Target-derived neurotrophic factors such as nerve growth factor (NGF) and neurotrophin-3 (NT-3) regulate sympathetic neuron survival. In the present study, NGF and NT-3 protein and transcript levels were examined in peripheral tissues in order to determine their role in neuronal atrophy observed in aging. The most obvious age-related alteration was a dramatic increase (up to 50-fold) in the proNGF species (25 kDa) in the superior cervical ganglion (SCG) and targets that atrophy in aging. In the iris, a tissue protected into old-age, proNGF was decreased. Alterations in NGF transcript generally paralleled changes in NGF protein, albeit to a lesser degree. Though NT-3 protein was significantly increased in SCG, only minor changes were observed in targets, even though NT-3 mRNA generally was decreased. In contrast, both NT-3 transcript and NT-3 precursors were increased in iris. The dramatic alterations in NGF precursor proteins and significant increases in NGF transcript, together with minimal changes in NT-3, indicate that altered NGF regulation, particularly the presence of proNGF, contributes to the degeneration of aged sympathetic neurons.
ALTERED NEUROTROPHIN EXPRESSION IN
AGED PERIPHERAL NEURONS AND TARGETS

A Thesis

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1. Introduction

1.1 Neurotrophins

Neurotrophins are glycoproteins that regulate the growth and differentiation of neurons during development [75] and maintain them into adulthood [23]. The neurotrophin family includes nerve growth factor (NGF), neurotrophin-3 (NT-3), brain derived neurotrophic factor (BDNF), and neurotrophin 4/5 (NT-4/5). Each neurotrophin gene encodes a mature neurotrophin sequence of approximately 120 amino acids, in which only 28 residues are invariant. Two pairs of anti-parallel beta-strands are connected at one end of the promoter by three highly flexible short hairpin loops [83] which have a role in the specificity of receptor binding [59]. Near the opposite end of the promoter, three disulfide bridges are clustered to form a cysteine knot motif that may provide molecular rigidity [83]. All neurotrophins activate cellular signaling cascades through binding to tyrosine kinase tropomyosin-related kinase (trk) receptors and the tumor necrosis factor p75 receptor [56].

The first characterized neurotrophin, NGF, as isolated from the male mouse submandibular gland, has a sedimentation coefficient of 7S and was determined to be composed of three subunit types, α, β, and γ, with the stoichiometry of α₂βγ₂ [40]. The function of the α subunit is unknown, whereas the β subunit (also referred to as the 2.5S subunit [87]), is responsible for the nerve-growth promoting activity of NGF. The γ subunit may be involved in the proteolytic processing of the 2.5S subunit precursor protein [62].

NGF plays a role in sympathetic neuron survival in vivo [26,76,77] and in vitro [31,50,82]. NGF knock-out mice exhibited excessive apoptosis and nearly complete elimination of sympathetic neurons beginning at E17.5 and continuing postnatally [26]. Dependence on NGF into adulthood was demonstrated when a three month treatment with NGF antiserum resulted in a loss of more than half of sympathetic superior cervical ganglion (SCG) neurons [101]. In addition, NGF overexpressing mice showed increased number and size of sympathetic neurons in vivo [28].

Neurotrophin-3 also is important for the survival of sympathetic neurons [46,119]. Analogous to NGF, NT-3 is a cysteine knot growth factor under the control of two promoters [74] forming a four stranded β sheet with three disulfide bonds [18,58]. Differences between NGF and NT-3 lie in the N- and C- termini, surface loops between β strands, and functional regions [18]. Antibody competition studies have revealed the involvement of NT-3 in
survival, transmission, and connectivity of mature sympathetic neurons [102]. Further, NT-3 deficient mice showed severe deficits in sensory and sympathetic neuronal populations [45]. NGF or NT-3 antiserum treatment for two weeks resulted in almost complete loss of sympathetic neurons in the superior cervical ganglion (SCG), suggesting that endogenous NGF and NT-3 may act together in the survival of postnatal sympathetic neurons. In addition, the administration of NGF during NT-3 antiserum treatment, or vice versa, inhibited neuronal death [111]. NT-3 also has been shown to stimulate sprouting of sympathetic neurons, and induced sympathetic basket formation around large sensory neurons after L5 spinal ligation, supporting a possible role for NT-3 in regeneration following injury [30].

1.2 Neurotrophin synthesis and processing

Nerve growth factor is translated from two alternatively spliced transcripts corresponding to 34 and 27 kDa prepro isoforms under two separate promoters and four exons [38]. Upon endoplasmic reticulum (ER) entry, the signal prepeptide is removed to produce 32 and 25 kDa species [27] capable of biological activity [103]. Here, NGF folds into its native structure \textit{in vivo} [8] with the propeptide facilitating the process [95]. The mature component stabilizes the structure of NGF in the propeptide region [66].

In other NGF processing, dibasic cleavage results in 22 or 18 kDa processing intermediates [8, 33, 62], and it was shown that N-glycosylation of the prosegment to 37 kDa, followed by carbohydrate chain trimming to 35 kDa, was necessary for efficient exit from the ER to the Golgi apparatus [105]. In the Golgi, further post-translational modifications can occur, including glycosylation and sulfonation. One potential glycosylation site exists on the mature region [88] and two sites reside within the prosegment. Although the function of such post-translational processing is not known, a role in protein folding and the regulation of protein secretion have been proposed [95,110]. Following post-translational modification, proNGF is cleaved to generate mature NGF protein products of 13.5 or 16.5 kDa [105].

In recent years, various protein products resulting from NGF processing have been reported in a variety of neuronal and non-neuronal tissues, and there appears to be a general tissue-specific NGF expression pattern throughout the nervous system and its target tissues. Most NGF used in previous experimental studies, particularly before the availability of recombinant forms of NGF, was extracted from male mouse submandibular glands, where
mature NGF is abundant [87]. Interestingly, only low levels of mature 2.5S NGF (13 or 16 kDa) protein have been observed in most other tissues. Instead, high molecular weight precursors appear to be the predominant species in central [4,34,41,91,96] and peripheral [12,52,92] neuronal and non-neuronal [12,20,22,29,34,35,78,81,86,89,96,117,118] tissues, CSF [51] and gastric juices [39]. Even commercial mouse and human NGF preparations have been reported to contain high molecular weight NGF forms [98]. In sympathetic neurons, which are the focus of the present study, NGF products recently were reported to be primarily 25 or 32 kDa proforms [12] and higher molecular weight species [12,52], some of which were believed to be postranslationally modified via glycosylation. Peripheral sympathetic target tissues, traditionally thought to be the site of NGF production, expressed little mature NGF, with higher molecular weight forms abundant [12]. These findings suggest that 2.5S NGF, although biologically active, may not be expressed in tissues in the abundant levels previously believed.

