ABSTRACT

NEUROTROPHIN EXPRESSION IN SYMPATHETIC NEURONS: INFLUENCES OF EXOGEOUS NGF AND AFFERENT INPUT

by Elizabeth Ellen Jones

The regulatory mechanisms that promote the survival of adult neurons remain unclear. Target-derived proteins called neurotrophins are known to play a role in the survival of sympathetic neurons, but their exact function is unknown. In the present study, expression of the neurotrophins nerve growth factor (NGF) and neurotrophin-3 (NT-3) in adult sympathetic ganglia and their targets was investigated. Using Western blot analysis, multiple precursor forms of both NGF and NT-3 were observed in neurons and blood vessel targets. In addition, using real time RT-PCR, transcripts for both NGF and NT-3 were detected in the ganglia, demonstrating the potential for neurotrophin synthesis in peripheral ganglia. The expression patterns of both NGF and NT-3 were found to be altered by both exogenous NGF and removal of pre-ganglionic input, suggesting the regulatory influences of both NGF and afferent input on neurotrophin expression and ultimately neuronal survival. Overall, this study advanced the understanding of neurotrophin regulation of neuronal survival.
NEUROTROPHIN EXPRESSION IN SYMPATHETIC NEURONS: INFLUENCES OF EXOGENOUS NGF AND AFFERENT INPUT

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INTRODUCTION

Neurotrophins

Neurotrophic factors are target-derived polypeptides that regulate the growth, differentiation and survival of specific neuronal cell populations during development, and maintain these neurons throughout adulthood [15,103]. Several protein members in this family include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Neurotrophins are characterized by a high number of “flexible hairpin loops”, in which most of their sequence variability exists, and three disulfide bridges which form into a distinct “cystine knot motif” [73,115]. Neurotrophins exist in vivo as dimers stabilized by a large hydrophobic interface [73].

The first discovered and accordingly most characterized member of the neurotrophin family is nerve growth factor (NGF). Isolated from a concentrated source, such as the male mouse submaxillary gland, NGF is comprised of three polypeptide subunits, α, β, and γ, of which the β subunit is known to be the mature, biologically active portion [155]. Both NGF and NT-3 have been shown to be released through both a constitutive [51,69,120] and a regulated secretory pathway [41,68,84,163]. These discrepancies have largely been attributed to experimental conditions in vitro, and their biological relevance remains somewhat elusive. In general, neurotrophins are bound to receptors at the neuron axon terminal and retrogradely transported to the cell body [122].

Numerous studies have demonstrated the trophic effects of NGF on sympathetic neurons. NGF has been shown to induce extensive neurite outgrowth in vitro, and to increase neuronal size and number in sympathetic ganglia during development [99,101,102]. Hypertrophy of sympathetic ganglia in NGF-overexpressing mice also has been observed [34]. Treatment of newborn mice with NGF anti-serum resulted in the loss of 93-99% of sympathetic nerve cells [100,101]. In the adult, studies from our laboratory have shown that exposure of sympathetic nerve endings to exogenous NGF results in a sprouting response [76], as well as increased tyrosine hydroxylase (TH) [113] and norepinephrine content [37].

More recently, NT-3 was cloned [44,106] and genetically isolated [107], and shown to have wide distribution throughout the central and peripheral nervous systems [82]. Structurally similar to NGF, NT-3 also exhibits trophic effects on sympathetic neurons. NT-3 stimulates
neurite outgrowth from sympathetic ganglia [10,43] and plays a role in the survival and maintenance of both neonatal [174,176] and mature [137,175] sympathetic and sensory neurons. During development, high levels of NT-3 expression have been detected associated with blood vessels adjacent to sympathetic ganglia, where sympathetic axons course with the vessels to their respective targets, thus suggesting a role for NT-3 in the guidance of those axons to their targets of innervation [53]. In addition, treatment with anti-NT-3 antibodies decreased the survival of postnatal sympathetic neurons by up to 80% [176], and NT-3 disrupted mutants showed similar neuronal deficits of 50% at birth [52,53].

Neurotrophin-3 has been shown to exert biological effects in sympathetic neurons through retrograde transport from the target tissue [137,175], but apparently it also can be anterogradely transported by retinotectal neurons [1,138,157], suggesting the possibility of neuron to target signaling [138]. NT-3 also plays a role in the differentiation of sympathetic neurons of the enteric nervous system [23] and cerebral cortex [5,23], and can induce axonal growth in the injured rat spinal cord [173].

Neurotrophin Biosynthesis and Processing

The mouse NGF gene spans a length of 45 kbs and contains 5 exons separated by 4 introns [141]. Two major transcripts, a “long” form coding for a 34 kDa precursor, and a “short” form coding for a 27 kDa precursor, were identified as the result of alternative RNA splicing [42,141]. Initially it was thought that only the long major transcript was predominant in the submaxillary gland of the mouse, while the short form was more abundant in other tissues [47,141], yet recent reports suggest that both precursor forms may be strongly expressed by some tissues [13]. Two minor precursors of NGF have been shown due to use of an alternative promoter and removal of an intron respectively [47]. The 13.5 kDa mature portion of NGF is translated from exon 1 at the C-terminus of each of the transcripts through an identical mechanism [40].

Traditionally, it has been accepted that neurotrophins are processed similarly to other pro-proteins and pro-hormones [98]. This follows that a pre-pro-protein initially is translated at a ribosome, and that the pre-portion (signal peptide) subsequently is cleaved upon entering the endoplasmic reticulum to yield the pro-protein. The pro- form can undergo further alterations in the trans-Golgi network and/or in secretory vesicles, and eventually be modified by removing the
FIGURE 1. Biosynthesis of two major nerve growth factor transcripts. Two major NGF transcripts have been identified, a longer transcript (A) encoding a 34 kDa peptide and a shorter one (B) coding for a 27 kDa precursor [42,141]. The signal sequence of each is cleaved upon entrance to the endoplasmic reticulum. Various glycosylations, enzymatic cleavage events, and sulphations can occur in the ER, Golgi complex, and cytoplasm to yield NGF protein species of various molecular weights.
N-terminal pro-domain to reveal mature neurotrophin. The most effective precursor-cleaving enzyme in this process has been shown to be a subtilisin/kexin convertase known as furin [17], with other members of this family, such as PACE4 and PC 5/6-B, also demonstrating considerable activity [139,140].

A range of neurotrophin intermediates of various molecular weights has been reported and attributed to various tissue-specific modifications, such as glycosylation, sulphonation, and enzymatic cleavage [12,31,132,133]. The biological significance of these intermediates and their unique processing is still not well understood. Although a role for the pro-NGF domain in assisting the mature NGF form both in proper conformational folding [51,120,130,131] and for correct sorting and processing [147] was described previously, more recent findings have begun to reshape the traditional views regarding the activity of neurotrophins and their precursors. Surprisingly, it was found that the primary form of NGF detected in mouse, rat and human brains was actually the pro-NGF precursor (32 kDa), with little or no mature NGF present [48]. Also, the pro- form has been shown to play a primary role in neuron apoptotic processes through binding to the p75 neurotrophin receptor [9]. These recent findings suggest a more active biological role for NGF precursors in typical survival mechanisms utilized by adult neurons.

**Neurotrophin Receptors**

The two classes of receptors known to bind neurotrophins include the tropomyosin-related kinase (trk) family of tyrosine kinase receptors and the p75 glycoprotein member of the tumor necrosis factor superfamily [126]. The trk family has historically been termed the high-affinity class, and includes trkA, trkB, and trkC, which bind NGF, BDNF and NT-4/5, and NT-3 respectively. The trk family of receptors shares 50% sequence homology between members [158].

