demonstrating that increased neuronal activity was beneficial were
performed in male rats (Dohanics, et al., 1996; Huang & Dellmann, 1996;
Shahar, et al., 2004), suggesting that there might be a gender-specific response
to the physiological activation on CNTFRα protein levels in the SON.

Numerous reports have demonstrated differential gender-specific
regulation of neurotrophins within the brain, including; BDNF, NGF, and
neurotrophin-3 (NT-3) (Bakos et al., 2009; Matsuki, Shirayama, Hashimoto,
Tanaka, & Minabe, 2001). Recently, McGregor et al. (2010) reported that CNTF
promoted contrasting effects on bone growth and remodeling in male and female
mice, indicating a possible hormonal influence on the actions of CNTF. Also, a
suggested role for CNTF in the reproductive axis has been proposed in the
female rat (Dozio et al., 2005; Stanley et al., 2000; Watanobe & Habu, 2001). Furthermore, gonadal steroids have long been implicated in the regulation of the magnocellular neurosecretory system (Amico, Seif, & Robinson, 1981; Skowsky, Swan, & Smith, 1979). Therefore, given the recognized influence of gonadal hormones on the SON (for review see Sladek & Somponpun, 2008), it would be of interest to determine possible hormonal interactions with CNTFRα as well as measuring CNTFRα protein levels within the rat SON throughout the female reproductive cycle.

Similar to the male NL, chronic salt-loading in the female NL did not affect the protein levels of CNTF or the CNTF receptor complex. Surprisingly, however, sustained lactation induced a reduction in LIFRβ protein levels in the NL. In the current study we demonstrated that LIFRβ is located on the axons and pituicytes within the NL; however, little is known about the general role of LIFRβ in the NL, or specifically, its role during lactation. In addition to being a receptor component for CNTF, LIFRβ is also involved in intracellular signaling for the following neurotrophins; leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin 1 (CT-1), and cardiotrophin-like cytokine (CLC) (Turnley & Bartlett, 2000). Thus, the down-regulation in LIFRβ protein levels in the female NL during lactation may not be a CNTF-dependent response. Therefore, future studies further characterizing the effects of lactation on cytokines that utilize LIFRβ would help in the understanding of the decrease of LIFRβ in the NL during lactation.
Maturing Rat Magnocellular Neurosecretory System

It has repeatedly been shown that the young brain can overcome injury by axonal reorganization (Crutcher & Chandler, 1985; Saporta & Phelps, 1985; Watt & Paden, 1991); however, it is well understood that the mature brain has a reduced capacity for functional or structural reorganization following injury (Crutcher, 1990; Scheff, Bernardo, & Cotman, 1978). While a robust sprouting response occurs in the magnocellular neurosecretory system of 35 day old rats, the same post-injury sprouting response does not occur in 125 day old rats (J.A. Watt, unpublished observation). Since CNTF is capable of inducing sprouting of injured motor neurons (Siegel, et al., 2000) and is implicated in the sprouting of injured MCNs in vitro (Vutskits, et al., 1998), we hypothesized that the reduced sprouting efficacy observed in mature rats was due to an age-dependent reduction in CNTF protein levels in the SON. Alternatively, a reduction in the proteins necessary for intracellular signaling in the mature SON, such as the CNTF receptor complex components or the Jak-STAT signaling proteins, might lead to a loss in sprouting efficacy. In this regard, it has been previously demonstrated that between 3 and 8 months of age there is a reduction in tSTAT3 protein expression in the rat brain (De-Fraja, Conti, Govoni, Battaini, & Cattaneo, 2000); however, it was not reported if this decrease occurs specifically in the magnocellular neurosecretory system. Interestingly, we demonstrated that from 35 to 125 days of age, there was a significant reduction in CNTFRα protein levels in the SON, with no changes in CNTF, LIFRβ, gp130, or tSTAT3 protein levels in the maturing SON or NL. Since CNTFRα is the specific receptor for CNTF and
CNTF signal transduction is unable to occur with only LIFRβ and gp130 (Davis, Aldrich, Stahl, et al., 1993), a reduction in CNTFRα could reduce the efficacy of CNTF signaling in the SON of the 125 day old rat, which may prevent the collateral sprouting response from occurring. Moreover, if the significant reduction in CNTFRα in the SON does prevent the collateral sprouting response from occurring, these data are suggestive that the collateral sprouting response is initiated in the SON since the mature NL exhibits consistent CNTFRα protein levels from 35 to 125 days of age.

In addition to the age-dependent decrease in CNTFRα protein levels in the SON, the protein levels of CNTF and the CNTF receptor complex did not increase in the contralateral SON or the axotomized NL following unilateral hypothalamic lesion in the 125 day old rat, while in the 35 day old rat, from which a sprouting response occurs following axotomy, all of these components are increased in the aforementioned regions. These data suggest that the lack of CNTFRα in the SON of the 125 day old rat precludes the heightened CNTF response, which we believe is necessary for the collateral sprouting response to occur. Interestingly, we did not see an increase in CNTF or the receptor components in the axotomized NL, which is the site of the degeneration of the axotomized axons. These data suggest that the increases in CNTF and the receptor components in the axotomized 35 day, 10 dpi NL are due to the sprouting event, and not the degeneration of the axotomized axons. However, the precise role of CNTF in the NL is still not known. To this point, there is evidence indicating that CNTF-immunoreactive perivascular cells of the NL are
neuronophagic during the axonal degeneration that follows partial denervation of the 35 day old rat NL (Lo et al., in preparation), indicating that CNTF may play an injury-dependent role in the NL. However, the neuronophagic role of CNTF in the 125 day old rat NL has not been reported. Altogether, these data provide evidence in support of the hypothesis that CNTF promotes the collateral sprouting response observed in the magnocellular neurosecretory system following partial denervation of the NL in the 35 day old rat. Studies in which stereotaxic infusion of CNTFRα and CNTF in to the contralateral SON following unilateral lesion in the 125 day rat are currently underway, and the results of these studies will provide valuable insight into the mechanisms mediating the collateral sprouting response.

Specific Aim / Conclusions

In conclusion, we provide the first evidence for activity-dependent regulation of CNTF in the CNS in the absence of injury. In addition, our data demonstrated that the increase in CNTF and CNTF-receptor complex observed in the sprouting SON is related to the sprouting response per se and not to the increased neurosecretory activity associated with the post-lesion response. We also demonstrated that the absence of immunoreactivity for the LIFRβ component of the CNTF receptor complex on magnocellular neurons further supports the hypothesis that CNTF is acting through an autocrine mechanism to enhance, indirectly, neuronal survival and axonal sprouting of magnocellular neurons. Moreover, we demonstrated that the absence of sprouting in the 125 day rat following unilateral hypothalamic lesion may be due to a deficit in
CNTFRα in the SON, which appears to affect the responsiveness of CNTF and the CNTF receptor complex in the contralateral SON and axotomized NL. Together, these support our hypothesis that the observed increase in CNTF and CNTF receptor complex is specifically related to the axonal sprouting response and supports neuronal sprouting through an astrocyte-dependent paracrine mechanism.

Specific Aim II

The Jak-STAT pathway is considered the canonical pathway for CNTF signal transduction (Bonni, et al., 1993; Bonni, et al., 1997; Darnell, et al., 1994). Moreover, our lab has demonstrated that exogenous rrCNTF injection into the SON in vivo activates STAT3 in astrocytes (Askvig et al., 2012b). Thus, these observations were extended to determine if pressure injection of exogenous rrCNTF into the SON resulted in a rapid and transient activation of the Jak-STAT pathway in vivo.

Others have demonstrated the neuroprotective effects that CNTF has on hypothalamic magnocellular neurons in organotypic cultures (House, et al., 2009; Rusnak, et al., 2002; Rusnak, et al., 2003; Shahar, et al., 2004; Vutskits, et al., 1998; Vutskits, et al., 2003). However, the mechanisms of these neuroprotective effects have not been reported. Therefore, utilizing hypothalamic organotypic cultures we determined that the Jak-STAT pathway is necessary to mediate CNTF-induced neuronal survival of oxytocinergic neurons. We utilized organotypic cultures because they exhibit several advantages over other in vitro culture systems primarily due to 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.

**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 Tyr\(^{705}\) (Bonni, et al., 1997; Darnell, et al., 1994; Wegenka, et al., 1993). We previously identified tSTAT3-immunoreactivity within the neurons and astrocyte cell bodies and processes throughout the SON (Askvig et al., 2012b). Moreover, tSTAT3-immunoreactivity was highly localized to the SON and not within the adjacent optic chiasm or surrounding hypothalamic region (Askvig et al., 2012b). These findings demonstrate specific localization of the Jak-STAT pathway to 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 the SON. This activation was previously demonstrated to be astrocytic and we did not observe pSTAT3 immunoreactivity in magnocellular neurons following rrCNTF injection (Askvig et al., 2012b). This is in accordance with our previous observation that magnocellular neurons in the SON are not immunoreactive for LIFRβ, which CNTF requires to mediate an intracellular signaling cascade (Davis, Aldrich, Stahl, et al., 1993). Consistent with our results, neurons of the hippocampus, neocortex, cerebellar cortex, and olfactory bulb express CNTFRα 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.

**Hypothalamic Organotypic Cultures**

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 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 oxytocinergic magnocellular neurons in the magnocellular neurosecretory system 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 oxytocinergic magnocellular neurons in the magnocellular neurosecretory system.

Others have reported that exogenous rrCNTF has a greater effect on vasopressinergic magnocellular neuron survival in the SON (Rusnak, et al., 2002) and the PVN (Vutskits, et al., 1998) compared to the observed effect on oxytocinergic magnocellular neurons. However, in our hands vasopressinergic magnocellular neurons did not survive in the presence of 25 ng/ml rrCNTF in the magnocellular neurosecretory system. 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 vasopressinergic magnocellular neurons survived at 255 mOsm/l but not at 300 and 330 mOsm/l culture media, while the survival of oxytocinergic 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 vasopressinergic magnocellular neuron survival in the magnocellular neurosecretory system.

Complex Administration

CNTFRα imparts its functionality either as a membrane bound receptor, which lacks a transmembrane sequence and is attached via a GPI-linkage or as a soluble receptor that results from the cleavage of the GPI-linked membrane-bound receptor in a phosphatidylinositol-specific phospholipase C (PI-PLC)-dependent mechanism (Davis, Aldrich, Ip, et al., 1993; Davis, et al., 1991). The enzymatic cleavage of membrane-bound CNTFRα releasing the soluble CNTFRα into the extracellular space is thought to occur in response to cellular injury, as demonstrated by its release from skeletal muscle following peripheral nerve injury (Davis, Aldrich, Ip, et al., 1993). While most examples of soluble receptors are thought to interfere with the binding of their ligands to membrane-bound receptors, soluble CNTFRα increases the biological activity of CNTF 30-fold (Ip, et al., 1993; Saggio, et al., 1995). Thus, soluble CNTFRα and CNTF
can stimulate cells that are not normally responsive to CNTF through the appropriate signal-transducing receptor components (gp130/LIFRβ) (Davis, Aldrich, Ip, et al., 1993), indicating that the GPI-linkage of CNTFRα may participate in a coordinated injury-release mechanism allowing soluble CNTFRα to potentially increase the functionality of CNTF.

Increasing evidence suggests that complex administration enhances the neuroprotective outcomes of CNTF signal transduction in the CNS. In cultured cortical neurons, complex protects against glutamate-mediated cytotoxicity as well as promoting nerve regeneration (Ozog, et al., 2004; Ozog, et al., 2008). However, our analyses demonstrated that complex administration did not increase the survival of the magnocellular neurons compared to the cultures receiving exogenous CNTF. Since astrocytes are the only cell type that contains all of the CNTF receptors, and the magnocellular neurons do not express LIFRβ, the astrocytes are the only cell phenotype that would be able to respond to CNTF, regardless of the presence of soluble CNTFRα. It has been previously demonstrated that all three receptor components are needed to elicit a CNTF signaling response (Davis, Aldrich, Stahl, et al., 1993); thus, it appears that administration of soluble CNTFRα only increases the efficacy of CNTF signaling if the target cell(s) lack the CNTFRα component and contained the gp130 and LIFRβ components.