With regard to NT-3 processing, the NT-3 protein is encoded by three exons upstream of a coding exon under the control of two promoters, and undergoes alternative splicing to generate up to six transcripts [64,74,106]. While little is known about the processing of NT-3 in vivo or in vitro [43,104], various NT-3 species have been detected in neuronal tissues including hippocampus [57,97] and cerebellum [57], as well as the pituitary gland [79]. Western blot analysis of hippocampus, dorsal root ganglion, and superior cervical ganglion in the adult rat revealed 120, 100, 80, 48, and 31 kDa species [97], while a 30 kDa proform was reported in the spinal cord [4], suggesting that NT-3 may undergo post-translational processing similar to NGF.

The identification of intracellular convertases responsible for cleaving neurotrophin precursors to yield the mature peptide has proven difficult. Furin-like proteases recognize two amino acid residues at the C-terminus of the NGF propeptide in vivo [94] and are leading candidates for intracellular cleavage. Additional support for furin as the protease responsible for the formation of mature neurotrophin comes from two cell lines. BSC40 kidney cells, which endogenously express furin, were shown to secrete mature NGF and NT-3, whereas LoVo colon carcinoma cells, which lack endogenous furin, secreted barely detectable amounts of the mature form [104, 105]. Additional members of the proprotein convertase family, including paired basic amino acid cleaving enzyme 4 (PACE4) and proprotein convertase 5/6-B (PC5/6-B), may also participate in neurotrophin processing [44,104,105].
1.3 Neurotrophin secretion and activity

The identification of various neurotrophin species that are secreted during processing is important to an understanding of neurotrophin biosynthesis. In typical target tissues, after leaving the trans-Golgi network, NGF is believed to be cleaved into the mature 2.5S (or β-NGF) species prior to secretion [40]. However, skeletal muscle [89] and sympathetic neurons [52] were shown to secrete high molecular weight NGF molecules, indicating cleavage may occur extracellularly. Similarly, proNT-3 can be secreted as 23, 33.5, and 35 kDa NT-3 species [104].

Even less clear is whether neurotrophin release is constitutive or regulated. Both constitutive- and regulated- secreting cells when infected with full length NGF, expressed a 35 kDa intracellular form, and secreted a 42.5 kDa glycosylated intermediate [105]. This suggests that NGF may be released through both constitutive and regulated pathways, which is mediated by the mature region [54]. In support of a regulated form of secretion, both NGF and NT-3 were shown to undergo processing to large dense core vesicles in vitro, with similar sorting of the short and long transcripts [115]. Thus, it appears that NGF and NT-3 may primarily follow a regulated secretory pathway, but also are capable of constitutive release.

Because proneurotrophins appear to be secreted under some circumstances [52,89,104, 105], the possibility of extracellular cleavage of secreted precursors to mature forms was explored. Consensus sites for the serine protease plasmin and selective matrix metalloproteinases, which are typically expressed in the synapse, were found on the proneurotrophins [73]. In that study, plasmin and matrix metalloproteinase-7 cleavage resulted in 13 and 17 kDa NGF forms, respectively [73], indicating that extracellular matrix metalloproteinases may mediate survival activities by acting on secreted proneurotrophins.

Elucidating any potential biological role for proneurotrophins also has proven difficult. Some researchers have demonstrated that proNGF may have neurotrophic activities [42,72,96], but other recent findings have shown that proNGF may be largely apoptotic [4,51,73,91].

1.4 Neurotrophin receptor binding

The biological effects resulting from the binding of mature neurotrophin (and proneurotrophin) to their trk and p75 receptors are just becoming evident. Neurotrophins utilize a two receptor system in which the trk receptor is largely responsible for neurotrophic activity, with the p75 receptor acting as an accessory receptor to modulate trk-ligand binding [6,7]. Yet,
when the p75 to trkA ratio favors p75, apoptosis may result [6]. Further, each of the
neurotrophins binds to at least one trk receptor with high specificity, while all neurotrophins,
including NGF and NT-3, bind to the p75 receptor [65].

It has been well established that mature NGF binds the trkA receptor with high
specificity [56,112], whereas binding studies examining proNGF have yielded variable results.
Cleavage resistant proNGF was shown to bind trkA to elicit primary neuronal sprouting of
sympathetic neurons [42]. Yet, in a different study, proNGF bound to p75 with high affinity,
reduced neurite outgrowth of PC12 and SCG neurons, and was at least 10 times more potent than
mature NGF at inducing apoptosis [73]. After injury, proNGF binding to p75 induced apoptosis
in vitro [51] and in vivo [4], while mature NGF showed no apoptotic induction [4].

The binding of NGF and proNGF to trkA or p75 is more complex than previously
believed. Recently, a neurotensin receptor, sortilin, which predominates in brain, spinal cord,
and muscle, was shown to be necessary for the p75-mediated proNGF apoptotic signal, though
sortilin did not affect binding of mature NGF to trkA, suggesting that sortilin may act as a co-
receptor to p75 [90]. Also, a closely related family member, SorCS3, interacts with both mature
and proNGF exclusive from other neurotrophins [114], while another p75-related protein,
neurotrophin receptor homolog-2 (NRH2), may regulate NGF binding to the trkA receptor.

1.5 Signal transduction

Neurotrophin binding to trk or p75 receptor results in receptor dimerization and
autophosphorylation, subsequently activating cellular signaling cascades responsible for either a
neurotrophic or apoptotic response [56]. Upon binding of the neurotrophin to the trk or p75
receptor, a clathrin-coated vesicle forms around the ligand-receptor complex [15,55], and once in
the signaling endosome, proceeds in a retrograde fashion along the microtubules using the
molecular dynein motor machinery [10,116]. Upon arrival in the cell soma, cellular responses
occur, such as cyclic adenosine monophosphate response element-binding protein (CREB)
activation [100], to regulate neuron survival.