Expression of the trkA receptor has been shown to be essential for normal sympathetic neuronal survival [46,146]. The first discovered member of the trk family, trkA was determined to be a 140 kD protein glycosylated from a 110 kD precursor [111,112]. The crystallized trkA-NGF binding domain was recently described [158], and additional analyses revealed that both the trkA-NGF [128,153] and the trkC-NT-3 [154] interactions were dependent upon specific IgG domains on the trk receptors. TrkA undergoes alternative splicing to yield both a neuronal and a non-neuronal isoform [4].
Following isolation and cloning of the 145 kD trkC receptor [90], trkC was found to be widely expressed in both the developing nervous system [91] as well as many non-neuronal tissues [91,149]. There are at least three trkC isoforms, each possessing distinct biological properties and substrate specificities [92]. Blocking trkC with a specific anti-serum resulted in the loss of 50% of developing sensory neurons in the dorsal root ganglia [97], however trkC null mutant mice showed no defects in sympathetic neurons in the superior cervical ganglia [46].

Early evidence of increasing trkA and decreasing trkC receptor expression throughout sympathetic and sensory development supported a role for these neurons “switching neurotrophin dependence” [14,32,46]. It was hypothesized that these neurons utilized NT-3/trkC binding during outgrowth, and then switched their dependence to the binding of NGF/trkA once they reached their target of innervation [33]. More recent evidence, based on actual levels of available neurotrophin rather than only levels of trk receptors, has argued against this hypothesis. It was shown that NT-3 null mutants suffer much greater sympathetic and sensory neuronal losses than that of the trkC null mutants, suggesting that NT-3 functions through an additional receptor or receptors [148,164]. Accordingly, it has been shown that NT-3 can bind [26] and activate [72] trkA, with an alternatively spliced isoform of trkA showing an enhanced response to NT-3 [24].

The p75 receptor was traditionally considered to be the low affinity receptor because it binds all of the neurotrophins similarly. Though the importance of the trk receptors to neurotrophin binding is fairly established, the role of the p75 receptor remains less clear. Some evidence has pointed to an important dual role for p75, serving as a key modulator of both neuronal survival and death [22,110]. p75 is a known mediator of the normal neuronal apoptosis that occurs during development [3,6,108] and it has been shown that p75-NGF binding alone does not induce the biological activity of NGF [55,59]. However, the highest affinity NGF binding occurs when trkA and p75 are co-expressed [67,88,105] and both receptors must be expressed for the uptake of NGF in sympathetic neurons to occur [56]. There is evidence to suggest that a specific p75:trkA ratio determines either an apoptotic or survival signal, with NGF regulating the levels of p75 to mediate survival [11,118,151]. Others have shown that trkA can interfere with and reduce p75-mediated apoptosis [109,152,169], and in the absence of p75, trkA will bind NT-3 [16].
The p75 receptor appears to mediate NGF-trkA induced survival using several mechanisms. It is hypothesized that p75 induces a conformational change in trkA to enhance NGF binding [45,70,171]. Also, p75 can regulate the mobility of trkA [162], increase the trafficking and kinetics of trkA at the cell surface [78], and inhibit trkA-NT-3 binding [119].

Recent studies of receptor binding to NGF precursors have revealed some surprising results. As expected, the pro-NGF peptide showed very low affinity for trkA, and in cultured sympathetic neurons expressing both trkA and p75, the mature NGF ligand led to survival signaling. Interestingly, the pro-NGF precursor resulted in neuronal death [96]. It was shown that the pro-NGF precursor had five times the affinity for p75 compared with the mature form, and that induction of apoptosis was ten times more effective for the pro- domain in cells lacking the trkA receptor [96]. These recent data suggest an alternative to the traditional beliefs regarding neurotrophin signaling and the trkA/p75 regulation of survival and cell death.

A neurotensin receptor known as sortilin appears to be necessary for the induction of apoptotic cell death [124]. Immunoprecipitation assays have shown that pro-NGF forms a complex with both p75 and sortilin receptors [124], implicating sortilin as both a co-receptor and molecular switch for pro-NGF induced apoptosis through the p75 receptor. Sympathetic neurons of the superior cervical ganglion express both the neurotensin receptor sortilin, and the neurotrophin receptor p75 [124]. Also, a novel transmembrane protein, neurotrophin receptor homolog-2 (NRH2), has been shown to modulate trkA/NGF binding affinity similarly to p75 [121]. Other reports link p75 with various pathological conditions, as p75 has been shown to bind aggregated β-amyloid [165] and prion proteins [36] as well as allowing the viral entry of rabies into cells [94].

Though the pro-NGF/p75 apoptotic signaling hypothesis represents a new approach to understanding the complex mechanisms governing neuronal cell survival and death, others have actually put forth the idea that the pro- form contributes to the biological survival activities that once were attributed to the mature NGF peptide. Support for this idea stems from findings that cholinergic neuron degeneration associated with Alzheimer’s disease results in a build-up of NGF pro form in the hippocampus and cortex [48]. Also, pro-NGF has been shown to exhibit neurotrophic activity in vitro, although to a lesser extent than the mature form [50]. Regardless of whether or not the pro- form functions in survival or apoptotic signaling processes, or in a
combination of the two, these new data warrant a reexamination of traditional views regarding the biological roles of neurotrophin precursor molecules.

**Signal Transduction**

Neurotrophin binding induces dimerization and phosphorylation of the trk receptor, which subsequently results in the initiation of signaling cascades to regulate survival activities in the nucleus [54]. The precise details of how these signals are propagated are not completely clear, however a few leading theories exist [20,167]. The most widely accepted hypothesis is that the NGF- phosphorylated(P)-trkA complexes are internalized and retrogradely transported to the cell body within clathrin-coated membrane derived signaling endosomes [61,62,71,150]. The endocytosis and retrograde transport of these neurotrophin-receptor complexes are believed to occur through interactions with dynamin [172] and association with actin and the dynein light chain [134,168,172]. It has been shown that autophosphorylation of specific tyrosine residues within the trk activation loops leads to NGF-dependent changes in receptor conformation [29]. Recently, the low affinity p75 receptor was shown to be internalized with NGF through clathrin coated pits at a much slower rate than the trk complexes, however the significance of these findings is yet to be determined [18]. The propagation of neurotrophin signaling also may involve retrograde signaling without transport of neurotrophins [104].

Though numerous details of neurotrophin signaling still remain unclear, many of the signaling molecules and pathways involved in neurotrophin-mediated neuronal survival have been identified. Following neurotrophin binding and autophosphorylation of the Trk receptor, associations with a number of dock adapter proteins, such as Shc, Grb2, PLCγ [80] and SHP-2 [125] are known to occur. The signals can then propagate to several pathways, including Ras/Raf, PI-3K/Akt, MEK/MAPK, IP3, and DAG [126]. The PI-3K pathway is often believed to be the major survival promoting route of neurotrophin signaling [79,80,87,156], and also has been shown to be necessary for retrograde transport of both NGF and NT-3 in sympathetic neurons [7,8,19,87,134]. Akt is the critical mediator of PI-3K induced survival [38], as PI3-K/Akt signaling was both necessary and sufficient for survival of NGF-dependent sympathetic neurons [27]. The signaling protein Ras acts to mediate survival indirectly by affecting various pathways such as PI3-K and MAPK, and has been shown to promote survival through suppression of the p53-BAX-JNK apoptotic pathway [79,114,123].
Internalized trkA/NGF complexes are necessary for the phosphorylation of cAMP response element binding protein (CREB) [136], which was ultimately shown to promote NGF-dependent survival in sympathetic neurons [135]. This survival likely occurs through increased NGF-induced expression of the immediate early gene c-fos [30,60,83]. The upregulation of c-fos then can contribute to the control of delayed early genes, such as tyrosine hydroxylase [57].