Temporal Response of Exogenous rrCNTF

It was previously demonstrated that hypothalamic organotypic cultures that received CNTF for the initial 7-10 days in culture had an increase in
magnocellular neuron survival; however, CNTF failed to rescue the injured magnocellular neurons when added to the culture media only for the last 7 days of the 14 day experimental period (Rusnak, et al., 2003). Moreover, it was recently demonstrated that a single pretreatment injection of exogenous CNTF promoted neuronal survival following kainate insult two days later (Bechstein, et al., 2012). These reports suggest that an acute dose of CNTF can reprogram the cell into producing the neuroprotective transcriptional products of CNTF that would result in prolonged neuronal survival. Thus, we exposed organotypic cultures to exogenous rrCNTF for either 24, 48, or 72 hours with the cultures being exposed to control media for the remaining days of the 14 day experimental period. Following these treatments, we did not see an increase in the survival of oxytocinergic neurons. One possible explanation may be due to the severity of the injury that occurs during the culture preparation. Similar to the unilateral hypothalamic lesion paradigm in vivo, we demonstrated that there was greater than a 90% loss of oxytocinergic neurons following 14 days in control media following the organotypic culture preparation. And it has been hypothesized that it takes 7-10 days for the axotomy-induced neuronal death to subside (Rusnak, et al., 2003). Therefore, exposure of exogenous rrCNTF for the first 1-3 days following axotomy may not be adequate to overcome the substantial injury, especially since the production and release of inflammatory cytokines and chemokines following the axotomy, termed the secondary cascade of cell death (Beattie, 2004; Beattie, Farooqui, & Bresnahan, 2000), will continue to result in neuronal death many days post-lesion. Therefore, the
organotypic cultures would need exogenous rrCNTF throughout the period of the secondary cascade of cell death, which supports the data of Rusnak et al. (2003).

The Role of the Jak-STAT Pathway in CNTF-Induced Neuroprotection

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 oxytocinergic 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; A. Muller, et al., 2009; K. 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 & 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) (E. Jones & Symes, 2000; Pitts, Wang, Jones, & Symes, 2001; Symes, et al., 1997; Symes, et al., 1994), connexin 43 (Ozog, et al., 2004; van Adel, et al., 2005), FGF-2 (Albrecht, et al., 2002; Wang, et al., 2008), FGF receptor 1 (FGFR-1) (F. Jiang, et al., 1999), insulin-like growth factor type 1 receptor (IGF-IR) (F. Jiang, et al., 1999), NGF, p75 low-affinity receptor (Semkova & Krieglstein, 1999), and glial cell-line derived neurotrophic factor (GDNF) (Krady 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 (Theodosis, et al., 1991), was demonstrated to be necessary for CNTF-mediated survival of oxytocinergic and vasopressinergic magnocellular neurons (Vutskits, et al., 2003), possibly through the mediation of astrocyte-neuronal interactions. Interestingly, while the CNTF-family member LIF also promotes the survival of oxytocinergic and vasopressinergic 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 motorneuron sprouting (Gurney, et al., 1992; Guthrie, et al., 1997; Kwon & Gurney, 1994; Oyesiku & Wigston, 1996; Siegel, et al., 2000; Simon, et al., 2010; Ulenkate, et al., 1994; Wright, et al., 2007; J. J. Xu, et al., 2009) and process outgrowth of retinal ganglion cells (Leibinger, et al., 2009; A. Muller, et al., 2007; A. Muller, et al., 2009). Furthermore, CNTF has been
implicated in hypothalamic magnocellular neuron sprouting \textit{in vitro} (Vutskits, et al., 1998). In the present study, our data suggests that CNTF induces process outgrowth from oxytocinergic 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.

\textit{Specific Aim II Conclusions}

In conclusion, we provide the first evidence for the CNTF-induced activation of the Jak-STAT pathway in the SON \textit{in vivo}. We demonstrated previously that the Jak-STAT activation occurs within the astrocytes of the SON, indicating that a paracrine role of neuroprotection for CNTF in the SON (Askvig et al., 2012b). Moreover, we demonstrated that the exogenous rrCNTF promoted the survival of oxytocinergic neurons in organotypic cultures, and that the Jak-STAT pathway was necessary for CNTF to promote neuronal survival.

\textit{Specific Aim III}

While the Jak-STAT pathway is considered the canonical CNTF signal transduction pathway, CNTF has been demonstrated to activate multiple intracellular signaling pathways (Bonni, et al., 1993; Cagnon & Braissant, 2009; Dolcet, et al., 2001; Gallagher, et al., 2007; Kassen, et al., 2009; Loy, et al., 2011; Lutticken, et al., 1994; A. Muller, et al., 2009; K. Park, et al., 2004;
Peterson, et al., 2000; Rhee, et al., 2004; Sango, et al., 2008; Symes, et al., 1994; Trimarchi, et al., 2009). This may occur because there are multiple points of convergence between the signaling components of the different pathways. Moreover, it appears that the activation of a specific pathway is dependent on a combination of various factors, such as; the stimulus, the cellular phenotype activated, and the research paradigm. However, we did not observe activation of the MAPK or PI3K-AKT pathways in the SON following pressure injection of exogenous rrCNTF. Although, our pharmacological inhibition studies demonstrated that the MAPK-ERK½ pathway is necessary for CNTF-mediated neuronal survival and the PI3K-AKT pathway is necessary to mediate CNTF-induced process outgrowth. Moreover, p38, JNK, and mTOR appear to be involved in the injury-induced death of the oxytocinergic neurons.

The Role of the MAPK-ERK½ Pathway in CNTF-Induced Neuronal Survival

In addition to the Jak-STAT pathway, CNTF has been demonstrated to activate the MAPK pathways via other SH2 domain-containing proteins, such as the phosphatases SHP2 and Shc, which are bound to the activated receptors (Giordano, et al., 1997; Stahl, et al., 1995). Numerous reports have demonstrated that the MAPK-ERK½ pathway promotes neuronal survival following injury (Chicoine & Bahr, 2007; Jover-Mengual, Zukin, & Etgen, 2007; Nagata, 1999), primarily through the inhibition of the pro-apoptotic molecule BAD and production of the anti-apoptotic molecules, Bcl-2 and Bcl-XL (Nagata, 1999). We did not observe activation of the MAPK-ERK½ pathway following injection of exogenous rrCNTF; however, our pharmacological inhibition studies
demonstrated that the MAPK-ERK½ pathway is necessary to mediate CNTF-induced neuronal survival in the magnocellular neurosecretory system. To date, however, it remains unclear whether the MAPK-ERK½ pathway is signaling in the astrocytes with the Jak-STAT pathway, or within the neurons.

One possibility for the function of the MAPK-ERK½ pathway is that it may lead to activation of the STAT3 molecule in the SON. Unlike Jak molecules, which are tyrosine kinases, members of the MAPK pathway are serine/threonine kinases. Thus, MAPK-dependent activation of STAT3 occurs at Ser{sup}727{sub} as opposed to the more commonly Jak-dependently phosphorylated Tyr{sup}705{sub} (Decker & Kovarik, 2000). However, following pressure injection of exogenous rrCNTF into the SON, we did not observe activation of STAT3 at the Ser{sup}727{sub} residue, STAT3 activation was only observed at the Tyr{sup}705{sub} residue. Thus, it appears that the MAPK-ERK½ pathway is not upstream of STAT3 activation in the astrocytes.

Reports have suggested that ligands that activate the Jak-STAT pathway also activate the MAPK-ERK½ pathway (Frank et al., 1995; Ihle & Kerr, 1995; Winston & Hunter, 1996). This mechanism appears to be dependent on Jak-mediated Ras or Raf activation, which are the initiating kinases for ERK½ activation. While Jak-mediated activation of STAT molecules has been reported to be Ras-independent, Jak-mediated activation of the MAPK-ERK½ pathway has been demonstrated to be Ras-dependent (Winston & Hunter, 1995). Others have demonstrated that Raf physically associates with Jak2, and Raf is tyrosine phosphorylated when co-expressed with Jak2 (K. Xia et al., 1996). We did not examine Ras or Raf activation in the SON or pharmacologically inhibit Ras or Raf
in organotypic cultures to determine the role that these signaling components have on neuronal survival. If these experiments demonstrated that Ras or Raf were involved in CNTF-induced neuronal survival, experiments utilizing the pharmacological inhibitor of Jak2, AG490, would have to be performed to determine if their involvement was Jak-independent or -dependent. Regardless, Jak may be activating STAT and MAPK-ERK\(^1/2\) pathway in the astrocytes to allow them to signal in parallel following exogenous CNTF administration to promote neuronal survival, although, we have yet to observe activation of the MAPK-ERK\(^1/2\) pathway in the SON following exogenous rrCNTF injection or determine if there is a signaling connection between Jak and the MAPK-ERK\(^1/2\) pathway.

An alternate explanation, however, is that the MAPK-ERK\(^1/2\) pathway may be downstream of the Jak-STAT pathway and signaling in the neurons to promote CNTF-mediated neuronal survival. In order for this to occur, there would have to be communication between the astrocytes and neurons, such as growth factors, cellular adhesion molecules, and/or transcription factors of the CNTF-induced astrocytic Jak-STAT pathway, which would induce the activation of the MAPK-ERK\(^1/2\) in the neurons to directly promote neuronal survival. Recent observations from our lab indicate that in response to exogenous rrCNTF, cultured cortical astrocytes produce and release a molecule that promoted neuronal survival in the magnocellular neurosecretory system (K.E. Behm, unpublished observation). However, we have yet to observe activation of a signaling component in the neurons following pressure injection of exogenous rrCNTF in to the SON in vivo. Moreover, the temporal and spatial involvement of
the MAPK-ERK\(\frac{1}{2}\) pathway in mediating CNTF-induced neuronal survival remains unknown. Therefore, future experiments utilizing small hairpin RNA (shRNA) lentiviral particles to silence the MAPK-ERK\(\frac{1}{2}\) pathway in organotypic cultures and dissociated magnocellular neurons co-cultured with astrocytes will test these hypotheses in order to determine the cellular phenotype and temporal activation of the MAPK-ERK\(\frac{1}{2}\) pathway following exogenous rrCNTF administration in the magnocellular neurosecretory system.

The Role of the p38-, JNK-MAPK, and mTOR Pathways in Injury-Induced Neuronal Death

The p38- and JNK-MAPK pathways are structurally similar, but functionally distinct, from the classic MAPKs (ERKs). These pathways are preferentially activated by environmental stresses such as UV radiation, heat and osmotic shock, and by pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin-1 (IL-1) (Tibbles & Woodgett, 1999). Others have demonstrated that CNTF, which is classified as a pro-inflammatory cytokine, is capable of activating the p38- (Loy, et al., 2011) and the JNK-MAPK pathways (Cagnon & Braissant, 2009). However, we did not observe activation of the p38- or JNK-MAPK pathways in the SON following pressure injection of exogenous rrCNTF. Moreover, pharmacological inhibition of the p38- and JNK-MAPK pathways did not affect CNTF-mediated neuronal survival in hypothalamic organotypic cultures. The SB203580 inhibitor only inhibits the p38\(\alpha\) and \(\beta\) isoforms and not the \(\gamma\) and \(\delta\) isoforms, and does not inhibit any of the JNK- or ERK-MAPK isoforms (English & Cobb, 2002). The SP600125 inhibitor has equal potency towards all three of the JNK isoforms (JNK1-3) and has been shown to
inhibit cJun in a dose dependent manner (Bennett, et al., 2001). Unexpectedly, when the organotypic cultures were cultured only in the presence of the inhibitors, there was an increase in the survival of oxytocinergic neurons. Similarly, when the cultures were cultured only in the presence of the mTOR inhibitors, rapamycin and torin-1, we observed a similar increase in the survival of oxytocinergic neurons. These data suggest that the p38-, JNK-MAPK, and mTOR pathways are involved in the post-axotomy responses that lead to neuronal death in the organotypic cultures.