The signaling molecules activated following neurotrophin binding typically affect the cell
body following retrograde transport, but also may act locally within the axon. Within the axon
terminal, trk activation may lead to the activation of several pathways, including the serine-
threonine kinase ras/mitogen activated protein kinase pathway (Ras/MAPK), tyrosine-threonine
kinase map/extracellular-regulated kinase kinase/extracellular-regulated kinase (MEK/Erk) pathway, and the phosphatidylinositol-3-kinase (PI3K) lipid signaling pathway [53]. Within the soma, similar pathways may be activated, including MAPK and PI3K, followed by lysosomal degradation [53]. In addition, PI3Ks may be involved in the regulation of NGF [3] and NT-3 [2] retrograde axonal transport, suggesting a possible regulatory function during transport.

The p75 signaling pathways are less understood. Neurotrophic survival signaling, resulting from p75 co-expression with trk, may occur through the Bcl-2, nuclear factor κB, PI3K, and MAPK pathways [19]. Apoptotic signaling may occur through the Jun amino-terminal kinase-p53-Bax pathway, neurotrophin receptor interacting factor, members of the tumor necrosis factor receptor-associated factor family, ceramides, and caspases [63].

Although proneurotrophin signaling is currently being investigated [42,73], there is evidence that proNGF may activate the p44/42 MAPK/Erk1/2 pathway [42].

1.6 The sympathetic model: effects of aging

The sympathetic superior cervical ganglion (SCG) of the rat is a collection of approximately 26,000 neuronal cell bodies [93]. Located near the bifurcation of the external and internal carotid arteries, the SCG projects post-ganglionic axons to various targets including the extracerebral blood vessels, pineal gland, submandibular/submaxillary salivary glands, iris, and external carotid arteries. Innervation to the SCG cell bodies originates from about 1,000 preganglionic neurons arising in the lateral horns of the spinal cord [93]. Each SCG neuron receives about ten synaptic contacts from one preganglionic neuron [84].

The sympathetic neurons in the SCG provide an excellent model for studying the degenerative processes that occur in the aged rat peripheral nervous system. Atrophy of the axons and dendrites of SCG neurons projecting to the cerebral blood vessels [113], submandibular gland [1], and pineal gland [68] has been noted. However, this atrophy is not only localized to the neurons of the SCG, for sympathetic innervation to the heart [16] and spleen [5] has been observed to decrease into old-age. Further, aged projections to the cerebral blood vessels show significant decreases in tyrosine hydroxylase immunoreactivity and norepinephrine content [32], suggesting alterations in neuronal function. In addition, in the cerebral blood vessel targets, an age-related thickening of the vascular wall and increased collagen fiber content, with a decreased number of perivascular sympathetic axonal contacts, has
been documented [60]. Yet, not all projections from the SCG show atrophy, as sympathetic innervation of aged iris does not show significant alteration and appears to undergo continued growth throughout aging [1]. Such findings suggest that sympathetic target tissues may have regulatory influences on their innervating neurons, and possibly mediate their survival. Though these targets can contribute to the regulation of their own sympathetic innervation, no significant age-related alterations in expression of survival proteins, such as NGF and NT-3, in the cerebral blood vessels or iris were noted [25].

1.7 Rationale and hypotheses

Recent studies in our lab, using a sensitive two site enzyme-linked immunosorbant assay (ELISA), revealed a significant increase in total NGF protein in the aged Fischer rat SCG compared to the young adult (Isaacson unpublished data). This was surprising in light of previous ELISA data, collected using a different series of antibodies, which showed a significant decrease in total NGF protein in the aged SCG [70]. Because recent Western blot analysis showed a relatively small amount of mature NGF protein (13.5 kDa species) and an abundance of higher molecular weight species in the SCG and peripheral target tissues of the young adult [12], we hypothesized that differences in the antibody sensitivity for various NGF species between the two ELISA systems may account for the discrepancies in ELISA values. Since Western blot analysis of aged tissues was necessary to resolve this issue, one goal of the present study was to examine the SCG and peripheral targets, such as the extracerebral blood vessels, external carotid artery, heart, pineal and submandibular glands, and iris, using NGF Western analysis to determine whether mature NGF and/or precursor species are altered in aged tissues.

In a previous study using ELISA, we observed a significant increase in NT-3 protein in the aged SCG and extracerebral blood vessel targets [61], providing preliminary evidence that NT-3 protein was altered in aged tissues. Because NT-3 undergoes processing similar to NGF [43,104], and the ELISA only detected total NT-3 protein levels, the second goal of the present study was to extend our ELISA data using NT-3 Western blot analysis to characterize the NT-3 protein processing forms present in young adult and aged SCG and peripheral targets. We hypothesized that changes in the aged SCG and peripheral tissue expression of the various NT-3 species would parallel the ELISA findings.
While Western blot analysis aids in the characterization of alterations in various neurotrophin processing forms, it is not possible to determine whether changes in protein expression in aged tissues were the result of altered transcription and/or post-translational processing. Using semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR), a method to examine the regulation of neurotrophin biosynthesis at the transcriptional level, no age-related alterations in NGF and NT-3 mRNA were reported in two SCG target tissues, the iris and extracerebral blood vessels, yet there was a trend toward increased NGF mRNA in the aged rat iris [25]. Therefore, a third goal of this study was to carry out a thorough analysis of the SCG and various sympathetic targets using the more sensitive relative real-time RT-PCR to determine whether transcription was altered, and to relate mRNA levels to any changes in protein content.
2. Methods

2.1 Animals

Fischer-344 virgin female rats (young: 3 months; aged: 24 months; NIA colony: Harlan Labs) were sacrificed via decapitation using a Harvard guillotine apparatus. Tissues were removed, snap frozen in liquid nitrogen, and stored at -80°C. Individual SCG or trigeminal ganglia, pineal glands, submandibular glands, external carotid arteries (immediately distal to the bifurcation of the common carotid), ventricular heart, spleen, and frontal cortex were processed separately. The extracerebral blood vessels (left and right internal carotid arteries, posterior communicating, middle cerebral, and anterior cerebral) were pooled from one animal. Left and right irides also were pooled for Western analysis, but processed individually for real-time RT-PCR analysis.