Though the early signaling events of NGF and NT-3 are similar [170], it has been shown that different neurotrophins can stimulate alternative interactions of the same signaling molecules [166], and that major differences may exist between NGF and NT-3 downstream signaling pathways [159,166]. Additional detailed studies of NT-3 signaling are necessary to elucidate the transduction pathways and molecules activated by this neurotrophin.

Superior cervical ganglion and the role of pre-ganglionic input

The superior cervical ganglion (SCG) is a cluster of approximately 26,000 neuronal cell bodies that receives innervation from pre-ganglionic neurons located in the spinal cord [129]. This ganglion is located near the bifurcation of the carotid artery into the external and internal carotid arteries. Approximately 1-10% of the neurons in the SCG extend axons into the cranial vault to contact the extracerebral vasculature [39,142]. Other intracranial targets of the SCG include the pineal gland [95] and iris [118].

The ability of these targets to synthesize neurotrophins has been verified through detection of NGF protein and mRNA in both the iris [66,145,161] and the pineal gland [85]. The superior cervical ganglion expresses trkA, trkC, and p75 neurotrophin receptor protein and mRNA [35], suggesting that neurotrophins that are synthesized and secreted from target tissues can bind to receptors on innervating axons from SCG cell bodies. Following binding, the neurotrophin-ligand complex is internalized and packaged within signaling endosomes, and retrogradely transported to the cell bodies [167]. A study examining the role of neurotrophins in sympathetic neuronal survival in vivo revealed a wide range in NGF requirements necessary for proper individual target innervation [58].

The sympathetic synapse is a complex system of convergence and divergence. Anatomical studies have revealed predominance in the number of sympathetic post-ganglionic neurons compared to pre-ganglions. For example, in the rat, the pre- to post-ganglionic neuron ratio is estimated to be approximately 1:15 [116]. Electrophysiological techniques have shown
that mammalian post-ganglionic cells in the SCG receive converging inputs, averaging about seven inputs per post-ganglionic neuron [116]. The functional significance of this arrangement remains unclear, though evidence has suggested bi-directional communication between pre- and post-ganglionic for maintenance of strong input connections and plasticity [116].

The functional role of pre-ganglionic input to the cell bodies in the SCG is unclear. Deafferentation of the SCG did not affect the density or distribution of post-ganglionic fibers projecting to the extracerebral vasculature [63], nor did it alter normal injury-induced sprouting [28]. Also, norepinephrine levels associated with the extracerebral blood vessels were unaffected following deafferentation of the SCG [77]. Yet, there is evidence that neurons in the SCG are affected by deafferentation. For example, recent data from our laboratory showed that a three week deafferentation of the SCG resulted in reduced levels of both TH mRNA and protein in the ganglion, and that TH immunoreactivity associated with extracerebral blood vessels was dramatically reduced [113]. Accordingly, levels of trkC and p75 neurotrophin receptor mRNA were significantly decreased [35]. Thus, there appears to be a series of events taking place in the neuron to ensure survival and proper functioning in the absence of afferent input, particularly over a three week period. One goal of the present study is to further characterize the compensatory mechanisms following deafferentation.

Rationale and Hypotheses

Our laboratory uses an in vivo model of NGF administration to examine the regulation and survival of adult rat sympathetic neurons (Figure 2). A chronic two-week infusion of NGF into the right lateral ventricle of the brain allows for NGF to flow throughout the ventricular system and to enter into the subarachnoid space. Sympathetic axons associated with the extracerebral vasculature are exposed to the exogenous NGF in the subarachnoid space, and therefore bind and retrogradely transport NGF protein to parent cell bodies located in the SCG. Following NGF administration, increased levels of NGF in the SCG have been verified using both NGF immunohistochemistry [142] and ELISA for NGF protein detection [86]. Trophic responses to this infusion, such as robust sprouting of sympathetic and sensory perivascular axons associated with extracerebral blood vessels [76], and significantly elevated levels of norepinephrine in these perivascular axons [74] have been documented. Also, upregulated trkA, trkC, and p75 neurotrophin receptor mRNA [35] and increased TH mRNA and protein [113] in
FIGURE 2. Schematic diagram depicting deafferentation procedure and in vivo model of NGF infusion. Shown is a coronal section of the adult rat brain at the level of Bregma, based on the stereotaxic rat atlas of Paxinos and Watson [127]. Pre-ganglionic axons arise from the spinal cord and synapse in the superior cervical ganglion. The site of deafferentation, or the removal of a small portion of the pre-ganglionic trunk, is represented with a X. The two-week intracerebroventricular infusion of NGF involves the insertion of a cannula with attached osmotic mini-pump into the right lateral ventricle of the brain. The infusate flows with the cerebrospinal fluid in the ventricular system and eventually enters the subarachnoid space, where it bathes the extracerebral blood vessels of the Circle of Willis. Sympathetic axons contacting these vessels bind NGF and retrogradely transport it to parent cell bodies in the superior cervical ganglion where it exerts biological effects.
the SCG have been observed.

The regulatory influences of pre-ganglionic input to post-ganglionic neurons located in the SCG also are a focus of investigation in our laboratory. The three-week deafferentation procedure involves removing a small section of the pre-ganglionic trunk to investigate the loss of this input on SCG neurons. As mentioned above, decreased p75 and trkC mRNA [35], and TH mRNA and protein expression [113] were observed following a three week deafferentation of the SCG, yet the morphology [76] and NE content [77] of the axons associated with blood vessel targets appear unchanged, suggesting that the function of these neurons is not altered by three week deafferentation. These data suggest that the activities of cell bodies of the SCG are influenced by afferent input, but also possess mechanisms to compensate for the loss of this input.

We also have observed various responses to NGF infusion by sympathetic neurons which have lost afferent input. For example, the accumulation of exogenous NGF typically observed following NGF infusion was blocked [35]. Also, though NGF restored the decreased p75 mRNA expression observed following deafferentation and generally increased p75 and trkA mRNA levels comparable to those receiving NGF only, trkC levels remained slightly depressed, similar to deafferentation only. In addition, though increases in TH mRNA were noted in these cases, both TH protein [113] and NE levels were similar to control values [77], and not increased as observed following NGF infusion alone. Because receptor mRNA levels were increased despite the absence of the NGF protein accumulation in the SCG [35], we postulated that other factors contributed to the observed alterations in receptor levels.

In the proposed study, we sought to examine protein expression of NGF and NT-3 associated with the SCG cell bodies and extracerebral blood vessel targets following deafferentation and/or NGF infusion using a sensitive two-site ELISA technique (Enzyme Linked ImmunoSorbant Assay), and qualitative Western blot analysis for NGF and NT-3. We expected alterations in neurotrophin expression to parallel the changes in receptor transcripts previously observed. Accordingly, based on previous findings, we predicted that NT-3 levels would be increased when deafferentation preceded the NGF infusion, but that NGF levels would remain comparable to vehicle.

Another primary aim of this research was to detect and measure NT-3 and NGF mRNA in the SCG using a relative real-time RT-PCR protocol. Though it is generally believed that
neurotrophins are target-derived, a recent report showed neurotrophin biosynthesis and secretion by SCG cell bodies [65]. Therefore, we wished to investigate the possibility of neurotrophin production in our model and to determine any effects of deafferentation and/or NGF infusion.

Because changes in trk mRNA expression were observed in the SCG following NGF infusion, protein expression of trkA receptor in the SCG was examined using a semi-quantitative Western blot technique. We predicted that the protein levels would correlate well with their relative mRNA levels. Therefore, we expected to detect an increase in trkA following NGF infusion only, and also when deafferentation preceded the infusion.

