Absence of the p75 Neurotrophin Receptor Alters the Pattern of Sympathosensory Sprouting in the Trigeminal Ganglia of Mice Overexpressing Nerve Growth Factor

Gregory S. Walsh, Karmen M. Krol, and Michael D. Kawaja

Department of Anatomy and Cell Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6

Sympathetic axons invade the trigeminal ganglia of mice overexpressing nerve growth factor (NGF) (NGF/p75−/− mice) and surround sensory neurons having intense NGF immunolabeling; the growth of these axons appears to be directional and specific (Walsh and Kawaja, 1998). In this investigation, we provide new insight into the neurochemical features and receptor requirements of this sympathosensory sprouting. Using double-antigen immunohistochemistry, we demonstrate that virtually all (98%) trigeminal neurons that exhibit a sympathetic plexus are trkA tyrosine kinase receptor (trkA)-positive. In addition, the majority (86%) of those neurons enveloped by sympathetic fibers is also calcitonin gene-related peptide (CGRP)-positive; a smaller number of plexuses (14%) surrounded other somata lacking this neuropeptide. Our results show that sympathosensory interactions form primarily between noradrenergic sympathetic efferents and the trkA/CGRP-expressing sensory somata.

Collateral sprouting of mature, undamaged sympathetic axons is dependent on the availability of the neurotrophin nerve growth factor (NGF). Tissues with augmented levels of NGF, as a consequence of damage or disease (Donohue et al., 1989; Aloe et al., 1992a, 1993; Zettler and Rush, 1993; Kapuscinski et al., 1996), display an increased density of sympathetic fibers (Mangiarua and Lee, 1990; Zettler et al., 1991; Aloe et al., 1992b; Falckh et al., 1992). Moreover, administration of NGF antibodies blocks the collateral sprouting response of sympathetic axons (Springer and Loy, 1985; Gloster and Diamond, 1992). A role for NGF in the guidance of elongating nerve fibers in vitro has been suggested (Letourneau, 1978; Gundersen and Barrett, 1979; Gundersen, 1985), as well as in vivo, because new growth of sympathetic axons occurs toward natural and unnatural sites of increased NGF content (Menesini-Chen et al., 1978; Edwards et al., 1989; Albers et al., 1994; Hassankhani et al., 1995; Ma et al., 1995; Kawaja and Crutcher, 1997).

Injury to peripheral nerves elicits the sprouting of sympathetic fibers into affected dorsal root ganglia (DRG) (McLachlan et al., 1993; Chung et al., 1996; Zhou et al., 1996; Ramer and Bisby, 1997a), where they form discrete projections to a subset of sensory somata and subsequently envelop them in a perineuronal (basket-like) plexus of fibers. These arborizations may represent the anatomical substrate for the functional coupling between sympathetic and sensory neurons that underlie the development of causalgia and sympathetically maintained pain states that result from nerve injury (Richards, 1967; Devor, 1983; Bonica, 1990). The molecular signal initiating this sympathetic sprouting response is most likely NGF, because a similar pattern of sprouting has been reported in the trigeminal ganglia of transgenic mice overexpressing NGF in skin (Davis et al., 1994) and in glial cells (Walsh and Kawaja, 1998), although recently leukemia inhibitory factor has also been shown to elicit sympathetic sprouting and basket formation in the intact DRG of adult rats (Thompson and Majithia, 1998).