2.2 Protein isolation and semi-quantitative Western blot analysis

Total protein was obtained by sonicating tissues in 0.01M Tris-HCL buffer (pH 7.4) containing 1% SDS and 1% protease inhibitor cocktail (Sigma-Aldrich). Following centrifugation, total protein concentration was determined using a BCA assay (Pierce Biotechnology, Inc.) and samples were prepared as described by Laemmli [71]. Samples (40µg for NGF, 10µg for NT-3) and 2.5S NGF (15ng; Harlan Labs) or recombinant human NT-3 (10ng; Chemicon) peptides were loaded and run on a 5% SDS-polyacrylamide gel electrophoresis (PAGE) stacking gel and 12% SDS-PAGE resolving gel (Owl Separation Systems). Proteins were transferred onto PVDF membrane overnight at 4°C and a total of 2,300 mAmps in transfer buffer (25mM Tris, 192mM glycine, 10% (v/v) methanol). On the outer lanes of each SDS-PAGE gel, Precision Plus protein unstained standards (Bio-Rad Laboratories) were loaded for estimation of molecular weight. The portion of the membrane containing the standards was cut from the rest of the membrane and processed separately to prevent non-specific binding of the StrepTactin-HRP conjugate to protein samples. The membrane containing protein was incubated in 8% non-fat dry milk diluted in Tris buffered saline containing Tween-20 (TBST) for 4 h at room temperature, incubated overnight at 4°C in either rabbit anti-NGF (H-20, 1:1000; Santa Cruz Biotechnology) or rabbit anti-NT-3 (N-20, 1:1000; Santa Cruz Biotechnology), rinsed with TBST, and incubated 2 h at room temperature in goat anti-rabbit HRP IgG (1:10,000 for NGF, 1:100,000 for NT-3; Chemicon). Concurrently, the
membrane containing the Precision Plus protein standards was incubated in 8% non-fat dry milk diluted in TBST for 4 h at room temperature, incubated overnight at 4°C in TBST, rinsed with TBST, and incubated 2 h at room temperature in Precision Protein StrepTactin-HRP Conjugate (1:500,000; Bio-Rad Laboratories). All membranes were rinsed with TBST, covered with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc.) for 5 min, and then placed in an autoradiography cassette. Film (Kodak BioMax MR or Pierce Chemiluminescent clear-blue) was exposed at multiple time increments to ensure detection within a linear range. Membrane containing protein was stripped and reprobed for actin (1:75,000; mouse anti-actin and 1:80,000 goat anti-mouse HRP IgG; Chemicon) to ensure samples of the same tissue type were loaded at comparable protein concentrations. Actin was not detected in the aged submandibular gland. Alternatives investigated include α1-Na,K ATPase (a gift from Dr. Paul F. James) and GAPDH (Novus Biologicals). However, these proteins were either not detected (α1-Na,K ATPase) or altered (GAPDH) in the aged submandibular gland. Antibody specificity was determined by omission of primary antibody from Western blot protocol. In addition, no signal was observed when membrane was incubated overnight at 4°C in solution containing NGF or NT-3 antibody with 10-fold excess of appropriate blocking peptide (Santa Cruz Biotechnology). Films were scanned into ImageQuant 5.2 (Amersham Biosciences) and densitometry readings for all bands obtained with local background subtracted. The densitometry of neurotrophin was normalized to actin and the ratio of aged versus young taken. For the submandibular gland, where actin was not detected in aged tissues and several other candidate ‘housekeeping proteins’ failed to prove useful, equal quantities of total protein loaded were presumed between young and aged, thus densitometry readings for all bands were taken and a ratio of aged versus young calculated without normalization to actin. Data for each tissue were collected from 3 to 5 animals per treatment and then were subjected to analysis using the Mann-Whitney test. Values are reported as mean ± SEM. Significance is reported at p<0.05.

2.3 RNA isolation and real-time assay

Following sonication in Trizol, total RNA was isolated through chloroform extraction, ethanol precipitation, and the Qiagen RNeasy Micro Isolation kit, and quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Inc.). 0.3µg total RNA diluted to a
volume of 22µL with DEPC-treated sterile water (Fisher Scientific) and 3µL random primers (500 µg/mL; Promega) were incubated 2 min at 90°C and allowed to ramp cool to 30°C. The samples were brought to a final reaction volume with 12µL DEPC-treated sterile water, 12µL 5X M-MLV RT buffer (Promega), 6µL 10mM PCR nucleotide mix (Promega), 1µL RNasin RNase Inhibitor (40u/µL, Promega), and 2µL M-MLV reverse transcriptase (200u/µL, Promega). Negative controls were performed concurrently for each RNA sample in which 2µL DEPC-treated water was added in the place of the M-MLV reverse transcriptase. Using a thermocycler, reaction mixtures were then incubated at 20°C for 15 min, 42°C for 2 h, and 90°C for 2 min. Resulting cDNA solutions were stored at -80°C. 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 [13]) and NT-3 (forward: 5’ - AGA ATT CCA GCC GAT GAT TG -3’ reverse: 5’ - AGC GTC TCT GTT GCC GTA GT - 3’; Tm=57°C) mRNA was measured and compared to glyceraldehyde-3-phosphate dehydrogenase (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) using the Rotorgene 3000 system (Corbett Research) and the Platinum qPCR Supermix UDG kit (Invitrogen). 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). No template controls were performed concurrently, in which 2µL DEPC-treated water was added in the place of the RT-PCR product. To verify NT-3 amplification, product from a representative NT-3 run was inserted into a plasmid vector and transformed using chemically competent One Shot Cells (Invitrogen). Plasmid DNA was isolated and digested in EcoR1 and sequenced on an ABI3100 Genetic Analyzer (Applied Biosystems). Data (n = 4) were subjected to analysis using the Mann-Whitney test and values were reported as mean ± SEM. Significance is reported at p<0.05.
3. Results

3.1 NGF and NT-3 protein expression in the young adult

Western blot analysis of tissues from the young adult revealed a characteristic pattern of NGF immunoreactivity that was consistent with previous findings from our lab [12]. As reported previously, weak expression of the mature form of NGF (13.5 kDa) was observed in these tissues, though, as shown in Figures 1-3 (NGF lanes), the antibody readily recognized the mature NGF species obtained from Harlan labs. In the young adult, NGF protein resulting from transcript A (32 kDa; proNGF-A) was typically the predominant species observed in pineal gland, extracerebral blood vessels, iris, submandibular gland, trigeminal ganglion (trig), and cortex, with NGF protein encoded by transcript B (25 kDa; proNGF-B) typically was more abundant in SCG, external carotid artery, and spleen (Figs. 1-3).