**The specific aims of the present study were to:**
1. Examine NGF and NT-3 protein and mRNA expression associated with sympathetic neurons using ELISA, Western blot analysis and real time RT-PCR and determine effects of deafferentation and/or exogenous NGF infusion.
2. Investigate trkA protein expression in the SCG using semi-quantitative Western blot analysis and determine effects of deafferentation and/or exogenous NGF infusion.
MATERIALS AND METHODS

Animals

A total of 70 Sprague Dawley female adult rats, 3 months of age, were purchased from Harlan Laboratories (Indianapolis, IN), and housed in the Miami University Animal Care Facility 2 per cage. The rats received food and water *ad libitum* while under a 12:12 hour light/dark cycle. Four treatment groups were used: 1) VEH: animals receiving no infusion, or a two-week infusion of cytochrome C, a protein that is similar in molecular size to NGF but with no biological effect (n=16: ELISA n=6; western n=6; RT-PCR n=4); 2) dVEH: animals receiving a three-week deafferentation or receiving a one-week deafferentation followed by a two-week cytochrome C infusion (n=19: ELISA n=9; western n=6; RT-PCR n=4); 3) NGF: animals receiving a two-week infusion of NGF (n=17: ELISA n=7; western n=6; RT-PCR n=4) 4) dNGF: animals receiving a one-week deafferentation followed by a two-week NGF infusion (n=18: ELISA n=8; western n=6; RT-PCR n=4).

Bilateral Deafferentation of Superior Cervical Ganglion

Animals were anesthetized via an intramuscular injection of Ketamine (80 mg/kg) and Rompun (14 mg/kg). Once reflexes ceased, the neck area was shaved and swabbed with alcohol and betadine. Protective gel was applied to each eye. A longitudinal incision was made along the neck and the skin was spread using retractors. The SCG was located at the bifurcation of the carotid artery, and a 3-5 mm section of the pre-ganglionic trunk was removed using microdissection scissors. The severed trunk was tucked underneath the SCG to prevent reinnervation. The wound was then closed using tissue glue (Nexaband, Phoenix, AZ). Animals survived for 3 weeks (deafferentation only) or received a cytochrome C pump or NGF pump one week following the deafferentation surgery. The closed wound was swabbed with an antibacterial topical powder (KenVet, Ashland, OH). Cheese puffs filled with 2-3 drops Tylenol® (acetaminophen: 100 mg/ml) were administered daily for one week as an analgesic.

Intracerebroventricular Infusion of NGF/ Cytochrome C

The protocol for the infusion of NGF or cytochrome C was based on the original methods of Williams et al. [160], and modified for use in our laboratory [74,75,77]. Animals were anesthetized and the dorsal region of the head shaved and treated with antiseptic as previously
described. The animal was placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) by securing the ear bars and nosepiece. A longitudinal incision along the dorsum of the skull was made. Sterile gauze and swabs were used to remove the connective tissue covering the cranium. Using a dremel (Dremel, Racine, WI) a small hole 1.0 mm lateral to Bregma was drilled [127]. Dura mater was removed using fine tipped forceps and a 27 gauge cannula (Popper and Sons Inc., New Hyde Park, NY) attached to an Alzet #2002 osmotic minipump reservoir (Alza Corp., Palo Alto, CA) was inserted 4.4 mm ventral from the brain surface [127] and secured using dental acrylic (Lang Dental Mfg. Co. Inc., Wheeling, IL). The pump reservoir was placed under the skin of the back. The incision was closed using 3-0 nylon sutures (JB Webster, Sterling, MA). Tylenol® (acetaminophen: 100 mg/ml) was administered as described above. The reservoir contained 200 µl of either cytochrome C (100 µg/ml; Sigma, St. Louis, MO) or mouse NGF (100 µg/ml: Accurate Inc., Westburg, NY). Bisbenzimide (Sigma, St. Louis, MO), a fluorescent marker, was added to the infusate to monitor placement of the cannula [74,77].

In all experiments, animals were sacrificed via decapitation using a rodent guillotine (Harvard Apparatus, Inc.; Holliston, MA). Left and right SCGs and extracerebral blood vessels located at the base of the brain (including left and right internal carotid, anterior, middle, and posterior cerebral, anterior and posterior communicating arteries and the basilar artery) were quickly dissected, snap frozen in liquid nitrogen, and stored at -80°C until use.

ELISA

The ELISA procedure for NT-3 and NGF was carried out using two-site E_max Immunoassay Systems purchased from Promega (Madison, WI). Tissue was processed according to manufacturer instructions coupled with a modified acid treatment protocol first described by Clemow et al. [25]. Briefly, ganglia for NT-3 analysis were weighed and sonicated for 10 s in 100 or 120 µl ice-cold lysis buffer (1 M NaCl, 20 mM Tris-HCl (pH=8.0), 2% NP-40, 10% glycerol, 1 mM PMSF, 10 µg/ml aprotinin, 1 µg/ml leupeptin and 0.5 mM sodium vanadate) depending on mass. For NGF ELISA, SCGs were sonicated in 200 µl lysis buffer. 1 µl of 1 N NaOH was added to each sample for every 40 µl of lysis buffer used. Samples were vortexed and allowed to sit undisturbed at room temperature for 5 min, and then centrifuged at 14,000g, 4°C, for 15 min. Supernatants were removed and 2x the previous NaOH vol of 1 N
HCl was added, samples again were vortexed, left undisturbed 5 min, and then centrifuged 30 min. Supernatants again were removed, the original vol of 1 N NaOH was again added, samples vortexed, left to sit 5 min, and centrifuged for 15 min. The supernatants were then drawn off and diluted 1:4 with ice-cold Dulbecco’s phosphate buffered saline (DPBS: 0.8% NaCl, 0.02% KCl, 0.02% KH₂PO₄, 0.115% Na₂HPO₄, 0.0133% CaCl₂ 2H₂O, 0.01% MgCl₂ 6H₂O, pH=7.35). Standards were prepared in duplicate as directed (NT-3: 4.7 – 300 pg/ml; NGF: 7.8 – 500 pg/ml) and all samples were assayed in triplicate. Samples tested for NGF were further diluted 1:4 (VEH and dVEH) or 1:8 (NGF and dNGF) in the plate with 1X block and sample buffer (supplied with kit). Absorbance from the colorimetric reaction of HRP and TMB at 450 nm was determined using a microplate reader and converted to pg NT-3/ml or pg NGF/ml with SOFTmax PRO software (Molecular Devices Inc, Sunnyvale, CA). Experimental recovery, determined by spiking SCG samples with NT-3 protein, was approximately 83%. Wells were tested without the coating antibody (anti-human NT-3 pAb or sheep anti-NGF pAb) in order to verify no detectable neurotrophin levels. Sample values were then normalized as pg/mg soluble protein (BCA protein assay kit, Pierce, Rockford, IL). Superior cervical ganglia were processed individually (NT-3 ELISA: VEH, n=6; dVEH, n=9; NGF, n=7, dNGF, n=8); (NGF ELISA: n=3 across the four treatment groups). For determination of NT-3 or NGF associated with extracerebral blood vessels, the Circle of Willis was processed and assayed as described above (NT-3: n=4 all treatments; NGF: VEH, n=3; dVEH, n=3; NGF, n=2, dNGF, n=3). NT-3 and NGF protein for vessel samples was expressed as pg/mg soluble protein. All comparisons were made across treatment groups, and two sample t-tests were performed, with significance reported at p<0.05.

Relative Real Time RT-PCR

RNA Isolation and Reverse Transcription

Total RNA was extracted from pooled (left and right) SCGs and DNased using the Ribopure™ kit per manufacturer instructions (Ambion, Austin, TX). Isolated RNA was quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Inc.; Rockland, DE). A 0.3 µg aliquot of total RNA was reverse transcribed in a two-step process in which a total volume of 20 µl containing 3 µl random hexamer primers (500 µg/ml; Promega, Madison, WI) was incubated 2 minutes at 90°C, and allowed to ramp cool to 30°C. The samples were brought
to a final reaction volume of 60 µl containing: 12 µl 5x buffer, 6 µl 10 mM dNTP mix, 1 µl RNasin, and 2 µl MMLV reverse transcriptase (Promega, Madison, WI). Negative controls were performed concurrently for each RNA sample in which 2 µl water was added instead of MMLV reverse transcriptase. Reaction mixtures then were incubated at 20°C for 15 minutes, 42°C for 2 hours and at 90°C for 2 minutes to inactivate the reverse transcriptase. The resulting cDNA solutions were stored at -80°C in two, 30 µl aliquots until use.