The relative contributions of the NGF receptors in mediating axon growth responses are not entirely clear. The trk tyrosine kinase receptor (trkA) is necessary and sufficient to confer NGF responsiveness (Koizumi et al., 1988; Loeb et al., 1991; Ibáñez et al., 1992) and mediate stereotypical NGF responses, such as neurite outgrowth (Loeb et al., 1991; Loeb and Greene, 1993; Peng et al., 1995) and growth cone turning (Galbo et al., 1997). The p75 neurotrophin receptor (p75NTR) was originally reported to function as a positive modulator of trkA activity (Benedetti et al., 1993; Barker and Shooter, 1994; Hantzopoulos et al., 1994; Verdi et al., 1994; Lachance et al., 1997). Recent experiments also indicate that p75NTR may play an autonomous signaling role (Dobrowsky et al., 1994; Carter et al., 1996; Casaccia-Bonnefil et al., 1996). Evidence is beginning to emerge that p75NTR plays a...
role in axonal growth responses. Disruption of NGF binding to p75NTR reduces, but does not inhibit, growth cone turning (Gallo et al., 1997), and a peptide analog of the cytoplasmic region of p75NTR modulates neurite outgrowth from PC12 cells (Dostaler et al., 1996). Targeted deletion of the p75NTR gene reduces neuronal sensitivity to NGF (Davies et al., 1993; Lee et al., 1994b) and perturbs the developmental innervation of selected peripheral targets by sympathetic axons (Lee et al., 1994a; Kawaja, 1998). The role of p75NTR in the collateral sprouting of mature sympathetic axons, however, is poorly understood.

Using transgenic mice that overexpress NGF among glial cells, we recently demonstrated that sympathetic axons form dense pericellular plexuses with only those trigeminal neurons that stain immunohistochemically for NGF (Walsh and Kawaja, 1998), indicating that sympathetic fibers selectively target a subpopulation of sensory somata that are NGF responsive. To provide added proof that this sprouting response is directional and specific, we have further characterized the neurochemical phenotype of those trigeminal neurons that exhibit sympathetic arborizations. Our results reveal that the majority of sympathetic plexuses forms around the trkA/calcitonin gene-related peptide (CGRP)-expressing population of sensory neurons in NGF transgenic mice. To investigate the role of p75NTR in this sympathetic sprouting response, we used a new line of transgenic mice that overexpresses comparable levels of glial NGF but lacks the functional expression of p75NTR (Coome et al., 1998). Specifically, the mice, but the directional growth of these collateral branches is perturbed in the absence of p75NTR expression. Specifically, the mice overexpress NGF among glial cells and a null mutation of p75NTR (Coome et al., 1998), (2) NGF/p75NTR−/− mice, which possess both an overexpression of NGF among glial cells and a null mutation of p75NTR (Coome et al., 1998), (3) C57Bl/6 mice, which are the backcross of the transgenic mice to C57Bl/6 and possess both trkA and CGRP immunoreactivities) for 2 hr at room temperature. For neuron counting, the ganglia were cut to completion, mounted on chrome alkum gelatin-coated slides, Nissl stained with thionin, dehydrated through a graded series of ethanol, cleared, and coverslipped for viewing under bright-field optics.

**Immunohistochemistry.** Sections of trigeminal ganglia were initially treated in 0.3% hydrogen peroxide (H2O2) in Tris-buffered saline (TBS), pH 7.4, for 30 min. They were then incubated in 10% bovine serum albumin (BSA) and 0.25% Triton X-100 in TBS. Endogenous avidin and biotin binding sites were blocked in two successive steps (Avidin-Biotin Blocking Kit, Vector Laboratories, Burlingame, CA). The sections were then incubated for 48 hr at room temperature in one of the following primary IgGs: sheep anti-rat tyrosine hydroxylase (TH) IgG (1:1000 dilution; Chemicon, Temecula, CA), rabbit anti-rat NGF IgG (1:1000; kindly provided by Dr. J. M. Conner) [for additional information concerning this antibody, see Conner et al. (1992), rabbit anti-synthetic trkA IgG (1:1000; Chemicon), and rabbit anti-synthetic CGRP IgG (1:1000; Chemicon). Primary antibodies were diluted in a standard solution containing 3% BSA and 0.25% Triton X-100 in TBS. For trkA immunohistochemistry, normal goat serum was used in place of BSA for all standard solutions. All control sections were processed in the absence of primary IgGs. After a rinse in TBS, the sections were incubated in the standard solution containing biotinylated rabbit anti-goat IgG (1:200; Vector Laboratories; for TH immunoreactivity) or biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories; for NGF, trkA, and CGRP immunoreactivities) for 2 hr at room temperature. They were then rinsed and incubated in avidin–biotin complex (Vector Laboratories) for 2 hr at room temperature and rinsed again. The sections were then reacted with a solution containing 0.05% diaminobenzidine (DAB) tetrahydrochloride, 0.04% nickel chloride, and 0.015% H2O2 in 0.1 M TBS. After the DAB reaction, sections were washed in TBS, dehydrated through a series of graded ethanol, coverslipped, viewed, and photographed under bright-field optics.