Contrary to the weak expression of mature NGF in these tissues, the mature NT-3 form (13.5 kDa) was the predominant species in most tissues (Figs. 1-3). However, mature NT-3 was only weakly detected in iris and cortex. Minor expression of large molecular weight NT-3 precursors of 36 (proNT-3) and 50 kDa also were observed in the SCG, pineal, extracerebral blood vessels, iris, trig, and cortex (Figs. 1-3). This is consistent with previous findings showing that the proNT-3 form migrated to approximately 35 kDa [79,104] along with higher molecular weight NT-3 species of 48-120 kDa [57,97]. Though the mature form was predominant in most tissues, in the iris and cortex, high molecular weight species of 43-50 kDa were predominant. (Figs. 2, 3). A 75 kDa NT-3 species was present only in the cortex (Fig. 3). Recombinant human NT-3 (10 ng; Chemicon), which was used as a positive control for NT-3 immunoreactivity, migrated to approximately 14.5 kDa (Figs. 1-3), in agreement with previous studies [79,109].

Though mature NT-3 was unchanged, a slightly different staining pattern of large molecular weight precursors was observed between the different lots of NT-3 antibody. All NT-3 blots shown here were obtained using antibody lot #C2505, which failed to detect the 36 kDa species. However, a different antibody lot (#J0103A) detected a 36 kDa species (proNT-3) in some tissues when 40µg total protein was loaded, though this isoform was 10 times less abundant than the mature NT-3 species. In addition, the 50 kDa form showed a weaker signal when using lot #J0103A, though all age-related trends were similar between the two lots.
Figure 1. NGF and NT-3 Western blot analysis of the superior cervical ganglion (SCG) from two young adult (y; 3 mos) and two aged (a; 24 mos) Fischer rats. A. Increases in 25 (proB), 32 (proA), 55, and 75 kDa NGF species were observed in the aged (40µg total protein). NGF: 15ng 2.5S NGF protein (Harlan) B. The 13.5 kDa mature NT-3 species was increased in aged SCG, with a decrease in 50 kDa NT-3 (10µg total protein). NT-3: 10ng recombinant human NT-3 peptide (Chemicon). For quantitation results, see Fig. 4.
Figure 2. Representative NGF and NT-3 Western blot analysis of young adult (y; 3 mos) and aged (a; 24 mos) target tissues of the Fischer rat SCG.  A. All aged targets, except iris, exhibited a significant increase in the 25 kDa NGF species.  Iris decreased in 22, 25, and 27 kDa NGF species; in pineal, 34, 50, and 75 kDa NGF species decreased (40µg total protein).  NGF: 15ng 2.5S NGF protein (Harlan)  B. Mature NT-3 (13.5 kDa) was unchanged in all aged tissues, except for a decrease in submandibular gland.  A 50 kDa NT-3 form decreased in pineal, but increased in iris (10µg total protein pineal, vessels, subm, 40µg iris ).  NT-3: 10ng recombinant human NT-3 peptide (Chemicon).  pineal: pineal gland; vessels: extracerebral blood vessels; subm: submandibular gland.  For quantitation results, see Fig. 4.
Figure 3. NGF and NT-3 Western blot analysis of young adult (y; 3 mos) and aged (a; 24 mos) trigeminal ganglion (trig) and other peripheral target tissues. A. Aged trig, external carotid artery, and heart exhibited a significant increase in 25 kDa NGF. The 20-22 kDa NGF was increased in aged spleen, an immune target with decreased sympathetic innervation (Bellinger et al. 1992). A 50 kDa NGF species decreased in the trig. Cortex shows no age-related changes (40µg total protein). NGF: 15ng 2.5S NGF protein (Harlan) B. Mature NT-3 (13.5 kDa) was increased in aged heart and spleen. A 43-50 kDa NT-3 species increased in cortex (10µg total protein trig, ext car, cortex, spleen, 40µg heart). NT-3: 10ng recombinant human NT-3 peptide (Chemicon). ext car: external carotid artery. For quantitation results, see Fig. 4.
Because the detection of the mature form of NT-3 also was similar between the antibody lots, and #J0103A showed immunoreactivity for both 36 and 50 kDa NT-3 forms, data from this antibody were used for our quantitative analysis at 40µg total protein (Fig. 4). However, because mature NT-3 was the predominant form in the tissues, we wanted to demonstrate the mature NT-3 band as clearly as possible for the detection of any age-related changes, and thus the immunoblots using 10µg total protein and lot #C2505 were chosen as representative blots in Figures 1-3.

3.2 Alterations in NGF and NT-3 protein expression in aged tissues

In the aged SCG, significant changes in both NGF and NT-3 protein were observed (Figs. 1, 4). The most obvious change in NGF was a dramatic increase in the 25 kDa proNGF-B species. The approximately 50-fold increase in proNGF-B was accompanied by a smaller, but significant, increase in 32 kDa proNGF-A (Figs. 1, 4). Higher molecular weight species of 55 and 75 kDa also were significantly increased (Fig. 1), while a 150 kDa form was significantly decreased (data not shown). In addition, mature NT-3 was increased by 3-fold, with significant decreases in the minor 50 kDa NT-3 and 36 kDa proNT-3 species (Figs. 1, 4).