**Real-Time Assay**

NGF [forward: 5’- GCC AAG GAC GCA GCT TTC TAT - 3’ reverse: 5’ - CGC AGT GAT CAG AGT GTA GAA CAA C - 3’; Tm=59°C [Bjorling, 2002 #276]] and NT-3 [forward: 5’ - AGA ATT CCA GCC GAT GTG -3’ reverse: 5’ - AGC GTC TCT GTT GCC GTA GT - 3’; Tm=57°C] mRNA were measured and compared to GAPDH [forward: 5’ - AGA CAG CCG CAT CTT CTT GT - 3’ reverse: 5’ - CCG ACC TTC ACC ATC TTG TC - 3’; Tm=57°C] (Integrated DNA Technologies, Coralville, IA) using the Rotorgene 3000 system (Corbett Research, Sydney, NSW, Australia) and the Platinum qPCR Supermix UDG kit (Invitrogen, Carlsbad, CA). NT-3 and GAPDH primer sets were designed specifically for real time RT-PCR using the Primer-3 program available online (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Negative reverse transcription controls and no template controls were included in each run. A melt curve analysis also was performed following each real-time run to verify strand separation of a single product, in which temperature was ramped from 72°C to 99°C (Figure 3a).

In order to investigate changes in neurotrophin transcripts, the ratio of neurotrophin mRNA to GAPDH mRNA was determined individually for every sample in each treatment group. Ratios obtained from VEH cases were averaged and set at 100%. Any deviation either above or below this ratio signified an increase or decrease in neurotrophin mRNA levels respectively. These data were subjected to analysis using a two-sample t-test and values were reported as mean ± SEM. Any significance was reported at p<0.05.
FIGURE 3. Validation of real-time RT-PCR method by automated sequencing of amplified product and melt curve analysis. (a) Shown is a representative melt curve analysis for a NT-3 real time RT-PCR run as plotted by the Rotorgene software. The graph is plotted as the derivative of fluorescence versus increasing temperature (°C). This step is routinely performed following the PCR portion of the assay to verify the presence of a single amplification product and involves the ramping of temperature from 72°C to 99°C. It can be seen that there is one major product, with strand separation at approximately 82°C.

(b) A 3% agarose gel was run to verify the correct insert size within the plasmid DNA isolated from bacterial colonies during the cloning procedure. A portion of each DNA sample was digested with EcoR1, and run alongside non-digested sample. In each case, the non-digested DNA (N) is in the left lane, with the digested sample in the right (D). Most of the digested samples showed a band at slightly larger than the 50 bp marker, at about where the 56 bp NT-3 product would be expected. Only those samples which showed this band were used for automated sequencing.
Cloning and Sequencing of NT-3 Product

As an additional control for the real time RT-PCR procedure, representative product from a NT-3 run was subjected to clean-up using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI). The product was then inserted into a pCR®2.1-TOPO® plasmid vector according to manufacturer instructions using a TOPO TA Cloning® kit and transformed using DH5α™-T1® chemically competent One Shot® Cells (Invitrogen, Carlsbad, CA). This vector contained an ampicillin resistance gene as well as a lacZ insert, and therefore the bacteria were plated on Luria-Bertani (LB) plates containing 50 µg/ml ampicillin and coated with 32 µl of 50 mg/ml X-Gal solution. Aliquots (50 µl and 200 µl) of bacteria containing the PCR product were spread on separate plates to ensure optimal colony growth. 50 µl from several controls (no vector, vector only, and control DNA plus vector) were plated as well. Following overnight incubation at 37°C, 12 white to light blue colonies were selected for analysis and grown overnight in 5 ml ampicillin containing LB media at 37°C while shaking. Lack of bacterial growth was noted on the no vector control plate.

Plasmid DNA was isolated using the Perfectprep® Plasmid Mini kit (Eppendorf; Hamburg, Germany) and quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Inc.; Rockland, DE). To verify correct insert size (56 bps), a 5 µl portion of each sample was digested in EcoR1 and both digested and non-digested aliquots were run on a 3% agarose gel containing ethidium bromide (1:20,000; Figure 3b). Both forward and reverse sequencing reactions were prepared using 0.4 pg DNA template, 5 pmoles of M13 primer, 4µl ET DYEnamic™ Terminator Reaction Mix, 4 µl 2.5X sequencing buffer (Amersham Biosciences; Piscataway, NJ). Samples were brought to a volume of 20 µl with sterile H2O, mixed by pipet, and cycled 25 times [20 sec @ 95°C, 15 sec @ 50°C, and 60 sec @ 60°C]. 2 µl Na+ Acetate/EDTA was added to each sample and washes of both 95% and 70% ethanol were performed. The pellet was allowed to air dry, and stored at -20°C until use.

Pellets were resuspended in 20µl MegaBACE solution (Amersham Biosciences; Piscataway, NJ) and sequenced on an ABI3100 Genetic Analyzer (Applied Biosystems; Foster City, CA). Data were analyzed with BioEdit software (Ibis Therapeutics; Carlsbad, CA), hand-checked for 100% base-to-base match, and genome searched using the BLAST program (www.ncbi.nlm.nih.gov/).
Western blot analysis

Protein Isolation and Assay

Total protein in the SCG was obtained by sonicating in 0.01 M Tris-HCL buffer (pH 7.4) containing 1% SDS and 1% protease inhibitor cocktail (Sigma, St. Louis, MO). Following centrifugation, protein concentrations were determined in the supernatant using a BCA assay (Pierce, Rockford, IL). Samples were prepared as described by Laemmli [89].

Semi-Quantitative Western Blot Analysis (trkA study)

Samples were loaded and run on a 5% SDS-polyacrylamide (PAGE) stacking gel and a 9% SDS-PAGE resolving gel at three pre-determined increasing amounts of protein (10 µg, 15 µg, and 20 µg) to ensure that bands for trkA and α1 Na, K-ATPase (internal control) were maintained in the linear range of detection. Protein then was transferred at a total of 2,500 mAmmps to PVDF membrane in 25 mM Tris buffer at 4°C (overnight). The membrane was incubated in 8% non-fat dry milk diluted in Tris buffered saline containing Tween-20 (TBST) for 4 h at room temperature. The membrane was incubated overnight at 4°C in rabbit anti-trkA (1:1000, Upstate Biotechnology, Lake Placid, NY) diluted in TBST. The membrane then was rinsed with TBST, incubated in goat anti-rabbit HRP IgG (1:20,000, Chemicon; Temecula, CA) for 2 h, rinsed, and covered with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) for 5 min. The membrane was placed in an autoradiography cassette and Kodak BioMax MR film was exposed at multiple time increments to ensure detection within a linear range. Membranes were stripped for 2 hours using elution buffer (Pierce, Rockford, IL) and reprobed for α1 Na, K-ATPase in the same manner as used for trkA (1° - mouse anti- α1 Na, K-ATPase, 1:1000, α6F, Developmental Studies Hybridoma Bank, Iowa City, IA; 2° - goat anti-mouse HRP IgG, 1:40,000, Chemicon). Banding specificity was determined by omission of primary antibody from Western blot protocol.