Our analysis demonstrated that the axotomy during the preparation of the organotypic cultures resulted in a greater than 90% loss of oxytocinergic and vasopressinergic neurons. It has been demonstrated previously that the vasopressinergic, but not oxytocinergic, magnocellular neurons in the PVN of organotypic cultures undergo neuronal death via apoptosis (Vutskits, et al., 1998). However, the anti-apoptotic agent, Bcl-xL, protected both oxytocinergic and vasopressinergic magnocellular neurons in the SON of organotypic cultures from degeneration, and an inhibitor of caspases, Z-VAD-fmk, rescued oxytocinergic magnocellular neurons in the SON (House, et al., 2006). These data suggest that the axotomy during the organotypic culture preparation results in an apoptotic response; however, due to the severe nature of the axotomy, a necrotic response cannot be discounted. The most quoted function of the p38- and JNK-MAPK pathways is its role in apoptosis (Cuenda & Rousseau, 2007; Dhanasekaran & Reddy, 2008). Moreover, because of the overlap of the MAPKKKs, specifically MKK4, involved in the p38- and JNK-MAPK pathways,
these pathways could be activated by the same stimulus. Thus, a possible explanation of the protection of the oxytocinergic neurons in the presence of the p38 and JNK inhibitors following axotomy is that both the p38- and JNK-MAPK pathways are activated by the injury to induce the apoptotic cascade in the neurons.

As mentioned above, the diverse signaling molecule, mTOR, was also protective of injured oxytocinergic neurons in the absence of exogenous rrCNTF. While mTOR has been primarily linked to the PI3K-AKT pathway, numerous reports have demonstrated mTOR signaling in the MAPK pathways (L. Chen, Liu, Luo, & Huang, 2008; S. Chen et al., 2011; Karassek et al., 2010; Kato et al., 2012; A. L. Miller, Garza, Johnson, & Thompson, 2007; B. Xu et al., 2011). While the main functions of mTOR signaling are involved in promoting process outgrowth, cell survival, cell proliferation, and cell growth, those functions primarily appear to be linked directly to the PI3K-AKT pathway (H. Zhou & Huang, 2010). However, when signaling within the MAPK pathways, mTOR activity may promote cell survival or cell death, depending on the stimulus (L. Chen, et al., 2008; S. Chen, et al., 2011; Karassek, et al., 2010; Kato, et al., 2012; A. L. Miller, et al., 2007; B. Xu, et al., 2011). Recently, a report demonstrated that mTORC1 signaling resulted in apoptosis through inhibition of AKT and selective activation of the JNK-MAPK pathway (Kato, et al., 2012). These reports suggest that JNK is capable of mediating an apoptotic response via mTOR, leading to our hypothesis that the following the axotomy during the organotypic culture preparation the p38- and JNK-MAPK pathways were co-
activated to induce an apoptotic cascade in the oxytocinergic neurons through the downstream signaling of mTOR.

Within the magnocellular neurosecretory system, understanding the specific mechanisms that regulate injury-induced neuronal apoptosis has not been well characterized. These novel data provide valuable insight into the mechanisms of axotomy-induced cell death that may lead to therapies that promote neuronal survival. However, exogenous rrCNTF administration resulted in more oxytocinergic magnocellular neurons surviving in the SON compared to inhibition of the p38-, JNK-MAPK, or mTOR pathways. Thus, it appears that targeting an extracellular neurotrophin receptor that would reprogram the cell’s transcriptional machinery into a neuroprotective mode may be more beneficial than inhibiting pro-apoptotic machinery. Until future in vivo analyses can be performed, this hypothesis remains unclear. Consequently, the therapeutic potential of the p38, JNK, and mTOR inhibitors will be tested in the SON in vivo utilizing the infundibular nerve crush paradigm, which results in the apoptotic death of 30-65% of the magnocellular neurons in the SON (Dohanics, et al., 1996; Shahar, et al., 2004). Following infundibular nerve crush, chronic infusion of the inhibitors, or exogenous rrCNTF, into the SON will determine if p38, JNK, and mTOR are involved in the post-injury apoptotic response and which therapeutic method has the greatest potential to promote neuronal survival in vivo.
The Role of the PI3K-AKT Pathway in CNTF-Induced Process Outgrowth

In addition to activating the Jak-STAT and MAPK pathways, reports have also demonstrated that CNTF can activate the PI3K-AKT pathway (Dolcet, et al., 2001; Gold, et al., 1994; Oh, et al., 1998). Moreover, it appears that CNTF-dependent activation of the PI3K-AKT pathway is a Jak-dependent mechanism (Dolcet, et al., 2001). In our analysis we did not observe activation of AKT following pressure injection of exogenous rrCNTF into the SON, however, we did not screen for activation of the PI3K signaling molecule. Pharmacological inhibition of PI3K did not affect CNTF-induced neuronal survival, indicating that CNTF does not utilize the PI3K-AKT pathway to promote oxytocinergic neuronal survival in the magnocellular neurosecretory system. Interestingly, there was a remarkable decrease in process outgrowth in the SON following pharmacological inhibition of PI3K compared to the CNTF-treated cultures. Although the tissue thickness and complexity of the processes prevented stereologic analysis of aspects of neurite outgrowth such as elongation, branching, and neurite caliber, we utilized quantitative optical densitometric stereological analysis using the grain counting function in image analysis software to determine the proportional area of neuronal processes which was corrected for the total number of neurons in the SON. This analysis resulted in a quantitative, yet conservative, analysis of process outgrowth, which confirmed our visual observation that there were fewer neuronal processes present in the SON following PI3K inhibition. Thus, these data suggest that the PI3K-AKT pathway is involved in regulating CNTF-mediated process outgrowth, but not involved in mediating neuronal survival.
It is not surprising that diverse intracellular signaling pathways mediate distinct neuroprotective processes in response to CNTF. Numerous reports have demonstrated divergent pathway mediation of CNTF-induced neuronal survival and process outgrowth (Dolcet, et al., 2001; Ozog, et al., 2008; K. Park, et al., 2004; Sango, et al., 2008). Similarities between those reports and our own observations indicate that the PI3K-AKT pathway plays a predominant role in mediating CNTF-induced process outgrowth in a variety of neuronal phenotypes. The PI3K-AKT pathway has been well-documented to promote neuronal survival, however, more recently the PI3K-AKT pathway has been revealed to be a key regulator in several aspects of process outgrowth, including elongation, branching, and neurite caliber (Read & Gorman, 2009). While the majority of the studies have focused on the role of AKT in mediating process outgrowth, our analysis directly implicates PI3K in CNTF-mediated process outgrowth. Since AKT is the primary downstream effector of PI3K, it is likely that AKT is also involved in mediating CNTF-induced process outgrowth. However, the direct role of AKT in mediating CNTF-induced process outgrowth has not been explored. There are many transcription factors that are downstream of PI3K that have been demonstrated to influence process outgrowth, including; CREB (Du & Montminy, 1998), NF-κB (Kane, et al., 1999; Romashkova & Makarov, 1999), and mTORC1 (Asnaghi, et al., 2004; Nave, et al., 1999). Not surprisingly, the transcriptional products of the PI3K-AKT pathway that directly mediate process outgrowth are cytoskeletal elements, such as microtubules (Kobayashi et al., 1997).
mTOR functions as two distinct signaling complexes, mTORC1 and mTORC2. These two complexes consist of unique mTOR-interacting proteins which determine their substrate specificity (H. Zhou & Huang, 2010). In a rapamycin-sensitive complex, mTORC1 consists of mTOR, raptor (regulatory associated protein of mTOR), DEPTOR, and GβL (Asnaghi, et al., 2004; Nave, et al., 1999), while mTORC2 consists of mTOR, Sin1, and rictor (rapamycin-insensitive companion of mTOR) (Jacinto et al., 2004). Interestingly, the roles of the mTOR complexes in the PI3K-AKT pathway are quite distinct. It is believed that mTORC2 functions upstream of AKT to assist in regulating maximal activity of AKT (Hresko & Mueckler, 2005; Sarbassov, et al., 2005), while mTORC1 is a downstream regulator of AKT activity (H. Zhou & Huang, 2010). It was demonstrated that PI3K-AKT-mTOR signaling has been shown to promote growth and branching of hippocampal neurons (Jaworski, Spangler, Seeburg, Hoogenraad, & Sheng, 2005). Our analysis demonstrated that pharmacological inhibition of mTORC1 and mTORC2 did not affect the survival or process outgrowth of oxytocinergic neurons, suggesting that mTOR is not involved in CNTF-mediated PI3K-AKT dependent process outgrowth of oxytocinergic neurons.

In addition to mTOR, NF-κB has been demonstrated to function as a transcription factor mediating process outgrowth in the PI3K-AKT pathway. NF-κB has been shown by several groups to regulate process outgrowth (S. J. Armstrong, Wiberg, Terenghi, & Kingham, 2008; Gutierrez, Hale, Dolcet, & Davies, 2005; O'Neil & Kaltschmidt, 1997; Sole et al., 2004). Our analysis
demonstrated that the NF-κB transcription factor was not necessary to mediate CNTF-induced neuronal survival or process outgrowth. Thus, the downstream effectors of the PI3K-AKT pathway that mediate CNTF-induced process outgrowth remain unknown. There are numerous downstream effectors of the PI3K-AKT pathway that have not been analyzed, including, but not limited to: CREB and δ-catenin, both of which have been demonstrated to play a role in process outgrowth (Read & Gorman, 2009). Furthermore, these data do not provide evidence for, or preclude, AKT as a signaling component involved in CNTF-mediated process outgrowth. To date, our evidence only indicates that PI3K is involved in CNTF-mediated process outgrowth. Therefore, future experiments will be performed that will determine the transcription factors and transcriptional products utilized in the PI3K pathway that mediate CNTF-induced process outgrowth. These experiments will provide clarity to the mechanisms that underlie CNTF-induced process outgrowth.

Similar to the MAPK-ERK½ pathway, it remains unclear whether the PI3K-AKT pathway is signaling in the astrocytes or within the neurons. Since the magnocellular neurons do not contain all of the CNTF receptor components, the immediate signaling response within the SON has to occur within astrocytes. While immediate activation of the Jak-STAT pathway in the astrocytes may induce an indirect response on the magnocellular neurons to elicit activation of the PI3K-AKT pathway that mediates process outgrowth, it is still unclear if the Jak-STAT pathway is involved in CNTF-mediated process outgrowth. Another hypothesis is that the PI3K-AKT pathway is activated in the astrocytes, and the
transcriptional products of this activation modulate the neurons to promote process outgrowth. Muller et al. (2009) demonstrated that astrocytes indirectly promoted process outgrowth via CNTF, however, it was mediated via the MAPK-ERK½ pathway as opposed to the PI3K-AKT pathway. Regardless, the ability of astrocytes to elicit an intracellular signaling response that leads to process outgrowth has been demonstrated. Future experiments utilizing dissociated magnocellular neurons co-cultured with astrocytes may help determine the temporal and spatial activity of the PI3K-AKT pathway in mediating CNTF-induced process outgrowth. Furthermore, in vivo experiments involving the infusion of the PI3K inhibitors into the sprouting SON following unilateral hypothalamic lesion in the 35 day old rat will directly test the hypothesis that the PI3K-AKT pathway is involved in the collateral sprouting response.