In the peripheral targets, age-related changes in NGF and NT-3 protein also were present. As in the SCG, the most dramatic change in NGF was a 6 to 43-fold increase in proNGF-B in major targets of SCG neurons, such as the pineal, extracerebral blood vessels, submandibular gland, and external carotid artery (Figs. 2-4). Mature NT-3 was unchanged in the pineal and extracerebral blood vessels, yet decreased in the submandibular gland (Figs. 2, 4). Similar to the SCG, the pineal and extracerebral blood vessels showed a significant decrease in the minor pro-NT-3 species (Fig. 4). Unlike other targets of the SCG, the pineal gland showed decreases in the 34, 50, and 55 kDa NGF (Fig. 2) and 50 kDa NT-3 species (Figs. 2, 4).

The iris was the one peripheral target of the SCG showing changes in NGF and NT-3 expression that were not consistent with the other target tissues. The 25 kDa NGF species, while dramatically increased in other tissues, was significantly decreased in the iris (Figs. 2, 4). In addition, the iris was the only peripheral tissue that showed significant decreases in all 22, 25, and 27 kDa NGF forms (Fig. 2). While most targets showed little or no change in the mature NT-3 and a decrease in NT-3 precursor species, the iris showed little mature NT-3 and an increase in the 50 kDa NT-3 species (Figs. 2, 4).
Figure 4. Semi-quantitative Western blot analysis of aged tissues. A. Significant increases (up to 50-fold) in the 25 kDa species (proNGF, transcript B) were observed in most aged tissues. The iris, however, had a significant decrease in the 25 kDa form. The 32 kDa form (proNGF-A) was largely unaffected, but was increased 2-fold in the SCG. The 33 kDa species in cortex most likely represents the 32 kDa proNGF-A reported previously by Fahnestock et al. [41]. B. Mature NT-3 (13.5 kDa) increased 3-fold in the SCG, while proNT-3 (36 kDa) decreased. ProNT-3 also decreased 6-10 fold in the pineal and vessels. Mature NT-3 decreased 5-fold in the submandibular gland, and increased marginally in heart and spleen. (*p<0.05) YA: young adult, SCG: superior cervical ganglion, pineal: pineal gland, vessels: extracerebral blood vessels, subm: submandibular gland, trig: trigeminal ganglion, ext car: external carotid artery.
Age-related changes were observed in the heart and spleen. Though these peripheral tissues are not direct targets of the SCG, they receive innervation from other sympathetic ganglia. Changes in NGF were similar to that typically observed in the SCG targets. ProNGF-B was increased by 29-fold in the heart, yet remained unchanged in the spleen. However, a 20-22 kDa NGF species was significantly increased in spleen. In contrast to unaltered or decreased NT-3 in SCG targets, mature NT-3 was significantly increased in both spleen and heart (Figs. 3, 4).

In the aged sensory trigeminal ganglion, as in most peripheral tissues examined, a dramatic 30-fold increase in proNGF-B was observed. No age-related changes in mature NT-3 or precursors were seen (Figs. 3, 4). Also, a 50 kDa NGF species showed a slight, but significant, decrease (Fig. 3).

Analysis of NGF protein expression in the aged rat cortex showed little mature NGF, though a 33 kDa species was strongly expressed (Figs. 3, 4). This isoform most likely parallels the 32 kDa species reported in a previous study [41]. There were no changes in proNGF in aged cortex as compared with young adult.

3.3 Neurotrophin mRNA expression in the aged SCG and peripheral tissues

To determine whether the age-related alterations in NGF or NT-3 protein content was the result of an upregulation in gene expression, real-time RT-PCR was carried out using RNA from young adult and aged SCG and each peripheral tissue. In the SCG, no changes in NGF or NT-3 transcript were evident, yet all other tissues examined, including the trigeminal ganglion, showed some alteration in NGF and/or NT-3 mRNA.

In many of the peripheral targets of the SCG, such as the extracerebral blood vessels, submandibular gland, and external carotid artery, levels of NGF mRNA were significantly increased (Fig. 5) and corresponded to the increase in the 25 kDa NGF proprotein (Fig. 4), though the increase in transcript was not as dramatic as the increase in protein. In contrast, changes in NT-3 mRNA expression (Fig. 5) did not always correlate well with alterations in protein (Fig. 4). The extracerebral blood vessels showed a significant decrease in NT-3 transcript that paralleled the decrease in proNT-3 protein expression. Though NT-3 protein decreased in the submandibular gland, NT-3 mRNA was unaltered. In the pineal gland, NGF
mRNA showed no correlation with the increase in proNGF-B protein, though a decrease in NT-3 transcript corresponded well to a decrease in NT-3 precursors (Figs. 4, 5).

As seen with protein content, the iris displayed different trends in mRNA expression when compared to the other targets. A decrease in NGF transcript (Fig. 5) paralleled the decreases in proNGF species (Fig. 4). Further, increased NT-3 transcript (Fig. 5) corresponded to the increase in 50 kDa NT-3 protein (Fig. 4).

In other tissues targeted by sympathetic neurons, such as the heart and spleen, alterations in transcript and protein did not correlate well. Unlike in the other targets, where alterations in NGF mRNA levels paralleled changes in NGF protein content, both NGF and NT-3 mRNA decreased in the aged spleen (Fig. 5), though protein expression was significantly increased. Likewise, in the heart, no change in NGF mRNA and a decrease in NT-3 mRNA (Fig. 5) contrasted with significant increases in both NGF and NT-3 protein (Fig. 4).

Transcript levels in the trigeminal ganglion were similar to the other peripheral tissues with increased NGF mRNA and decreased NT-3 mRNA (Fig. 5). The increase in NGF transcript paralleled the increase in proNGF (Fig. 4).
Figure 5. Real-time RT-PCR analysis of NGF (A) and NT-3 (B) mRNA expression. Age-related alterations were evident in most target tissues examined. Though no changes were observed in the SCG, in most other tissues susceptible to atrophy, NGF mRNA was significantly increased. In addition, most tissues also showed either decreased NT-3 mRNA or no change. The iris, however, showed a decrease in NGF mRNA and an increase in NT-3 transcript. (*p<0.05) SCG: superior cervical ganglion, pineal: pineal gland, vessels: extracerebral blood vessels, subm: submandibular gland, trig: trigeminal ganglion, ext car: external carotid artery.
4. Discussion

4.1 Neurotrophin expression and age-related alterations in the periphery

These results show for the first time that, similar to the young adult [12], the mature 13.5 kDa NGF species is only weakly expressed in peripheral targets of the aged rat, and that higher molecular weight forms are predominant in these tissues. In addition, we present the first documentation that, unlike NGF, NT-3 is predominantly expressed as the mature 13.5 kDa form in most young and aged peripheral tissues, with higher molecular weight species only weakly expressed. Two tissues, the iris and cortex, failed to show this trend in NT-3 protein, and high molecular weight NT-3 species were predominant over the relatively weak expression of mature NT-3. The strong expression of mature NT-3 in these tissues supports the idea that, in the periphery, mature NT-3 may play a more important role in survival activities when compared to the large molecular weight precursor species.