Films were scanned into ImageQuant 5.2 analysis software and densitometry readings were obtained for all bands. Only films in which the three increasing protein amounts for each treatment were linear were used for data analysis. The ratio of trkA protein to α1 Na, K-ATPase protein was determined across treatment groups (VEH, n=4; dVEH, n=4; NGF, n=4; dNGF, n=4). The ratio from the VEH group was set at 100%. Any deviation either above or below this ratio signified an increase or decrease in trkA protein levels respectively. The results for each
treatment group were averaged and subjected to a two-sample t-test and values were reported as mean ± SEM. Significance was reported at p<0.05.

Qualitative NT-3 and NGF Western Blotting

A modified protocol for western blotting was carried out for NT-3 and NGF. A 12% SDS-PAGE resolving gel was used to achieve better band separation. Also, a single protein amount was loaded for NT-3 and NGF (40 µg), since quantitative comparison was not desired. The following primary antibodies were used: goat anti-NT-3 (L-15, 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit anti-NGF (H-20, 1:1000; Santa Cruz Biotechnology). Secondary antibodies used were rabbit anti-goat HRP IgG (1:10,000; Chemicon) and goat anti-rabbit HRP IgG (1:10,000; Chemicon) respectively. The SCG (n=3) was examined across four treatments each for NGF and NT-3. Exploratory blots for NGF (n=3) and NT-3 (n=1) associated with the extracerebral blood vessels also were performed. Membranes were stripped and reprobed for β-actin (1:40,000; mouse anti- β-actin and 1:20,000 goat anti-mouse HRP IgG; Chemicon) as an internal control.
RESULTS

NGF and NT-3 expression: effects of exogenous NGF

As expected, a significant increase in NGF protein levels was observed associated with the extracerebral blood vessels (196%, p=0.0009; Figure 4a), as well as in the SCG (270%, p=0.04; Figure 4c) following NGF infusion. This increase in NGF protein signified successful uptake of infused NGF protein by sympathetic endings in the subarachnoid space and accumulation of exogenous NGF in the SCG. These findings are largely confirmatory of previous data obtained from our laboratory using the Crutcher ELISA protocol [35]. However, the control values for NGF protein in the SCG obtained using the Promega ELISA system were consistently higher than those previously reported using the Crutcher assay. For example, mean NGF protein levels using the Crutcher ELISA were approximately 22 pg/mg wet weight [35] compared with 113 pg/mg wet weight using the Promega assay.

Analysis of NGF protein expression in the SCG and blood vessels using a qualitative Western blot assay showed prominent NGF forms of varying molecular weights [blood vessels: 22, 25, 30, and 50 kDa (Figure 6a); SCG: 25, 30, 50, and 75 kDa (Figure 6b)]. Interestingly, the 13 and 16 kDa mature forms of NGF were weakly expressed in these blots compared to the larger molecular weight species, and only were noticeable following very long film exposure times. A clear increase in the expression of a 50 and 53 kDa species of NGF was associated with vessels from NGF-infused cases, corresponding to the increase shown using the ELISA assay. However, no qualitative changes in NGF protein expression were noted in the SCG that might parallel the significant increase detected using ELISA in NGF-infused cases (Figure 6b).

Real-time RT-PCR analysis revealed the presence of NGF transcripts in the SCG, in agreement with previous reports [65,144]. Following a two week NGF infusion, the level of mRNA transcripts in the SCG was unchanged, suggesting that relative mRNA expression was not affected by the presence of exogenous NGF (Figure 7a).

While NT-3 levels associated with the target extracerebral blood vessels were unchanged following NGF infusion (Figure 4b), ELISA analysis of NT-3 protein revealed a significant increase in the SCG (53%, p=0.007; Figure 4d). Qualitative Western blot analysis of the blood vessels and SCG revealed multiple prominent NT-3 species [blood vessels: 13, 16, 18, 22, 42, and 75 kDa (Figure 8a); SCG: 13, 16, 27, and 42 kDa (Figure 8b)] including the mature forms of NT-3. However, no noticeable changes in NT-3 expression were noted in the NGF-infused cases.
FIGURE 4. Quantification of neurotrophin protein levels in the SCG and extracerebral blood vessel targets using the Promega Emax ELISA system. Measurements of NGF and NT-3 protein levels in the extracerebral blood vessels (a,b) and SCG (c, d) were obtained using Emax ELISA kits from Promega. No significant changes were observed in NGF or NT-3 protein in the blood vessels or SCG following a 3 week deafferentation (dVEH). Following NGF only (NGF), NGF and NT-3 protein levels were significantly elevated in the SCG (by 270% and 53% respectively). When deafferentation preceded the infusion (dNGF), NGF and NT-3 levels remained significantly increased (413% and 83%). While NGF levels associated with the blood vessels were elevated in NGF cases by 196% and dNGF cases by 147%, NT-3 protein levels were unchanged in these treatments. *p<0.05, **p<0.01.
FIGURE 5. Determination of NGF protein levels in the superior cervical ganglion (SCG) using the Crutcher ELISA method. Previous results obtained in the laboratory using the NGF ELISA protocol developed by Crutcher revealed a significant increase of NGF protein in the SCG following a two-week NGF infusion (DeCouto et al. 2003). This increase was absent when deafferentation preceded the NGF infusion. [VEH: (vehicle), either no surgery or a two-week cyto C infusion; dVEH: three week deafferentation procedure; NGF: two-week NGF infusion; dNGF: one week deafferentation followed by two week NGF infusion].
FIGURE 6. Qualitative Western blot analyses of NGF protein located in the extracerebral blood vessels and SCG. NGF forms of varying molecular weights were observed associated with extracerebral blood vessels and SCG. a) In the extracerebral blood vessels, changes in 50 and 53 kDa NGF species were observed following NGF treatment (bracket). b) When deafferentation preceded the NGF infusion (dNGF), apparent increases in 25, 30, and 75 kDa NGF bands were observed in the SCG (brackets). 40 µg of protein was loaded for each case and all blots were probed for actin (43 kDa) as a loading control. In (b), 25 ng of 2.5S NGF peptide served as a control for the mature (13 kDa) form of NGF. Note that the mature forms of NGF (13 and 16 kDa) were not apparent in either blot.
Figure 7. Neurotrophin mRNA expression in the superior cervical ganglion (SCG). Real time RT-PCR analysis revealed a significant increase in NGF mRNA by 84% following deafferentation (dVEH), and was still apparent when deafferentation was followed by NGF infusion (dNGF, 174% compared to vehicle). Also, though NT-3 mRNA levels were significantly decreased (39%) following a 2-week in vivo intracerebroventricular infusion of NGF (NGF), when deafferentation preceded the infusion, a robust response was observed (dNGF, 206%) (**p<0.01; *p<0.05).
FIGURE 8. Qualitative Western blot analyses of NT-3 protein located in the extracerebral blood vessels and SCG. Following deafferentation (dVEH), an increase in a 22 kDa form (bracket) of NT-3 is apparent in the SCG. Note the strong appearance of mature NT-3 forms (approximately 16 and 13 kDa) in both the blood vessels and SCG. 40 µg of protein was loaded for each case and all blots were probed for actin (43 kDa) as a loading control.
to parallel the increase detected using ELISA. NT-3 mRNA expression was detected in the SCG using real-time RT-PCR analysis. Following NGF infusion, levels of NT-3 transcript were significantly decreased in the SCG (39%, p=0.001; Figure 7b), indicating that the accumulation of infused NGF in the SCG influenced both NT-3 protein and mRNA levels in the SCG.

**NGF and NT-3 expression: effects of deafferentation**

When pre-ganglionic input to the SCG was removed, no changes in NGF protein levels associated with the extracerebral blood vessels or SCG were observed (Figures 4a,c). In addition, qualitative Western analysis showed no apparent alterations in the various molecular weight NGF species found in either tissue type due to the deafferentation procedure (Figure 6). However, NGF mRNA in the SCG was significantly elevated following a three week deafferentation (84%, p=0.02; Figure 7a), suggesting a profound influence of afferent input on NGF mRNA expression.