Specific Aim III Conclusions

In addition to the Jak-STAT pathway, we demonstrated that the MAPK-ERK½ pathway was also necessary to promote CNTF-induced neuronal survival of oxytocinergic neurons in the magnocellular neurosecretory system. To date, however, it remains unclear whether the MAPK-ERK½ pathway is signaling in the astrocytes with the Jak-STAT pathway, or within the neurons. Interestingly, we discovered that the p38-, JNK-MAPK, and mTOR pathways were involved in the apoptotic cascade of the oxytocinergic neurons following axotomy. These observations provide another therapeutic target to promote neuronal survival in post-injury conditions. However, the full potential of the pharmacological inhibition of these pathways in vivo needs to be explored. It has been previously
demonstrated that CNTF has been implicated in hypothalamic magnocellular neuron sprouting \textit{in vitro} (Vutskits, et al., 1998), however, we demonstrated the first quantitative evidence indicating that CNTF promotes process outgrowth from oxytocinergic magnocellular neurons in the SON. Moreover, we demonstrated that the PI3K signaling component was necessary to facilitate CNTF-induced process outgrowth. Altogether, these data indicate that diverse intracellular signaling pathways mediate distinct neuroprotective processes in response to CNTF. The results of these studies will provide the basis for future studies that will continue to elucidate the mechanisms underlying CNTF-mediated neuroprotection in the CNS. The long-term goal is that these mechanisms will lead to the development of future therapeutic strategies that will target nervous system injuries or disorders that result in neurodegenerative processes.
Figure 1. Intracellular Signaling Pathways Activated by CNTF. Schematic representation of the activation and interconnectivity of intracellular signaling pathways, including the Jak-STAT, MAPK, and PI3K-AKT pathways, which can be activated by CNTF.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host Species</th>
<th>Source</th>
<th>IHC</th>
<th>WB</th>
</tr>
</thead>
<tbody>
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<td>Akt</td>
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<td>Cell Signaling</td>
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<td>1:2000</td>
</tr>
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<td>1:2000</td>
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<td>pAkt (Thr(^{320}))</td>
<td>rabbit</td>
<td>Cell Signaling</td>
<td>NA</td>
<td>1:5000</td>
</tr>
<tr>
<td>β-actin</td>
<td>mouse</td>
<td>Serotec</td>
<td>NA</td>
<td>1:50,000</td>
</tr>
<tr>
<td>c-Jun</td>
<td>rabbit</td>
<td>Cell Signaling</td>
<td>NA</td>
<td>1:6000</td>
</tr>
<tr>
<td>p-c-Jun(Ser(^{202}))</td>
<td>rabbit</td>
<td>Cell Signaling</td>
<td>NA</td>
<td>1:3000</td>
</tr>
<tr>
<td>CNTF</td>
<td>goat</td>
<td>R&amp;D Systems</td>
<td>1:100</td>
<td>NA</td>
</tr>
<tr>
<td>CNTF</td>
<td>goat</td>
<td>R&amp;D Systems</td>
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<td>NA</td>
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<td>CREB</td>
<td>rabbit</td>
<td>BD Pharmigen</td>
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<td>Cell Signaling</td>
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<td>1:3000</td>
</tr>
<tr>
<td>ERK(^{1/2})</td>
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<td>Cell Signaling</td>
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<td>1:3000</td>
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<td>pERK(^{1/2}(Thr^{183}/Tyr^{185}))</td>
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<td>Cell Signaling</td>
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</tr>
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<td>GFAP</td>
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<td>Sigma</td>
<td>1:1000</td>
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</tr>
<tr>
<td>GFAP</td>
<td>goat</td>
<td>Sigma</td>
<td>1:1000</td>
<td>NA</td>
</tr>
<tr>
<td>gp130</td>
<td>rabbit</td>
<td>Santa Cruz</td>
<td>1:200</td>
<td>1:5000</td>
</tr>
<tr>
<td>pJak1(Tyr(^{102/105}))</td>
<td>rabbit</td>
<td>R&amp;D Systems</td>
<td>NA</td>
<td>1:1000</td>
</tr>
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<td>Jak2</td>
<td>rabbit</td>
<td>Cell Signaling</td>
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<td>1:2000</td>
</tr>
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<td>pJak2(Tyr(^{100/103}))</td>
<td>rabbit</td>
<td>Abcam</td>
<td>NA</td>
<td>1:2000</td>
</tr>
<tr>
<td>JNK</td>
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<td>R&amp;D Systems</td>
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<td>1:2000</td>
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<tr>
<td>pJNK(Tyr(^{103/105}))</td>
<td>rabbit</td>
<td>R&amp;D Systems</td>
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<td>1:2000</td>
</tr>
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<td>LIFR(^{A}(H-220))</td>
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<td>Santa Cruz</td>
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<tr>
<td>LIFR(^{B}(C-19))</td>
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<td>Santa Cruz</td>
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<td>MEK(^{1/2})</td>
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<td>Cell Signaling</td>
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<td>pMEK(^{1/2}(Ser^{211}))</td>
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<td>oxytocin</td>
<td>guinea pig</td>
<td>Peninsula Labs</td>
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<td>oxytocin-neurophysin</td>
<td>mouse</td>
<td>Hal Gainer lab</td>
<td>1:500</td>
<td>NA</td>
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<td>p38MAPK</td>
<td>rabbit</td>
<td>Cell Signaling</td>
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<tr>
<td>p-p38MAPK(Thr(^{180}/Tyr^{182}))</td>
<td>rabbit</td>
<td>Cell Signaling</td>
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<td>1:2000</td>
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<td>s100β</td>
<td>mouse</td>
<td>Millipore</td>
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<td>NA</td>
</tr>
<tr>
<td>STAT1</td>
<td>rabbit</td>
<td>Cell Signaling</td>
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<td>1:3000</td>
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<td>pSTAT1 (Ser(^{221}))</td>
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<td>Cell Signaling</td>
<td>NA</td>
<td>1:2000</td>
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<tr>
<td>pSTAT1 (Tyr(^{118}))</td>
<td>rabbit</td>
<td>Cell Signaling</td>
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<tr>
<td>STAT3</td>
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<td>Cell Signaling</td>
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<td>1:3000</td>
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<td>pSTAT3 (Ser(^{221}))</td>
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<td>Cell Signaling</td>
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<td>1:5000</td>
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<td>pSTAT3 (Tyr(^{118}))</td>
<td>rabbit</td>
<td>Cell Signaling</td>
<td>1:300</td>
<td>1:1000</td>
</tr>
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<td>Tyk2</td>
<td>rabbit</td>
<td>Cell Signaling</td>
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<td>1:2000</td>
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<td>pTyk2(Tyr(^{1054/1055}))</td>
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<td>Cell Signaling</td>
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<tr>
<td>vasopressin</td>
<td>guinea pig</td>
<td>Peninsula Labs</td>
<td>1:1000</td>
<td>NA</td>
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<td>vasopressin-neurophysin</td>
<td>mouse</td>
<td>Hal Gainer lab</td>
<td>1:500</td>
<td>NA</td>
</tr>
</tbody>
</table>