Our findings suggest that an increase in the 25 kDa proNGF correlates with a loss of sympathetic innervation, and that a decrease in this species may be protective. Changes in this NGF isoform were the most obvious age-related alteration detected in this study. All aged target tissues, except iris, showed a dramatic increase in the 25 kDa proNGF-B species. Interestingly, these are targets that showed significantly decreased sympathetic innervation in aging [1,16,68,113]. Although there are no reports of decreased sympathetic innervation in the aged external carotid artery, this vessel showed a significant 6-fold increase in proNGF-B. In comparison, the age-related changes in NT-3 protein were relatively small. Alterations in mature NT-3 in aged targets only were observed in the submandibular gland (decreased) and heart (increased). ProNT-3, though a minor species, showed a drastic (up to 10-fold) reduction in SCG targets, such as the vessels and pineal gland, yet this was not a trend across all tissues that lost sympathetic innervation in aging.

The predominance of proNGF may have a detrimental effect on survival. The functional importance of the proneurotrophins in neuron survival remains largely unknown, though it has been suggested that 40-60% of all NGF secreted by target tissues and central neurons is in the proform [80]. Recently, an increase in proNGF was reported in the cortex of individuals with Alzheimer’s disease (AD) and mild cognitive impairment [41,91,92]. Altered pro-brain derived neurotrophic factor also was observed in parietal cortex of AD patients [85]. Therefore, it would appear that an accumulation of proneurotrophin is related to disease and neuronal apoptosis.
Here, for the first time, we show that a dramatic increase in proNGF correlates well with a known susceptibility to decreased sympathetic innervation of aged target tissues.

The alterations in proNGF-B in the target tissues paralleled smaller, albeit significant, increases in NGF mRNA expression. The dramatic increases in NGF protein with less obvious increases in mRNA indicate that processing of NGF protein is greatly affected and is altered to a greater degree than NGF transcription in aged peripheral target tissues. In contrast to NGF, alterations in NT-3 mRNA did not correlate well with mature NT-3 protein. However, changes in NT-3 transcription did parallel changes in NT-3 precursor protein in the pineal and extracerebral blood vessels, where a significant decrease in NT-3 mRNA paralleled a drastic decrease in proNT-3 protein.

Both NGF and NT-3 expression in the iris were in direct contrast to that of the other target tissues. While the 25 kDa proNGF-B greatly increased in most peripheral tissues, prepro- and pro- NGF-B in the iris was dramatically decreased (up to 28-fold), a tissue that maintains sympathetic innervation into old-age [1]. A small, but significant decrease in NGF mRNA paralleled this decrease in protein. Also, in contrast to other targets, mature NT-3 was only weakly expressed, with higher molecular weight NT-3 precursor species predominant. A 50 kDa NT-3 species was significantly increased only in the aged iris, and an increase in NT-3 protein expression paralleled a significant increase in NT-3 mRNA. Given that the iris is the only peripheral tissue to have increased NT-3 mRNA and NT-3 precursors, it may be that, unlike proNGF, an increase in NT-3 precursor protein, though a minor form in most peripheral tissues, may serve a protective role in the aged.

The importance of other NGF species is unclear. For example, in the spleen, a tissue known to undergo an age-related loss of sympathetic innervation [5], a two-fold increase in a 20-22 kDa dibasic cleavage product was observed, but this form was significantly decreased in the iris, a tissue that maintains sympathetic innervation into old-age [1]. This suggests the 20-22 kDa NGF species may represent an isoform contributing to neuron survival.

Though most sympathetic targets showed increased 20-25 kDa NGF protein species, suggesting altered NGF processing, NT-3 was unchanged or slightly increased in the aged. This suggests that NT-3 processing at the target level was unaltered. Thus, our data provide evidence that NT-3 cannot compensate for the dramatic alterations in NGF protein expression in these tissues.
4.2 Alterations in neurotrophin expression within the aged SCG may influence neuron survival

Curiously, in addition to changes in the targets, the SCG showed a drastic 50-fold increase in 25 kDa proNGF-B, as well as alterations in higher molecular weight NGF species. This finding parallels previous ELISA data from our lab showing an increase in NGF content in the aged SCG [Isaacson, unpublished data]. Further, total NT-3 protein detected using ELISA [61] parallels findings of the present study using Western blot, where mature NT-3 was significantly increased (3-fold) in the aged SCG.

While the source of these high molecular weight forms in the SCG, a set of neuronal cell bodies that typically derives neurotrophins from peripheral targets in vivo, remains unknown, these products may originate from biosynthesis within SCG neurons and/or glial cells, rather than being target-derived. Indeed, SCG neurons have been shown to secrete larger NGF forms [52] which are reportedly N-glycosylated [91,96].

There also is evidence to support the idea that these high molecular weight species detected in the SCG may represent proNGF forms that have been retrogradely transported from target synapses to the SCG cell bodies. ProNGF binds both p75 and trk receptors [4,42,51,72,73,91,96], can be secreted in vitro [37,105], and may comprise up to 40-60% of secreted neurotrophin [80]. Thus, it is possible that the high molecular weight species may be derived from retrogradely transported target-derived neurotrophin (mature or pro-) that, upon reaching the cell bodies, subsequently is directed to Golgi processing through endocytic ligand recycling [14,17]. Therefore, the changes observed in high molecular weight species in the SCG with no corresponding alteration in transcript suggests that alterations in post-translational processing, endocytic recycling, and/or transport of NGF may occur in the aged SCG.