No changes were detected in NT-3 protein levels in the blood vessels or SCG using ELISA (Figures 4b, 4d), nor were NT-3 mRNA levels significantly affected following the removal of afferent input (Figure 7b). However, qualitative NT-3 Western analysis indicated increased expression of a 22 kDa species in the SCG following deafferentation, with expression of other molecular weight forms unchanged (Figure 8b). Thus, removal of pre-ganglionic input altered the typical NT-3 protein expression in the SCG, even though no changes in NT-3 protein were detected using ELISA (see Figure 4d).

**Response following deafferentation and NGF infusion**

When NGF infusion followed deafferentation, NGF protein levels were found to be elevated by 147% in the extracerebral blood vessels, indicating that the protein was readily available to associated sympathetic axon terminals (p=0.04; Figure 4b). A robust increase in NGF protein also was observed in the SCG when removal of afferent input preceded the infusion (413%, p=0.009; Figure 4d), suggesting that deafferentation did not affect the NGF response. This is in contrast with the Crutcher ELISA protocol (see Figure 5), where the significant increase in NGF observed in the SCG following NGF infusion was blocked when deafferentation preceded the infusion. Yet, consistent with the findings obtained using the Promega assay, qualitative Western blot analysis revealed obvious increases in the expression of 25, 30 and 75
kDa NGF species in the SCG from dNGF cases (Figure 6), suggesting that the Promega ELISA assay may have recognized NGF forms that were not readily identified by the Crutcher ELISA assay.

NGF mRNA levels were significantly increased in the SCG following deafferentation and subsequent NGF infusion (174%, p=0.02; Figure 7). This increase is similar to deafferentation only, suggesting a greater influence of afferent input (when compared with exogenous NGF) on NGF mRNA expression.

As with NGF infusion only, no changes in NT-3 protein levels were detected in the extracerebral blood vessels in dNGF cases, and NT-3 protein levels were significantly upregulated in the SCG when deafferentation preceded the NGF infusion (83%, p=0.002; Figure 4d), indicating that removal of afferent input did not affect the upregulation of NT-3 protein levels in the presence of exogenous NGF. Though alterations were observed following deafferentation only, no obvious alterations were observed in NT-3 Western blots in either the SCG or target blood vessels in the dNGF treatment (Figure 8).

NT-3 mRNA in the SCG was significantly increased when afferent input was removed prior to the NGF infusion (206%, p=0.03; Figure 7), showing a reversal of the effect observed with NGF infusion alone. Overall, these data suggest a role for afferent input in the regulation of both NGF and NT-3 mRNA in the SCG.

**TrkA neurotrophin receptor protein expression: effects of deafferentation and/or NGF infusion**

The expression of the 140 kDa trkA species was examined using semi-quantitative Western blot analysis. No significant alterations in trkA protein expression were detected following deafferentation or NGF infusion (Figure 9). Also, no changes were noted when deafferentation preceded the NGF infusion, suggesting that relative levels of trkA receptor protein were not affected by removal of afferent input and/or exposure to exogenous NGF.
FIGURE 9. TrkA protein expression in the SCG determined by semi-quantitative western blot analysis. An increasing linear gradient of protein (10, 15, and 20 µg) was loaded for each treatment. Each 140 kDa trkA band was normalized for the respective 100 kDa Na⁺/K⁺ ATPase band, and then compared to vehicle using densitometry readings. No significant alterations in trkA protein levels were detected with this procedure.
DISCUSSION

Several complementary techniques, including ELISA, Western blot analysis, and real time RT-PCR, were utilized to investigate the regulatory influences of afferent input and NGF infusion on neurotrophin expression in adult sympathetic ganglia. Neurons of the SCG and their intracranial axonal projections to the extracerebral blood vessels were used as a model system to investigate the regulation of NGF and NT-3 expression. One finding of primary importance was that exposure of sympathetic axon terminals to exogenous NGF peptide resulted in subsequent upregulation of NT-3 within the ganglion. This novel result is suggestive of a cooperative interaction between two survival-promoting neurotrophins associated with sympathetic neurons.

A second major finding was the detection of neurotrophin transcripts in the SCG and the plasticity of mRNA expression following experimental manipulation. While NGF mRNA previously has been reported in the SCG [144], there have been no reports of alterations in NGF transcripts in adult sympathetic ganglia. In addition, the presence of NT-3 mRNA in the SCG described here is the first report of NT-3 mRNA in sympathetic ganglia. Though neurotrophins in peripheral ganglia are commonly thought to be target-derived, our results demonstrate that cell bodies in the superior cervical ganglion likely possess the capability to produce neurotrophic proteins such as NGF and NT-3. The alterations in neurotrophin expression observed in the present study represent an example of neuronal plasticity that ultimately ensures survival and suggest that NGF and NT-3 expression is dynamic, and will undergo alterations to meet ever-changing environmental demands.

Taken together, these data show that the longstanding views regarding the mechanisms of neurotrophin action are more complex than previously believed. Through an examination of the regulatory influences of pre-ganglionic input and exogenous NGF exposure on neurotrophin expression in sympathetic neurons on the SCG, we have the opportunity to investigate the intricate processes involved in neurotrophin action and thus, neuronal survival.

Neurotrophin expression: effects of nerve growth factor infusion (NGF)

Following a two week infusion of NGF, increased amounts of NGF protein were detected in the extracerebral blood vessels associated with the Circle of Willis. These data are in agreement with previous findings [142] and show that our infusion procedure exposes the sympathetic cerebrovascular axon terminals, which extend from cell bodies in the superior
cervical ganglion, to exogenous NGF. Accordingly, NGF levels in the SCG were dramatically increased following the NGF treatment. This result is consistent with previous ELISA data and supports the idea that exogenous NGF accumulates in the SCG following NGF infusion [35]. However, it must be noted that, because NGF mRNA was expressed in the ganglion, it is possible that exposure to exogenous NGF at the level of the axon terminal may lead to an upregulation of NGF protein production at the level of the SCG. This is not likely the case, however, because previous immunohistochemical data obtained using an antibody that recognized only the exogeneously administered NGF protein form demonstrated an accumulation of NGF in SCG cell bodies following the infusion procedure [142].

The trophic biological effects of NGF on sympathetic neurons are well documented, and include in vitro neurite outgrowth and in vivo increases in neuron size and number during development [99,101,102]. Studies from our laboratory have revealed extensive sprouting by sympathetic cerebrovascular axons following a two week NGF infusion [76]. Additionally, our previous data have demonstrated the regulatory influences of NGF infusion on overall sympathetic phenotype, showing upregulation of both tyrosine hydroxylase (TH) mRNA and protein in the SCG [113] as well as norepinephine (NE) content associated with the extracerebral blood vessels [37,74,77,113].

The present study provided novel insight concerning the regulatory influences of NGF on NT-3 expression. Following the two week NGF infusion, levels of NT-3 protein in the SCG were increased along with those of NGF. Other accounts of one neurotrophin affecting levels of another neurotrophin have been reported. For example, NGF treatment was shown to upregulate the transcription of BDNF in sensory neurons of the dorsal root ganglion [2,81,117], while NT-3 infusion decreased BDNF mRNA [81]. In newly generated granule neurons of the mammalian cerebellum, BDNF signaling specifically induced the transcription of NT-3 [143]. Also, exposure of lamina cribrosa cells and astrocytes of the optic nerve to various neurotrophins resulted in increased secretion of NGF, while the same treatment decreased BDNF secretion [93]. Demonstrating a possible competitive interaction, transgenic mice overexpressing NGF showed decreased levels of NT-3 in the superior cervical ganglion [64]. Additionally, two neurotrophins may behave in a synergistic fashion. For example, a greater overall effect on axonal guidance of neurons in the dorsal root ganglion was reported when NGF and NT-3 were combined than with either neurotrophin alone [21].
It is not clear whether the additional NT-3 protein observed in NGF-infused cases was produced at the level of the blood vessels and retrogradely transported to the SCG, or if NT-3 was synthesized within the ganglion. NT-3 protein levels in the extracerebral blood vessels, a primary target of sympathetic neurons of the SCG, were not altered, yet the rate of NT-3 retrograde transport to the SCG may have increased, thus masking an increase at the level of the target blood vessels. Alternatively, the production of NT-3 may have been upregulated at targets other than the extracerebral blood vessels, such as the iris, pineal gland or spleen, and NT-3 from these targets then was transported to the ganglion. It is also possible that, because NT-3 transcripts were found in the SCG (see below), the increased NT-3 present in the SCG following NGF infusion was a result of increased NT-3 biosynthesis within the ganglion.