IHC = immunohistochemistry; NA = not applicable; p = phosphorylated; WB = Western blot
<table>
<thead>
<tr>
<th>Inhibitor (Concentration Used)</th>
<th>Pathway</th>
<th>Site of Action</th>
<th>Cross Reactivity?</th>
<th>Source Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG490 (50, 100 µM)</td>
<td>Jak-STAT</td>
<td>Jak2</td>
<td>Can inhibit Jak3, but Jak3 is only hematopoetically expressed</td>
<td>Calbiochem #658401-5MG</td>
</tr>
<tr>
<td>Bay 11-7082 (15 µM)</td>
<td>NF-κB transcription factor</td>
<td>Inhibits phosphorylation of IkBα</td>
<td>No mention on tech. sheet.</td>
<td>Calbiochem #196870-10MG</td>
</tr>
<tr>
<td>Cucurbitacin I (aka. JSI-124) (10 µM)</td>
<td>Jak-STAT</td>
<td>Suppresses tyrosine phosphorylation of STAT3</td>
<td>Does not affect Ras, AKT, JNK, or ERK½</td>
<td>Calbiochem #238590-1MG</td>
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<tr>
<td>LY294002 (15 µM)</td>
<td>PI3K-AKT</td>
<td>Acts on ATP binding site of PI3K</td>
<td>Does not effect, EGF receptor kinase, MAPK, PKC, PI-4 kinase, S6 kinase, or c-Src.</td>
<td>Calbiochem #440202</td>
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<tr>
<td>LY303511 (15 µM)</td>
<td>Control molecule for LY294002</td>
<td>Contains single atom substitution in morpholine ring compared to LY294002</td>
<td>Does not inhibit PI3K even at concentrations of 100 µM.</td>
<td>Calbiochem #440203</td>
</tr>
<tr>
<td>PD184352 (5 µM)</td>
<td>MAPK-ERK</td>
<td>Inhibitor of MEK. Specific for ERK½ and not ERK5.</td>
<td>Does not inhibit ERK5 (PD98059 and U0126 can, see reference), more specific for ERK½.</td>
<td>Santa Cruz #sc-202759</td>
</tr>
<tr>
<td>PD98059 (5 µM)</td>
<td>MAPK-ERK</td>
<td>Acts by inhibiting activation of MEK and subsequent phosphorylation of MAPK substrates. Is the same inhibitor as U 0126 except they are from different generation of production.</td>
<td>Has no effect on phosphorylation of Trk or Src, or PI3K. Can inhibit ERK5 as well as ERK½.</td>
<td>Calbiochem #513000</td>
</tr>
<tr>
<td>Rapamycin (10 µM)</td>
<td>mTOR</td>
<td>Inhibitor of mTORC1. Selectively inhibits phosphorylation and activation of p70 S6 kinase</td>
<td>No mention on technical sheet.</td>
<td>Calbiochem #553210</td>
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<tr>
<td>SB203580 (75 µM)</td>
<td>p38 MAPK</td>
<td>A highly specific, potent, cell permeable, selective, reversible, and ATP-competitive inhibitor of p38 MAPK</td>
<td>Does not significantly inhibit JNK or p42 MAPK at 100 µM.</td>
<td>Calbiochem #559389</td>
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<tr>
<td>Inhibitor (Concentration Used)</td>
<td>Pathway</td>
<td>Site of Action</td>
<td>Cross Reactivity?</td>
<td>Source Company</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------</td>
<td>---------------</td>
<td>-------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>SC-514 (aka. Iκk-2 inhibitor) (20 μM)</td>
<td>NF-κB transcription factor</td>
<td>Acts as a potent, reversible, ATP-competitive, and highly selective inhibitor of Iκk-2(β).</td>
<td>Does not affect MAPK signaling pathways.</td>
<td>Calbiochem # 401479</td>
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<tr>
<td>SP600125 (aka. JNK Inhibitor II) (50 μM)</td>
<td>JNK MAPK</td>
<td>A potent, cell-permeable, selective, reversible and ATP-competitive inhibitor of c-Jun N-terminal kinase (JNK).</td>
<td>Exhibits over 300 fold greater selectivity for JNK as compared to ERK 1 and p38 MAPK.</td>
<td>Calbiochem # 420119</td>
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<tr>
<td>Torin-1 (500 nM)</td>
<td>mTOR</td>
<td>A potent and selective mTOR inhibitor, for mTORC1 and mTORC2.</td>
<td>Displays 200 fold selectivity for mTOR over DNA-PK, ATM, and hVps34.</td>
<td>Tocris (R&amp;D Systems) #4247</td>
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<tr>
<td>U 0124 (1 μM)</td>
<td>Control molecule for UO126.</td>
<td>Useful negative control for U 0126.</td>
<td>Does not inhibit MEK activity even at 100 μM.</td>
<td>Calbiochem # 662006</td>
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<tr>
<td>U 0126 (1 μM)</td>
<td>MAPK-ERK</td>
<td>A potent and specific inhibitor of MEK1 and MEK2.</td>
<td>Has very little effect on Abl, Cdk2, Cdk4, ERK, JNK, MEKK, MKK-4/SEK, MKK-6, PKC, and Raf. Can inhibit ERK5 as well as ERK1%.</td>
<td>Calbiochem # 662005</td>
</tr>
<tr>
<td>Wortmannin (aka. KY12420) (1 μM)</td>
<td>PI3K-AKT</td>
<td>A potent, selective, and irreversible inhibitor of PI3K. Blocks the catalytic activity of PI3K without affecting upstream signaling effects.</td>
<td>Can block phospholipase D, myosin light chain kinase, and PI4K at high concentrations.</td>
<td>Calbiochem # 681675</td>
</tr>
</tbody>
</table>
Figure 2. Pharmacological Inhibition of the Jak-STAT Pathway. Schematic illustration demonstrating the pharmacological inhibitors of the Jak-STAT pathway, AG490 and curcubitacin I, and their site of action.
Figure 3. Pharmacological Inhibition of the MAPK Pathway. Schematic illustration demonstrating the pharmacological inhibitors of the MAPK pathways, U0126, PD98059, PD184352, SB203580, and SP600125, and their sites of action.
Figure 4. Pharmacological Inhibition of the PI3K-AKT Pathway. Schematic illustration demonstrating the pharmacological inhibitors of the PI3K-AKT pathway, LY294002 and wortmannin, and mTOR, rapamycin and torin-1, and their sites of action.
Figure 5. Pharmacological Inhibition of NF-κB. Schematic illustration demonstrating the pharmacological inhibitors of NF-κB, bay 11-7082 and sc-514, and their site of action.
Figure 6. Visual Confirmation of Successful Unilateral Hypothalamic Lesion. 
(A) Cresyl violet counterstained image confirming unilateral hypothalamic lesion. The lesion tract (arrows) is visible just lateral to the III ventricle and medial to the ipsilateral SON and cuts through the optic chiasm. Also apparent is the non-injured, contralateral SON which contains more neurons than the lesioned SON. (B) Immunofluorescently stained image for anti-oxytocin in the median eminence. The lack of oxytocin-immunoreactivity from the right hemisphere is indicative of a successful unilateral hypothalamic lesion while the intact magnocellular neurons on the contralateral hemisphere send oxytocinergic axons that course through the internal zone of the median eminence (arrows). OC, optic chiasm. SON, supraoptic nucleus. Scale bar A=300 μm; B=100 μm.
Figure 7. Magnocellular Neuron Survival Following Unilateral Hypothalamic Lesion in the 35 Day Rat SON. Immunohistochemical labeling of oxytocinergic and vasopressinergic magnocellular neurons were used to identify individual magnocellular neurons. Cell counts demonstrated no significant difference in the number of oxytocinergic or vasopressinergic neurons in the SON contralateral to the unilateral hypothalamic lesion. However, at 10 dpi the numbers of oxytocinergic and vasopressinergic neurons were reduced by 85% and 90% respectively in the axotomized SON. Column bars and error bars represent the mean and SD of each group. Each group is comprised of a minimum of six sections sampled from each of 5 animals. ***p<0.0001.
Figure 8. Pre-Adsorption Controls for CNTF and CNTFRα Western Blots. (A) Normal staining pattern for anti-CNTF. In addition to the expected band at 23 kDa, there are two additional bands between 36 and 55 kDa which are consistent with literature reports. (B) Following the pre-adsorption of the anti-CNTF antibody with a 10 molar excess of exogenous rrCNTF, there was a dramatic reduction in band intensity. (C) Normal staining pattern for anti-CNTFRα. In addition to the band at the expected molecular weight of 40 kDa, there was an additional band at 60 kDa which correlates to glycosylated CNTFRα. (D) Following the pre-adsorption of the anti-CNTFRα antibody with a 10 molar excess of exogenous rrCNTFRα, there was the complete absence of any bands. These controls indicate that the anti-CNTF and -CNTFRα antibodies are specific for their appropriate antigens.
Figure 9. CNTF and CNTF Receptor Complex are Increased at 10dpl in the 35 Day Rat SON. Western blot analysis demonstrated a significant increase in CNTF (A), CNTFRα (B), LIFRβ (C), and gp130 (D) protein levels in the axotomized SON compared to age-matched control. Within the sprouting SON contralateral to the lesion, CNTF (A), CNTFRα (B), LIFRβ (C), and gp130 (D) protein levels were significantly increased compared to age-matched control. Column bars and error bars represent the mean and SD of 5 groups. Each group represents isolated SON pooled from six rats. **p<0.01, ***p<0.0001.
Figure 10. CNTF and CNTF Receptor Complex are Increased at 10dpi in the 35 Day Rat NL. Western blot analysis demonstrated a significant increase in CNTF (A), CNTFRα (B), LIFRβ (C), and gp130 (D) protein levels in the axotomized NL compared to age-matched control. Column bars and error bars represent the mean and SD of 5 groups. Each group represents isolated SON pooled from six rats. *p<0.05, **p<0.01, ***p<0.0001.
Figure 11. α- and βII-Tubulin Protein Levels in the 35 Day Old Rat SON and NL at 10dpl. Western blot analysis demonstrated no significant difference in α- (A) or βII-tubulin (B) protein levels in the SON at 10dpl. Similarly, no significant difference was observed in α- (C) or βII-tubulin (D) protein levels in the NL at 10dpl. Column bars and error bars represent the mean and SD of 5 groups. Each group represents isolated SON pooled from six rats.
Figure 12. Chronic Salt-Loading Decreased CNTF Protein Levels in the Male Rat SON. (A) Western blot analysis revealed a significant decrease in CNTF protein levels in the male salt-loaded SON compared to the age-matched control SON. Moreover, Western blot analysis revealed no change in CNTFRα (B), LIFRβ (C), and gp130 (D) protein levels in the male salt-loaded SON compared to the age-matched control SON. Column bars and error bars represent the mean and SD of 5 groups. Each group represents isolated SON pooled from six rats. ***p<0.0001.
Figure 13. Chronic Salt-Loading Did Not Affect Protein Levels of CNTF or the CNTF Receptor Complex in the Male Rat NL. (A) Western blot analysis revealed no change in CNTF (A), CNTFRα (B), LIFRβ (C), or gp130 (D) protein levels in the male salt-loaded NL compared to the age-matched control NL. Column bars and error bars represent the mean and SD of each group. Each group represents isolated SON pooled from six rats.
Figure 14. Chronic Physiological Activation Increased CNTFRα Protein Levels in the Female Rat SON. (A) Western blot analysis revealed no significant difference in CNTF protein levels in the female lactating or female salt-loaded SON compared to the age-matched control SON. (B) However, both lactation and chronic salt-loading significantly increased CNTFRα protein levels in the female rat SON compared to age-matched control SON. Moreover, Western blot analysis revealed no change in LIFRβ (C) or gp130 (D) protein levels in the female lactating or female salt-loaded SON compared to the age-matched control SON. Column bars and error bars represent the mean and SD of 5 groups. Each group represents isolated SON pooled from six rats. **p<0.01, ***p<0.0001.
Figure 15. Lactation Decreased Protein Levels of LIFRβ in the Female Rat NL. Western blot analysis revealed no change in CNTF (A) or CNTFRα (B) protein levels in the lactating or female salt-loaded NL compared to the age-matched control NL. However, LIFRβ (C) protein levels in the lactating NL were significantly decreased from age-matched control and female salt-loaded LIFRβ protein levels. gp130 (D) protein levels in the lactating or female salt-loaded NL were not significantly different from age-matched control NL. Column bars and error bars represent the mean and SD of 5 groups. Each group represents isolated SON pooled from six rats. ***p<0.0001.
Figure 16. CNTF Protein Levels Did Not Change in the Maturing SON or NL. Western blot analysis revealed no change in CNTF protein levels in the control SON (A) or control NL (B) from 35 to 125 days of age. Column bars and error bars represent the mean and SD of 5 groups. Each group represents isolated SON pooled from six rats.
Figure 17. CNTFRα Protein Levels Decreased in the Maturing SON. (A) Western blot analysis revealed a significant decrease in CNTFRα protein levels in the control SON from 35 to 125 days of age. (B) However, no significant difference was observed in the control NL from 35 to 125 days of age. Column bars and error bars represent the mean and SD of 5 groups. Each group represents isolated SON pooled from six rats. *p<0.05 **p<0.01.
Figure 18. LIFRβ Protein Levels Did Not Change in the Maturing SON or NL. Western blot analysis revealed no change in LIFRβ protein levels in the control SON (A) or control NL (B) from 35 to 125 days of age. Column bars and error bars represent the mean and SD of 5 groups. Each group represents isolated SON pooled from six rats.
Figure 19. gp130 Protein Levels Did Not Change in the Maturing SON or NL. Western blot analysis revealed no change in gp130 protein levels in the control SON (A) or control NL (B) from 35 to 125 days of age. Column bars and error bars represent the mean and SD of 5 groups. Each group represents isolated SON pooled from six rats.
Figure 20. Total-STAT3 Protein Levels Did Not Change in the Maturing SON or NL. Western blot analysis revealed no change in total-STAT3 (tSTAT3) protein levels in the control SON (A) or control NL (B) from 35 to 125 days of age. Column bars and error bars represent the mean and SD of 5 groups. Each group represents isolated SON pooled from six rats.
Figure 21. CNTF and CNTF Receptor Complex are Increased at 10dpl in the 125 Day Rat Axotomized SON. Western blot analysis demonstrated a significant increase in CNTF (A), CNTFRα (B), LIFRβ (C), and gp130 (D) protein levels were not significantly different compared to age-matched control. Column bars and error bars represent the mean and SD of 5 groups. Each group represents isolated SON pooled from six rats. *p<0.05, **p<0.01, ***p<0.0001.
Figure 22. CNTF and CNTF Receptor Complex Did Not Increase at 10dpl in the 125 Day Rat NL. Western blot analysis demonstrated no significant difference in CNTF (A), CNTFRα (B), LIFRβ (C), and gp130 (D) protein levels in the 125 day axotomized NL compared to age-matched control. Column bars and error bars represent the mean and SD of 5 groups. Each group represents isolated SON pooled from six rats.
Figure 23. Magnocellular Neuron Survival Following Unilateral Hypothalamic Lesion in the 125 Day Rat SON. Immunohistochemical labeling of oxytocinergic and vasopressinergic magnocellular neurons were used to identify individual magnocellular neurons. Cell counts demonstrated no significant difference in the number of oxytocinergic or vasopressinergic neurons in the SON contralateral to the unilateral hypothalamic lesion. However, at 10 dpi the numbers of oxytocinergic and vasopressinergic neurons were reduced by 88% and 93% respectively in the axotomized SON. Column bars and error bars represent the mean and SD of each group. Each group is comprised of a minimum of six sections sampled from each of [n] animals. ***p<0.0001.
α and βII-Tubulin Protein Levels in the 125 Day Old Rat SON and NL at 10dpl. Western blot analysis demonstrated no significant difference in α- (A) or βII-tubulin (B) protein levels in the SON at 10dpl. Similarly, no significant difference was observed in α- (C) or βII-tubulin (D) protein levels in the NL at 10dpl. Column bars and error bars represent the mean and SD of 5 groups. Each group represents isolated SON pooled from six rats.
Differential Expression of CNTF Receptor Complex on Astrocytes and Magnocellular Neurons. Dual fluorescent colocalization of anti-gp130 (A), with anti-VP (B), revealed co-localization in vasopressinergic neurons (C, arrows). Note the gp130-immunoreactive profiles in presumptive astrocytes in the ventral glial limitans (VGL) of the SON (A, C, arrowheads). Similar observations were observed with anti-OT (not shown). Immunocytochemical analysis also demonstrated colocalization of anti-gp130 (D), with GFAP-immunoreactive astrocytes (E), of the SON (F, arrows). Also present are presumptive magnocellular neurons that are immunopositive for gp130 (D, F, asterisks). Unlike gp130, there was no observable colocalization of anti-LIFRβ in the magnocellular neurons of the SON (G-I). Note the LIFRβ-immunoreactive profiles surrounding blood vessels (v) in the SON (G, I, arrowheads) which do not colocalize with anti-VP (H) or anti-OT (not shown). However, strong LIFRβ-immunoreactivity in the ventral glial limitans (VGL) of the SON (J), revealed extensive colocalization of anti-LIFRβ (J), with anti-GFAP (K), throughout the entire SON (L, arrows). As a control for the dual label fluorescence immunohistochemistry, incubations with the primary antibodies were followed by incubation with the species specific fluorescent-conjugated secondary antibodies for the opposite primary antibody. These controls demonstrated an absence of immunoreactivity in the rat SON (M-N). OC, optic chiasm; OT, oxytocin; v, blood vessel; VGL, ventral glial limitans; VP, vasopressin. Scale bars A-L= 50 μm, M-N= 100 μm.
The CNTF Receptor Complex is Localized to Axons and Pituicytes in the NL. Dual-label immunohistochemistry demonstrated that CNTFRα- (A-C), LIFRβ- (I-K), and gp130- (Q-S) immunoreactive profiles are localized to oxytocinergic and vasopressinergic (not shown) axons. However, it is apparent that there are immunoreactive profiles that do not co-localize with the axonal profiles. We further determined that CNTFRα- (E-G), LIFRβ- (M-O), and gp130- (U-W) immunoreactive profiles also are localized to s100β-immunoreactive pituicytes, which are the astrocyte population of the NL. The controls demonstrated a decrease or complete lack of immunoreactivity present. The pre-adsorption controls for anti-CNTFRα with Texas red (D) and FITC (H) demonstrated a strong decrease in immunoreactivity, indicating that the anti-CNTFRα antibody is specific for CNTFRα. Moreover, negative controls for LIFRβ (L and P), and gp130 (T and X) demonstrated a lack of immunoreactivity, indicating that the secondary antibody was specific for species of the primary antibody. OT, oxytocin. Scale bars =10 μm.
Figure 27. 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 (Tyr705) 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 (Tyr705) levels at 3 hrs post-CNTF-injection were significantly decreased from the pSTAT3 (Tyr705) levels at 1 hr post-CNTF-injection. Administration of AG490 (10 mg/kg IP) 1 hour prior to rrCNTF injection significantly decreased the pSTAT3 (Tyr705) levels to levels that are not significantly different from vehicle-infused control SON but still significantly elevated from contralateral control SON. (B) Western blot analysis demonstrated no activation of pSTAT3 (Ser727) in the SON following pressure injection of exogenous rrCNTF. Column bars and error bars represent the mean and SD 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. pSTAT3, phosphorylated STAT3; ROD, relative optical density; tSTAT3, total STAT3. *p<0.05, ***p<0.0001.
Figure 28. Exogenous rrCNTF Did Not Activate STAT1, Tyk2, or Jak2. Western blot analysis of dissected SON samples demonstrated that acute pressure injection of 100 ng/μl rrCNTF did not result in activation of STAT1 (Tyr701) (A), STAT1 (Ser727) (B), Jak2 (C), or Tyk2 (D) in the SON. However, AG490 administration significantly decreased the level of pJak2 compared to all other groups (C). Column bars and error bars represent the mean and SD 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; pSTAT1, phosphorylated STAT1; pTyk2, phosphorylated Tyk2; ROD, relative optical density; tJak2, total Jak2; tSTAT1, total STAT1; tTyk2, total Tyk2. ***p<0.0001.
Figure 29. Qualitative Survey of Jak-STAT Pathway. A qualitative Western blot assessment of the Jak-STAT pathway was performed at various time points. Similar to what was observed at 1 and 3 hours post rrCNTF injection, we did not see activation of pSTAT3 (Ser727) at 15, 30, 45 minutes, 6, 12, 24, or 48 hours post rrCNTF injection. The bands present in the pSTAT3 (Tyr705) survey extend the quantitative observations observed at 1 and 3 hours post rrCNTF injection. pSTAT3 (Tyr705) activation is apparent by 30 and 45 minutes post rrCNTF injection and visible bands of pSTAT3 (Tyr705) are still apparent at 6 and 12 hours post rrCNTF injection; however, by 24 and 48 hours post rrCNTF injection we no longer see pSTAT3 (Tyr705) bands in the SON. Consistent with the levels of pSTAT1 (Ser727) and (Tyr701), pJak2, and pTyk2 at 1 and 3 hours post rrCNTF injection, we did not see an apparent increase in activation of these band sizes at any of the time points surveyed. pJak2, phosphorylated Jak2; pSTAT1, phosphorylated STAT1; pSTAT3, phosphorylated STAT3; pTyk2, phosphorylated Tyk2; tJak2, total Jak2; tSTAT1, total STAT1; tSTAT3, total STAT3; tTyk2, total Tyk2.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Time Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSTAT3 Ser727</td>
<td>15 min, 30 min, 45 min, 6 hr, 12 hr, 24 hr, 48 hr</td>
</tr>
<tr>
<td>pSTAT3 Tyr705</td>
<td></td>
</tr>
<tr>
<td>tSTAT3</td>
<td></td>
</tr>
<tr>
<td>pSTAT1 Ser727</td>
<td></td>
</tr>
<tr>
<td>pSTAT1 Tyr701</td>
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<td>tJak2</td>
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</tr>
<tr>
<td>β-actin</td>
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</table>