**Double-antigen immunohistochemistry.** To investigate whether TH- and NGF transgenic mice were trkA-positive and CGRP-positive, double-immunolabeling for TH and CGRP was performed, using the method of Levey et al. (1986). Briefly, sections were stained for the first antigen and reacted with DAB as the chromagen reagent, as described for single antigen immunohistochemistry. After the DAB reaction, sections were reacted in 0.3% H2O2 in TBS for 10 min to eliminate any remaining peroxidase activity. Sections were then stained for the second antigen as per single-antigen immunohistochemistry up to and including the incubation in the avidin–biotin complex. Sections were then rinsed in 0.01 M phosphate buffer, pH 6.6, for 15 min, and transferred to a solution containing 0.01% benzidine dihydrochloride (BDHC) and 0.025% sodium nitroferricyanide in 0.01 M phosphate buffer, pH 6.6. After 10 min, the reaction was initiated by adding H2O2 to a final concentration of 0.005% in fresh BDHC solution. The reaction was terminated after 5–10 min by rinsing with cold 0.1 M phosphate buffer, pH 6.6, and then rapidly dehydrated through a series of ethanol, coverslipped, viewed, and photographed under bright-field optics. This technique yields a diffuse, brown-colored reaction product for the first antigen, and a granular, blue-colored reaction product for the second antigen.

**Electron microscopy.** For electron microscopy, anesthetized NGF/p75NTR−/− mice (n = 2) and NGF/p75NTR+/− mice (n = 2) were perfused transcardially with a solution containing 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The trigeminal ganglia were dissected out, minced into smaller pieces, and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 2 hr at room temperature. The tissues were rinsed, dehydrated through a graded
series of methanols, cleared in propylene oxide, and embedded in a mixture of Araldite and Epon. Ultrathin sections of the tissues were cut on a Sorvall Ultramicrotome, and the sections were collected on copper grids, stained with uranyl acetate and lead citrate, and viewed and photographed in a Hitachi 7000 transmission electron microscope.

**Quantitative analysis.** For neuron counting, complete series of Nissl-stained trigeminal ganglia were taken from C57Bl/6 mice (n = 3), BALB/c mice (n = 4), NGF/p75

\[ \text{number of TH-wrapped neurons.} \]

were scored for the presence or absence of trkA or CGRP immunoreactivity. Neuronal profiles with pericellular TH-immunostained axons in sections 50 μm apart throughout the entire ganglia. Then, images of the ganglia were captured directly from the microscope at 40× objective using a Sony CCD color video camera and imported into an image analysis software package (Bioquant/TCW, R & M Biometrics, Nashville). The perimeter of nuclei profiles of randomly selected trigeminal neurons from each genotype were outlined manually using a computer mouse, and the computer measured the nuclear area. Diameter was subsequently determined on the assumption that the nuclei are approximately circular. Results were tested for significance by one-way ANOVA, and comparisons between groups were made using a post hoc Newman–Keuls test.

The proportion of TH-wrapped neurons that were trkA positive and CGRP positive was determined from sections of trigeminal ganglia, taken from NGF/p75

\[ \text{mice, and double-immunostained for TH/trkA and TH/CGRP, respectively. For TH/trkA-immunostained sections, TH-wrapped neurons were sampled from six sections (50 μm apart) of ganglia per animal (n = 3), for a total of 402 neurons; for TH/CGRP-immunostained sections, TH-wrapped neurons were sampled from four sections (100 μm apart) of ganglia per animal (n = 3), for a total of 317 neurons. Neuronal profiles with pericellular TH-immunostained axons were scored for the presence or absence of trkA or CGRP immunoreactivity, and the data were represented as a percentage of the total number of TH-wrapped neurons.} \]