In this study, we detected NT-3 mRNA in the SCG, showing that NT-3 may be synthesized by cells (neuronal or non-neuronal) in the ganglion. Yet, we observed that, following the two week NGF infusion procedure, NT-3 mRNA was significantly decreased when compared to vehicle. This decrease may result from a negative feedback response resulting from the upregulation of NT-3 protein, and might serve to gradually stabilize NT-3 levels over an extended time period. Further studies are necessary to quantify NT-3 mRNA expression at the level of targets, such as the extracerebral blood vessels. In situ hybridization studies of both the SCG and blood vessels for NT-3 mRNA would help to localize NT-3 biosynthesis. Also, once the origin of NT-3 is determined, it will be important to discern whether NT-3 has an autocrine and/or paracrine function in the ganglion. Regardless, our studies show, for the first time, that exogenous NGF can influence NT-3 expression in adult sympathetic neurons.

Investigations of neurotrophin expression in the SCG and extracerebral blood vessels using qualitative Western analysis showed no major alterations in NGF or NT-3 protein species in the SCG following NGF infusion. Blots of the extracerebral blood vessels, however, showed noticeable alterations in the 50 and 53 kDa NGF species. The functional significance of these changes in the blood vessel tissues is unclear, but it is apparent that exogenous NGF somehow altered NGF protein expression in these targets. It is possible that the 13 kDa infused NGF may undergo modifications at the target prior to release. The biological relevance of these higher weight molecular species of NGF and NT-3 in sympathetic ganglia and their targets is currently under investigation [13,48,49].
Neurotrophin expression: effects of deafferentation (dVEH)

A three week removal of pre-ganglionic input revealed no changes in the protein levels of NGF or NT-3 associated with the SCG or extracerebral blood vessels. This finding might initially suggest that deafferentation did not affect neurotrophin expression in the neurons. Indeed, morphological observations using electron microscopy have shown no changes in the number or appearance of perivascular axons associated with the extracerebral blood vessels [77] following a 3 week deafferentation, and others have reported that deafferentation did not affect the density or distribution of post-ganglionic fibers projecting to the target extracerebral blood vessels [63]. Though previous data from our laboratory have shown decreased levels of TH mRNA and protein in the SCG following deafferentation [113], no overall alterations in norepinephrine (NE) levels were observed [77]. However, we have detected changes in deafferented SCG cell bodies, such as a decrease in trkC and p75 mRNA [35] and increase in NGF mRNA (present study). Also, our qualitative Western analysis indicated an apparent upregulation in the 22 kDa species of NT-3.

Therefore, while it does not appear that removal of pre-ganglionic input had a major detrimental effect on the overall function of sympathetic innervation to target tissues, the neuron appeared to undergo compensatory changes in order to ensure survival following the removal of afferent input. Consequently, it can not be assumed that the role of afferent input is minimal or unimportant. Rather, it is more likely that the neurons in the SCG have multiple redundant mechanisms in place to compensate for this loss. For example, the upregulation of NGF mRNA detected in the SCG when afferent input is removed may indicate an attempt by the neurons to survive and maintain functional capabilities over the three week period while TH mRNA and protein are decreased. It is possible that the loss of this neuronal activity may result in a loss of NGF derived from targets, and thus NGF must be produced within the ganglion. The long term consequences of deafferentation on neuron maintenance and survival in the SCG will need to be explored.

Neurotrophin expression: effects of deafferentation followed by NGF infusion (dNGF)

Though deafferentation alone did not seem to cause critical changes in overall neuron function, we sought to determine if deafferentation would influence the normal neuronal
response to NGF infusion. Previously, we have shown that the increased NE generally associated with NGF infusion is absent (with NE levels similar to control) when afferent input precedes the NGF infusion period [77]. One of the major findings in this study was a significant increase in NGF and NT-3 transcripts when deafferentation preceded the infusion. These alterations were in contrast to those detected following NGF infusion alone, in which NT-3 mRNA was significantly depressed and NGF mRNA was not altered when compared to vehicle. Also, the increase in NT-3 protein in the SCG in dNGF cases was more robust than with NGF infusion alone. Therefore, while neuronal function appeared to be intact, with normal NE levels in the axons, the typical regulatory mechanisms may have been altered.

Another example of a possible alteration in NGF response due to deafferentation can be seen when comparing ELISA and Western analysis of dNGF cases. Qualitative Western analysis of dNGF cases revealed apparent increases in 25, 30, and 75 kDa NGF species in the SCG when afferent input was removed prior to the infusion, suggesting an alteration in NGF expression. Interestingly, the NGF only and dNGF cases showed similar increases in NGF protein using ELISA even though there were obvious changes in the expression of high molecular weight NGF precursors only in the dNGF cases. These findings speak to the need for different types of measurements in order to accurately detect such changes in protein expression.

It is not clear why the Crutcher ELISA showed no change in NGF protein in dNGF cases while the Promega assay showed a robust increase. There may be differences in antibody specificity between the two systems, such that the Promega antibodies recognized the larger NGF species. Further investigations of the expression of the larger molecular weight neurotrophin species and processing intermediates, using both Western analysis and ELISA, will help in our understanding of the differences in these two ELISA protocols.

Neurotrophin receptor expression: trkA protein in the SCG

Investigations into changes in the protein expression of the preferential NGF receptor trkA were performed using semi-quantitative Western blot analysis. In a previous study from our laboratory, trkA mRNA expression was significantly increased following NGF infusion [35]. Yet we did not detect any significant changes in trkA protein in the SCG following NGF infusion or deafferentation. It may be that the technique was not sensitive enough to detect small changes in trkA protein levels. Another explanation may be that, while overall trkA levels were
unchanged, the amount of phosphorylated trkA receptor was increased. Alternatively, although overall protein levels in the SCG were not affected, increased trkA protein may have been immediately transported from SCG cell bodies to the axon terminals. Indeed, immunohistochemical data from our laboratory have shown a significant increase in trkA protein associated with the extracerebral blood vessels following NGF infusion (unpublished observations).

SUMMARY AND CONCLUSIONS

We have demonstrated that exposure of axon terminals to exogenous NGF results in a significant increase in NT-3 protein with a decrease in NT-3 mRNA in the SCG after a two week infusion period. Also, our laboratory has reported alterations in the SCG response to this NGF infusion when it is preceded by removal of afferent input. It would be of great interest to investigate these changes at various time periods, in order to discern if the alterations are transient and temporal in nature. Also, it would be useful to employ in situ hybridization experiments in order to determine which cells of the SCG are expressing neurotrophin mRNA and are undergoing changes following treatment. In addition, pioneering studies into the intricate means of neurotrophin processing will be necessary to further understand the biological relevance of the multiple molecular weight species and intermediates found to be uniquely present in various tissues. One example might be to examine levels of furin, the most effective neurotrophin convertase. Finally, it will be crucial to determine the pattern of neurotrophin mRNA in target tissues, and to document changes following experimental conditions.
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