Post acute rrCNTF pressure injection.
Figure 30. Preservation of the Cytoarchitecture in Hypothalamic Organotypic Cultures. (A) A representative micrograph demonstrating that oxytocinergic magnocellular neurons maintains its in vivo cytoarchitecture in culture and contains two dendrites (thick arrows) with an axon (thin arrows) branching from one of the dendrites. (B) A representative micrograph montage demonstrating the preservation of the specific magnocellular nuclei after being in culture for 14 days. The organization of the oxytocinergic 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. (C) Analysis of control organotypic culture media demonstrated that the osmolality and pH did not change during the entire experimental period, with levels maintained around 310 mmol/kg and 8.1, respectively. PVN, paraventricular nucleus; SON, supraoptic nucleus. Scale bar A=50 μm, B=300 μm.
Organotypic Culture Media

Days in Culture
Table 3. Survival of rat hypothalamic oxytocinergic (OT) and vasopressinergic (VP) neurons in the SON, PVN, and ACC *in vitro*.

<table>
<thead>
<tr>
<th>Nuclei/Phenotype</th>
<th>Intact P6</th>
<th>Control</th>
<th>10 ng/ml rrCNTF</th>
<th>25 ng/ml rrCNTF</th>
<th>50 ng/ml rrCNTF</th>
</tr>
</thead>
<tbody>
<tr>
<td>SON/OT</td>
<td>878.6±305.3 (15)</td>
<td>20.74±29.95 (17)</td>
<td>62.7±41.02 (15)</td>
<td>162.4±65.36 (15)***</td>
<td>151.3±74.14 (17)***</td>
</tr>
<tr>
<td>SON/VP</td>
<td>529.1±271.2 (14)</td>
<td>3.88±4.45 (16)</td>
<td>8.39±9.0 (18)</td>
<td>10.97±13.68 (15)</td>
<td>13.92±11.62 (19)*</td>
</tr>
<tr>
<td>PVN/OT</td>
<td>1092±345.5 (15)</td>
<td>70.71±44.85 (17)</td>
<td>53.3±22.0 (15)</td>
<td>122.3±54.68 (15)***</td>
<td>162.8±38.53 (17)***</td>
</tr>
<tr>
<td>PVN/VP</td>
<td>267.8±123.4 (14)</td>
<td>36.13±26.32 (16)</td>
<td>31.4±27.13 (18)</td>
<td>19.7±18.22 (15) *</td>
<td>10.7±9.27 (19) **</td>
</tr>
<tr>
<td>ACC/OT</td>
<td>917.7±243.8 (15)</td>
<td>3.85±4.9 (17)</td>
<td>18.9±27.8 (15)</td>
<td>74.57±55.1 (15)***</td>
<td>74.91±56.57 (17)***</td>
</tr>
<tr>
<td>ACC/VP</td>
<td>248.8±118.9 (14)</td>
<td>0.75±1.28 (16)</td>
<td>0.44±0.78 (18)</td>
<td>1.07±1.62 (15)</td>
<td>1.47±1.72 (19)</td>
</tr>
</tbody>
</table>

Mean = cells/filter ±SD (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).
Figure 31. Exogenous rrCNTF Promotes Survival of Oxytocinergic Neurons in Vitro. Administration of 25 ng/ml rrCNTF for 14 days significantly increased the number of surviving oxytocinergic magnocellular neurons in the SON (A), PVN (C), and ACC (E). However, only a slight increase in the number of surviving vasopressinergic magnocellular neurons was observed in the SON following treatment with 50 ng/ml rrCNTF for 14 days (B), while there was no significant increase in the number of surviving vasopressinergic neurons in the PVN (D), or ACC (F). Column bars and error bars represent the mean and SD of [n] groups. ACC, accessory nuclei; PVN, paraventricular nucleus; SON, supraoptic nucleus. *p<0.05, **p<0.01, ***p<0.0001.
Figure 32. Soluble CNTFRα Does Not Enhance CNTF-Mediated Neuroprotection in Vitro. Administration of 25 ng/ml rrCNTF + 250 ng/ml rrCNTFRα (complex) for 14 days did not significantly increase the number of surviving oxytocinergic magnocellular neurons in the SON (A), PVN, (C), or ACC (E). We did not observe a significant increase in the number of vasopressinergic neurons in the SON (B), or ACC (F), following complex treatment; although, we did observe a significant increase in the number of surviving vasopressinergic neurons in the PVN following complex administration compared to exogenous rrCNTF administration (D). However, there was a significant decrease in the number of surviving vasopressinergic neurons in the PVN following exogenous rrCNTF administration (D), and complex administration recovers the amount of vasopressinergic neurons in the PVN to control levels (D). Column bars and error bars represent the mean and SD of [n] groups. ACC, accessory nuclei; PVN, paraventricular nucleus; SON, supraoptic nucleus. *p<0.05, **p<0.01, ***p<0.0001.
Oxytocinergic Neurons in ACC