The changes in NGF and NT-3 expression may underlie observed alterations in tyrosine hydroxylase (TH) expression in aged sympathetic neurons. Perivascular axon terminals associated with the extracerebral blood vessels showed a significant age-related decrease in TH protein [113, 32] and norepinephrine (NE) [32], and axon collaterals around the aged pineal gland also showed decreased TH [67]. Further, a decline in TH protein and NE in nerve fibers has been observed in another sympathetic target, the aged spleen, by 17 months and which progressed through aging [5]. In contrast, the cell bodies of the aged SCG show increased TH protein and mRNA [69, Isaacson unpublished data], suggesting that TH may not be appropriately
transported to the nerve ending. While the exact relationship between the increase in proNGF and loss of TH at the targets remains to be determined, it is possible that the alteration in NGF protein may somehow contribute to the loss of appropriate TH shuttling to select subsets of sympathetic nerve terminals.

4.3 The critical balance of proNGF expression in aged tissues and neuronal innervation

The abundance of proNGF and higher molecular weight forms in most aged tissues examined seems to result, at least in part, from altered processing of the NGF protein. Changes in NGF forms larger than 34 kDa suggest alterations in glycosylation, deglycosylation, sulfonation, or other post-translational modifications. Changes in 25, 27, 32, and 34 kDa proNGF and the 22 kDa dibasic cleavage product suggest impaired processing by furin or other proprotein convertases [44,73,105].

Elucidation of the biological activity of various NGF species has proven difficult. ProNGF and higher molecular weight species may have neurotrophic activities through trkA [42, 72,96], while proNGF and a 53 kDa glycosylated species have also been shown to induce apoptosis through the p75 receptor [4,51,73,91] with a 5-fold greater affinity than mature NGF [73]. Adding complexity, there have been recent reports that several transmembrane proteins, including sortilin [90] and SorCS3 [114], may act as co-receptors to regulate the binding of proNGF to trkA or p75. Although proNGF may have neurotrophic activity, it has at least 5-fold less activity than mature NGF [42].

It has been suggested that the ratio of proNGF to mature NGF may determine cell survival [21]. The drastic increase in proNGF in tissues, such as the pineal, extracerebral blood vessels, submandibular gland, and heart, known to undergo atrophy in the aged, together with the marked decrease of proNGF in the iris, which is reportedly protected in aging, support this idea. Further, in the current study, the increase in proNGF derived from transcript B not only shifts the proNGF:mature NGF ratio in favor of proNGF, but also alters the predominant pro-species in most tissues from proNGF-A [12] to proNGF-B.

4.4 Implications of age-related changes in proNGF expression in peripheral target tissues

There have been reports that the balance between trkA and p75 is critical for the survival of sympathetic neurons, with an increase in p75 contributing to apoptosis [6]. However, an age-
related decrease in the expression of p75 with no change in trkA, thus favoring trkA, was observed in the SCG and its projection to the extracerebral blood vessels [25]. These neurons show vulnerability to atrophy [25], but also express a high trkA:p75 ratio, suggesting that a better indicator of susceptibility to degeneration may be the accumulation of proNGF, as observed in the present study. Indeed, the occupancy of less than 30% of p75 receptors is sufficient to induce apoptosis [73], suggesting that an accumulation of even small amounts of proNGF, regardless of the amount of p75 present, may play an important role in mediating susceptibility to age-related atrophy. Interestingly, this idea is supported by previous findings that aged sympathetic iridial projections showed a 3-fold higher expression of p75 than the vulnerable aged cerebral vessel projecting neurons [25], even though iridial tissues showed a marked decrease in proNGF-B.

4.5 The influence of the sensory trigeminal ganglion on sympathetic neuron survival

The dramatic changes in proNGF-B seen in aged target tissues also were observed in the sensory trigeminal ganglion, while there was no change in mature NT-3. The increase in 25 kDa proNGF in sensory neurons suggests that, similar to sympathetic neurons, either these neurons also derive pro-forms from the target tissues, or that at least some sensory neurons undergo age-related alterations in neurotrophin biosynthesis and/or processing. The sensory trigeminal neurons innervate many of the same targets as the SCG neurons and are responsive to neurotrophins such as NGF and NT-3 [36,99,107,108]. It is believed that sensory and sympathetic neurons compete for neurotrophic factors at the target level. Using ELISA, and here with Western blot, a significant increase in NGF protein was observed in the aged trigeminal ganglion [24, Isaacson unpublished data], despite a decrease in trk receptor and unaltered p75 protein expression [9]. While the aged trigeminal ganglion maintains responsiveness to NGF into old age [49], and shows no significant changes in the number of neurons [11], a decrease in the innervation of select targets, including the iris, have been noted [47,48,49]. It is unknown whether the increase in 25 kDa proNGF-B is the result of the nearly 3-fold increase in NGF mRNA, or due to altered post-translational processing and/or retrograde transport.
5. Conclusion

In summary, we demonstrated a relative abundance of NGF proforms in the aged SCG and peripheral target tissues. Mature NT-3 is predominant in most tissues examined, with low expression of proforms and little alteration in the aged, indicating that an increase in the expression of proNGF forms may make aged neurons more susceptible to neuronal atrophy. Our observations support the idea that the balance between proNGF and mature NGF, when favoring proNGF, may result in neuronal atrophy. The expression of various biosynthetic products with differential activities may help to explain the apparent lack of correlation between total NGF expression and changes in innervation of some aged tissues [24,68]. While NGF showed clear alterations correlating to vulnerability, NT-3 remained largely unchanged in target tissues although it was dramatically increased in the SCG. The precise species secreted by peripheral targets, as well as their fate, have yet to be determined. While it would appear some proneurotropins and high molecular weight species can directly interact with p75 and trkA receptors [4,42,51,72,73,91,96], others may be extracellularly cleaved to mature forms by the serine protease plasmin and selective matrix metalloproteinases, which are typically expressed in the synapse [73]. Thus, additional studies characterizing the precise nature of the higher molecular weight NGF species, the neurotrophin forms secreted by target tissues, and biological effects of the high molecular weight species, proNGF, and processing intermediates on neuronal cells will provide further insight into the mechanisms by which neurotrophins elicit neuron survival.
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