Vasopressinergic Neurons in ACC

Oxytocinergic Neurons in PVN

Vasopressinergic Neurons in PVN

Oxytocinergic Neurons in SON

Vasopressinergic Neurons in SON

Oxytocinergic Neurons in ACC

Vasopressinergic Neurons in ACC
Figure 33. Micrograph Montages of Magnocellular Neurosecretory System Nuclei in Hypothalamic Organotypic Cultures Immunohistochemically Stained for Oxytocinergic Neurons. (A) Micrograph montage of magnocellular neurosecretory system nuclei of a representative explant slice fixed and immunohistochemically stained for oxytocinergic neurons at the time of sacrifice (post-natal day 6). Note the hypothalamo-neurohypophysial tract (A, arrows), which contains the magnocellular neuron axons that project to the NL. (B) Micrograph of oxytocinergic 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 oxytocinergic neurons from a representative explant slice that received 25 ng/ml rrCNTF treatment for 14 days. Note the substantial increase in oxytocinergic neurons in the SON and PVN as well as the maintenance of process density following rrCNTF treatment (arrows) compared to control (B). (D) Montage of oxytocinergic neurons from a representative explant slice that receive complex (25 ng/ml rrCNTF + 250 ng/ml rrCNTFRα) for 14 days. Similar to the culture receiving only exogenous rrCNTF, the culture receiving complex for 14 days also displayed a prominent increase in oxytocinergic neuron number and process density throughout the magnocellular neurosecretory system nuclei, particularly processes extending between the nuclei (arrows). 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 between the images. ACC, accessory nuclei; PVN, paraventricular nucleus; SON, supraoptic nucleus. Scale bar=300 μm.
• " •  - ACC
• •
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1 ? :; *  . 7
SON complex
204
Figure 34. Micrograph Montages of Magnocellular Neurosecretory System Nuclei in Hypothalamic Organotypic Cultures Immunohistochemically Stained for Vasopressinergic Neurons. (A) Micrograph montage of magnocellular neurosecretory system nuclei of a representative explant slice that received control media for 14 days. While there are an observable number of vasopressinergic neurons in the PVN, the majority of the neurons that are present appear to be parvocellular neurons, as opposed to the larger, more prominent, magnocellular neurons. (B) Montage of vasopressinergic neurons from a representative explant slice that received 25 ng/ml rrCNTF treatment for 14 days. It is apparent that there was not a significant increase in the amount of vasopressinergic neurons following exogenous rrCNTF treatment. Note that the representative images were not obtained from the same level of the magnocellular neurosecretory system. The micrograph in (B) is slightly more rostral than the micrograph in (A), which is apparent by the presence of the suprachiasmatic nucleus. PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus. Scale bar=300 µm.
Figure 35. Exogenous rrCNTF for Seven Days Enhanced Survival of Oxytocinergic Neurons in the PVN and ACC. Administration of 25 ng/ml rrCNTF for 7 days did not significantly increase the number of surviving oxytocinergic magnocellular neurons in the SON (A). However, 7 days of exogenous rrCNTF administration enhanced the survival of oxytocinergic neurons in the PVN (C) and ACC (E). Vasopressinergic magnocellular neuron survival in the SON (B), PVN (D), and ACC (F) were significantly increased with 7 days of exogenous rrCNTF administration compared to both the 7 day control and 14 day rrCNTF groups. Column bars and error bars represent the mean and SD of \([n]\) groups. ACC, accessory nuclei; PVN, paraventricular nucleus; SON, supraoptic nucleus. *\(p<0.05\), **\(p<0.01\), ***\(p<0.0001\).
Figure 36. Acute Exposure to Exogenous rrCNTF Does Not Promote Survival of Oxytocinergic Neurons. Our results demonstrated that 24, 48, or 72 hours of acute exposure to exogenous rrCNTF did not result in a significant increase in the number of oxytocinergic neurons in the SON (A), PVN (B), or ACC (C) compared to control. Column bars and error bars represent the mean and SD of [n] groups. ACC, accessory nuclei; PVN, paraventricular nucleus; SON, supraoptic nucleus. *p<0.05, **p<0.01, ***p<0.0001.
Figure 37. pSTAT3 Activation In Vitro Occurs in Astrocytes in the SON. In the control treated cultures, pSTAT3 activation in the SON occurs exclusively in GFAP-immunoreactive astrocytes (arrows, A-C) and not oxytocinergic magnocellular neurons of the SON (asterisks, D-F). Similarly, after a 1 hour exogenous rrCNTF treatment, pSTAT3 activation only occurred in GFAP-immunoreactive astrocytes (arrows, G-I) and not in oxytocinergic magnocellular neurons of the SON (asterisks, J-L). GFAP, glial fibrillary acidic protein; OT, oxytocinergic neuron; pSTAT3, phosphorylated STAT3. Scale bar=20 μm.
Figure 38. The Jak-STAT Pathway is Necessary to Mediate CNTF-Induced Survival of Oxytocinergic Neurons. Immunohistochemical neuronal cell counts demonstrated that exogenous rrCNTF promoted the survival of oxytocinergic neurons while inhibition of the Jak-STAT pathway with AG490 or cucurbitacin I significantly reduced the number of surviving oxytocinergic neurons in the SON (A), PVN (B), and ACC (C). Column bars and error bars represent the mean and SD of [n] groups. ACC, accessory nuclei; Cucu I, cucurbitacin I; PVN, paraventricular nucleus; SON, supraoptic nucleus. * p<0.05, ***p<0.0001.
Figure 39. Micrograph Montages of Hypothalamic Organotypic Cultures Following Jak-STAT Inhibition. (A) Micrograph montage of magnocellular neurosecretory system nuclei of a representative explant slice that received 25 ng/ml reverse sequence CNTF. Note the preservation of the hypothalamic nuclei and the few neurons present in the SON. (B) Micrograph of oxytocinergic magnocellular neurons of a representative explant slice that received 25 ng/ml rrCNTF treatment for 14 days. Note the substantial increase in oxytocinergic neurons in the SON, PVN, and ACC as well as the maintenance of process density following rrCNTF treatment compared to control (A). After pharmacological inhibition of the Jak-STAT pathway with AG490 (C) and curcibitaclin I (D), it is apparent that the number of surviving oxytocinergic neurons is consistent with what is observed in the control group (A). 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 between the images. ACC, accessary nuclei; PVN, paraventricular nucleus; SON, supraoptic nucleus. Scale bar =300 μm.
reverse sequence CNTF

SON PVN PVN ACC

SON

rrCNTF

SON

rrCNTF + AG490

SON

rrCNTF + cucurbitacin I
Figure 40. Micrographs of Individual SON Following Inhibition of the Jak-STAT Pathway. Note the substantial increase in neuron numbers in the SON following rrCNTF administration (D) compared to the control media group (A), and the groups receiving the pharmacological inhibition of the Jak-STAT pathway (B, C). Moreover, the maintenance of process density in the rrCNTF group (D) is apparent compared to the control group (A) and the Jak-STAT inhibited SON (B, C). Scale bar =100 μm.
Figure 41. Exogenous rrCNTF Did Not Activate ERK½ or MEK½. Western blot analysis of dissected SON samples demonstrated that acute pressure injection of 100 ng/μl rrCNTF did not result in activation of ERK½ (A) or MEK½ (B) in the SON. Column bars and error bars represent the mean and SD 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. pERK1, phosphorylated ERK1; pERK2, phosphorylated ERK2; pMEK½, phosphorylated MEK½; ROD, relative optical density; tERK1, total ERK1; tERK2, total ERK2; tMEK½, total MEK½.
Figure 42. Exogenous rrCNTF Did Not Activate p38 or JNK. Western blot analysis of dissected SON samples demonstrated that acute pressure injection of 100 ng/μl rrCNTF did not result in activation of p38 (A) or JNK (B) in the SON. Column bars and error bars represent the mean and SD 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. p46 pJNK, phosphorylated JNK1; p54 pJNK, phosphorylated JNK2; p46 tJNK, total JNK1; p54 tJNK, total JNK2; p-p38, phosphorylated p38; ROD, relative optical density; t-p38, total p38.
Figure 43. Qualitative Survey of MAPK Pathways. A qualitative Western blot assessment of the MAPK pathways was performed at various time points. Similar to what was observed at 1 and 3 hours post rrCNTF injection, we did not see activation of pERK½, MEK½, p38, or JNK at 15, 30, 45 minutes, 6, 12, 24, or 48 hours post rrCNTF injection. p46 pJNK, phosphorylated JNK1; p54 pJNK, phosphorylated JNK2; p46 tJNK, total JNK1; p54 tJNK, total JNK2; pERK1, phosphorylated ERK1; pERK2, phosphorylated ERK2; pMEK½, phosphorylated MEK½; p-p38, phosphorylated p38; tERK1, total ERK1; tERK2, total ERK2; tMEK½, total MEK½; t-p38, total p38.
Figure 44. Exogenous rrCNTF Did Not Activate AKT in the SON. Western blot analysis of dissected SON samples demonstrated that acute pressure injection of 100 ng/μl rrCNTF did not result in activation of AKT (Thr308) (A) or AKT (Ser473) (B) in the SON. Column bars and error bars represent the mean and SD 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. pAKT, phosphorylated AKT; ROD, relative optical density; tAKT, total AKT.
Exogenous rrCNTF did not Activate CREB or cJun in the SON. Western blot analysis of dissected SON samples demonstrated that acute pressure injection of 100 ng/μl rrCNTF did not result in activation of CREB (A) or cJun (B) in the SON. Column bars and error bars represent the mean and SD 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. p-cJun, phosphorylated cJun; pCREB, phosphorylated CREB; ROD, relative optical density; t-cJun, total cJun; tCREB, total CREB.
Figure 46. Qualitative Survey of AKT, CREB, and cJun. A qualitative Western blot assessment of the AKT, CREB, and cJun was performed at various time points. Similar to what was observed at 1 and 3 hours post rrCNTF injection, we did not see activation of AKT, CREB, or cJun at 15, 30, 45 minutes, 6, 12, 24, or 48 hours post rrCNTF injection. pAKT, phosphorylated AKT; p-cJun, phosphorylated cJun; pCREB, phosphorylated CREB; tAKT, total AKT; t-cJun, total cJun; tCREB, total CREB.
Figure 47. The MAPK-ERK½ Pathway is Necessary to Mediate the CNTF-Induced Survival of Oxytocinergic Neurons. Immunohistochemical neuronal cell counts demonstrated that exogenous rrCNTF promoted the survival of oxytocinergic neurons while inhibition of the MAPK-ERK½ pathway with U0126, PD98059, and PD184352 significantly reduced the number of surviving oxytocinergic neurons in the SON (A), PVN (B), and ACC (C). Column bars and error bars represent the mean and SD of [n] groups. ACC, accessory nuclei; PVN, paraventricular nucleus; SON, supraoptic nucleus. ***p<0.0001.
Figure 48. Micrograph Montages of Hypothalamic Organotypic Cultures Following MAPK-ERK½ Inhibition. (A) Micrograph montage of magnocellular neurosecretory system nuclei of a representative explant slice that received control media for 14 days. Note the preservation of the hypothalamic nuclei and the small number of neurons present in the SON. (B) Micrograph of oxytocinergic magnocellular neurons of a representative explant slice that received 25 ng/ml rrCNTF for 14 days. Note the substantial increase in oxytocinergic neurons in the SON, PVN, and ACC as well as the maintenance of process density following rrCNTF treatment compared to control (A). After pharmacological inhibition of the MAPK-ERK½ pathway with U0126 (C), PD98059 (D), and PD184352 (E), it is apparent that the number of surviving oxytocinergic neurons is consistent with what is observed in the control group (A). 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 between the images. ACC, accessory nuclei; PVN, paraventricular nucleus; SON, supraoptic nucleus. Scale bar = 300 μm.
Figure 49. Micrographs of Individual SON Following Inhibition of the MAPK-ERK½ Pathway. Note the substantial increase in neuron numbers in the SON following rrCNTF administration (D) compared to the groups receiving the pharmacological inhibition of the MAPK-ERK½ pathway (A,B, C). Scale bar =100 μm.
Figure 50. The p38- and JNK-MAPK Pathways Are Not Necessary to Mediate the CNTF-Induced Survival of Oxytocinergic Neurons. Immunohistochemical neuronal cell counts demonstrated that exogenous rrCNTF promoted the survival of oxytocinergic neurons while inhibition of the p38- and JNK-MAPK pathways with SB203580 and SP600125, respectively, did not affect the number of surviving oxytocinergic neurons in the SON (A), PVN (B), and ACC (C). However, when cultures were treated with the inhibitors alone, there was a significant increase in the number of surviving oxytocinergic neurons in the SON (A), PVN (B), and ACC (C), compared to control. Column bars and error bars represent the mean and SD of [n] groups. ACC, accessory nuclei; PVN, paraventricular nucleus; SON, supraoptic nucleus. *p<0.05, ***p<0.0001.
Figure 51. Micrograph Montages of Hypothalamic Organotypic Cultures Following p38- and JNK-MAPK Inhibition. (A) Micrograph montage of magnocellular neurosecretory system nuclei of a representative explant slice that received SB203580 for 14 days. Note the prominent processes and preservation of oxytocinergic neurons. (B) Micrograph of oxytocinergic magnocellular neurons of a representative explant slice that received pharmacological inhibition of the p38-MAPK pathway with SB203580 in the presence of 25 ng/ml rrCNTF for 14 days. Note the slight increase in the number of oxytocinergic neurons and processes in the SON, PVN, and ACC following inhibition of the p38-MAPK pathway in the presence of rrCNTF compared to SB203580 (A). (C) Micrograph montage of magnocellular neurosecretory system nuclei of a representative explant slice that received SP600125 for 14 days. Note the prominent processes and preservation of oxytocinergic neurons. (D) Micrograph of oxytocinergic magnocellular neurons of a representative explant slice that received pharmacological inhibition of the JNK-MAPK pathway with SP600125 in the presence of 25 ng/ml rrCNTF for 14 days. Note the slight increase in the number of oxytocinergic neurons and processes in the SON, PVN, and ACC following inhibition of the JNK-MAPK pathway in the presence of rrCNTF compared to SP600125 (C). The representative images were obtained from approximately the same level of the magnocellular neurosecretory system, which is apparent when comparing the III ventricle between the images. ACC, accessory nuclei; PVN, paraventricular nucleus; SON, supraoptic nucleus. Scale bar =300 μm.
Figure 52. Micrographs of Individual SON Following Inhibition of the p38- and JNK-MAPK Pathways. Note the slight increase in neuron numbers and process density in the SON following inhibition of the p38 (B) and JNK (D) pathways in the presence of rrCNTF compared to the groups receiving the inhibitors alone (A and C). Scale bar =100 µm.
Figure 53. The PI3K-AKT Pathway is Not Necessary to Mediate the CNTF-Induced Survival of Oxytocinergic Neurons. Immunohistochemical neuronal cell counts demonstrated that exogenous rrCNTF promoted the survival of oxytocinergic neurons while inhibition of the PI3K-AKT pathway with LY294002 and wortmannin did not affect the number of surviving oxytocinergic neurons in the SON (A), PVN (B), and ACC (C). Column bars and error bars represent the mean and SD of [n] groups. ACC, accessory nuclei; PVN, paraventricular nucleus; SON, supraoptic nucleus. *p<0.05, **p<0.01, ***p<0.0001.
# Oxytocinergic Neurons

## A. SON

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</tr>
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<td>25 ng/ml rCNTF</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>15 µM LY303511</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>15 µM LY294002</td>
<td>20 ± 3</td>
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## B. PVN

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<td>20 ± 3</td>
</tr>
<tr>
<td>15 µM LY303511</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>15 µM LY294002</td>
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</table>

## C. ACC

<table>
<thead>
<tr>
<th>Treatment</th>
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</thead>
<tbody>
<tr>
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<td>25 ng/ml rCNTF</td>
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</tr>
<tr>
<td>15 µM LY303511</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>15 µM LY294002</td>
<td>20 ± 3</td>
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Figure 54. Micrograph Montages of Hypothalamic Organotypic Cultures Following PI3K Inhibition. When visibly comparing the number of oxytocinergic neurons between rrCNTF (B), the rrCNTF plus LY294002 (C), and the rrCNTF plus wortmannin (D), groups, it is apparent that there are similar numbers of oxytocinergic neurons present, and these groups all have more oxytocinergic neurons than the control group (A). Furthermore, what is visually apparent is that there are fewer neuronal processes present in the groups receiving PI3K inhibition compared to the rrCNTF group, even though they have the same number of surviving oxytocinergic neurons. 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 between the images. ACC, accessory nuclei; PVN, paraventricular nucleus; SON, supraoptic nucleus. Scale bar =300 μm.
Figure 55. Micrographs of Individual SON Following Inhibition of the PI3K-AKT Pathway. Note the increase in neuron numbers and process density in the SON from control (A) to the rrCNTF group (B). The number of oxytocinergic neurons following inhibition of PI3K (C, D) is not different than the rrCNTF group (B); however, visibly there are fewer processes in the PI3K-inhibited groups (C, D) compared to the rrCNTF group (B). Scale bar =100 μm.
Figure 56. Micrographs Illustrating the Steps Involved in Quantifying the Area Occupied by Neuronal Processes in the SON. (A) The image of the SON, captured such that the entire SON is centered in the bottom of the image. (B) A highlighted SON is shown following setting of the density. The density has been set to highlight the neuronal processes and cell bodies, however, note that the analysis is a conservative estimate of the proportional area of neuronal processes because not all of the neuronal processes are highlighted (arrowheads, A, B). (C) A highlighted SON is shown following the setting of the density to highlight just the neuronal somata. Note that similar to their immunoreactive profiles, the nuclei are not highlighted by the density setting (arrows, A, C). Scale bar =100 μm.
Figure 57. The PI3K-AKT Pathway is Necessary to Mediate the CNTF-Induced Process Outgrowth of Oxytocinergic Neurons in the SON. The ratio of process proportional area to total number of neurons in the SON demonstrated that following administration of exogenous rrCNTF there was a significant increase in the proportional area of neuronal processes compared to control. The LY303511 molecule served as a control molecule for the LY294002 inhibitor and the proportional area of neuronal processes in the LY303511 in conjunction with 25 ng/ml rrCNTF group did not differ from the 25 ng/ml rrCNTF group. However, when the PI3K inhibitors, LY294002 and wortmannin, were administered to the organotypic cultures in the presence of 25 ng/ml rrCNTF, there was a significant reduction in the proportional area of neuronal processes in the SON compared to the 25 ng/ml rrCNTF group. Moreover, the LY294002 plus 25 ng/ml rrCNTF group was significantly reduced from control values. Column bars and error bars represent the mean and SD of [n] groups. **p<0.01, ***p<0.0001.
mTOR is Not Necessary to Mediate the CNTF-Induced Survival of Oxytocinergic Neurons. Immunohistochemical neuronal cell counts demonstrated that exogenous rrCNTF promoted the survival of oxytocinergic neurons while inhibition of mTOR with rapamycin and torin-1 did not affect the number of surviving oxytocinergic neurons in the SON (A), PVN (B), and ACC (C). However, when cultures were treated with the inhibitors alone, there was a significant increase in the number of surviving oxytocinergic neurons in the SON (A), PVN (B), and ACC (C), compared to control. Column bars and error bars represent the mean and SD of [n] groups. ACC, accessory nuclei; PVN, paraventricular nucleus; SON, supraoptic nucleus. ***p<0.0001.
Figure 59. Micrograph Montages of Hypothalamic Organotypic Cultures Following mTOR Inhibition. (A) Micrograph montage of magnocellular neurosecretory system nuclei of a representative explant slice that received rapamycin for 14 days. Note the preservation of oxytocinergic neurons. (B) Micrograph of oxytocinergic magnocellular neurons of a representative explant slice that received pharmacological inhibition of mTOR with rapamycin in the presence of 25 ng/ml rrCNTF for 14 days. Note the slight increase in the number of oxytocinergic neurons and processes in the SON, PVN, and ACC following inhibition of mTOR in the presence of rrCNTF compared to rapamycin (A). (C) Micrograph montage of magnocellular neurosecretory system nuclei of a representative explant slice that received torin-1 for 14 days. Note the prominent processes and preservation of oxytocinergic neurons. (D) Micrograph of oxytocinergic magnocellular neurons of a representative explant slice that received pharmacological inhibition of mTOR with torin-1 in the presence of 25 ng/ml rrCNTF for 14 days. Note the slight increase in the number of oxytocinergic neurons and processes in the SON, PVN, and ACC following inhibition of mTOR in the presence of rrCNTF compared to torin-1 (C). The representative images were obtained from approximately the same level of the magnocellular neurosecretory system, which is apparent when comparing the III ventricle between the images. ACC, accessory nuclei; PVN, paraventricular nucleus; SON, supraoptic nucleus. Scale bar = 300 μm.
A

ACC

SON

rapamycin

B

ACC

SON

rrCNTF + rapamycin

C

ACC

SON

torin-1

D

ACC

SON

rrCNTF + torin-1
Figure 60. Micrographs of Individual SON Following Inhibition of mTOR. Note the slight increase in neuron numbers and process density in the SON following inhibition of mTOR with rapamycin (B) and torin-1 (D) in the presence of rrCNTF compared to the groups receiving the inhibitors alone (A and C). Scale bar =100 μm.
Figure 61. NF-κB is Not Necessary to Mediate the CNTF-Induced Survival of Oxytocinergic Neurons. Immunohistochemical neuronal cell counts demonstrated that exogenous rrCNTF promoted the survival of oxytocinergic neurons while inhibition of NF-κB with bay 11-7082 and sc-514 did not affect the number of surviving oxytocinergic neurons in the SON (A), PVN (B), and ACC (C). However, when cultures were treated with sc-514 alone, there was a significant increase in the number of surviving oxytocinergic neurons in the SON (A), PVN (B), and ACC (C), compared to control. Column bars and error bars represent the mean and SD of [n] groups. ACC, accessory nuclei; PVN, paraventricular nucleus; SON, supraoptic nucleus. **p<0.01, ***p<0.0001.
Figure 62. Micrograph Montages of Hypothalamic Organotypic Cultures Following NF-κB Inhibition. (A) Micrograph montage of magnocellular neurosecretory system nuclei of a representative explant slice that received control media for 14 days. Note the preservation of oxytocinergic neurons in their respective nuclei. (B) Micrograph montage of a representative explant slice that received 25 ng/ml rrCNTF for 14 days. The significant increase in the number of oxytocinergic neurons compared to control (A) is obvious, as well as the visual increase in the process density. Micrograph of oxytocinergic magnocellular neurons of a representative explant slice that received pharmacological inhibition of NF-κB with bay 11-7082 (C) and sc-514 (D) in the presence of 25 ng/ml rrCNTF for 14 days. Note the similar appearance of processes and neurons compared to the rrCNTF group (B). The representative images were obtained from approximately the same level of the magnocellular neurosecretory system, which is apparent when comparing the III ventricle between the images. ACC, accessory nuclei; PVN, paraventricular nucleus; SON, supraoptic nucleus. Scale bar =300 μm.
25 ng/ml rrCNTF

rrCNTF + bay 11-7082

rrCNTF + sc-514
Figure 63. Micrographs of Individual SON Following Inhibition of NF-κB. Note the increase in neuron numbers and process density in the SON from control (A) to the rrCNTF group (B). The number and process density of oxytocinergic neurons following inhibition of NF-κB (C, D) appears not to be different than the rrCNTF group (B). Scale bar =100 μm.
Figure 64. Consistency of Control Groups Throughout the Study. Throughout all of the experiments, there was no statistical difference in the number of oxytocinergic neurons in control SON (A), PVN (B), or ACC (C). Column bars and error bars represent the mean and SD of [n] groups.
Figure 65. Consistency of rrCNTF Groups Throughout the Study. Throughout all of the experiments, there was no statistical difference in the number of oxytocinergic neurons in the rrCNTF-treated SON (A), PVN (B), or ACC (C). Column bars and error bars represent the mean and SD of [n] groups.
REFERENCES


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