The cell size of trigeminal neurons surrounded by TH–IR axons in NGF/p75

\[ \text{mice was quantified. Images were captured directly from the microscope at 40× objective using a Sony CCD color video camera and imported into an image analysis software package (as before). The perimeter of neuronal profiles with pericellular TH–IR axons was outlined manually using a computer mouse, and the computer measured the neuronal area. Diameter was subsequently determined on the assumption that the trigeminal neurons are approximately circular. Perimeters of TH–IR neurons were traced from six sections (100 μm apart) of ganglia per animal (n = 3), for a total of 160 neurons. To compare the cell size distribution of TH-wrapped neurons with the cell size distribution of the total trigeminal neuron population of C57Bl/6 and NGF/p75

\[ \text{mice, we used} \]

\[ \text{perimeters of randomly selected Nissl-stained neurons (displaying a prominent nucleolus) were traced from two sections (100 μm apart) of ganglia per animal (n = 3), for a total of 823 and 554 neurons, respectively. Cell sizes were plotted as relative frequencies, and statistical differences in the mean diameter of cell size distributions were determined using a one-way ANOVA, and comparisons between groups were made using a post hoc Newman–Keuls test.} \]

Sections of trigeminal ganglia stained immunohistochemically for TH were used to measure the area occupied by TH–IR axons within the ganglionrich peripheral ganglia of NGF/p75

\[ \text{mice, project only to those sensory neurons displaying NGF immunostaining (Walsh and Kawaja, 1998). We postulated that neurons exhibiting a TH–IR plexus must express the high molecular weight neurotrophin receptor trkA, because NGF responsiveness is dependent on expression of this receptor (Loeb et al., 1991; Loeb and Greene, 1993). To confirm this hypothesis, we used simultaneous double immunohistochemistry to demonstrate the coincidence of TH–IR sympathetic plexuses with trkA–IR trigeminal somata in sections of ganglia taken from NGF/p75

\[ \text{mice. Trigeminal neurons displaying a prominent TH–IR perineuronal plexus were scored for the presence or absence of trkA immunoreactivity (Fig. 1A,B). This analysis revealed that virtually all TH–IR perineuronal plexuses (98%; 394 of 402) surrounded neurons exhibiting trkA immunoreactivity; not all trkA-positive trigeminal neurons, however, were surrounded by TH–IR fibers. These results indicate that, in agreement with Davis et al. (1998), sympathetic axons are attracted specifically to trkA-expressing sensory neurons that appear to accumulate high levels of NGF within their cell bodies.} \]

Averill et al. (1995) determined that the majority (92%) of trkA-expressing DRG neurons co-express the neuropeptide CGRP in rats. Thus, we reasoned that CGRP immunoreactivity would also identify those trigeminal neurons exhibiting a TH-positive perineuronal plexus. The proportion of TH-wrapped neurons showing CGRP immunoreactivity was determined using sections of trigeminal ganglia of NGF/p75

\[ \text{mice double-immunostained for TH and CGRP. Many sensory neurons surrounded by TH–IR fibers possessed variable intensities of CGRP immunoreactivity (Fig. 1C–G); not all CGRP–IR sensory neurons, however, were surrounded by sympathetic fibers. Quantitative analysis revealed that most TH–IR plexuses (86%; 273 of 317) were associated with sensory somata immunopositive for CGRP; no distinction was made between those somata possessing weak to strong immunolabeling for this neuropeptide. Interestingly, a small but significant number of TH–IR perineuronal plexuses (14%; 44 of 317) formed around sensory somata that displayed no detectable immunoreactivity for CGRP (Fig. 1H). These findings demonstrate that sympathetic axons selectively target the trkA-positive/CGRP-positive population of trigeminal sensory neurons, presumably those having unmyelinated axons and subserving nociception (Lawson, 1992). To further characterize the population of trigeminal neurons that exhibit a TH–IR perineuronal plexus, we determined the size/frequency distribution of both the trigeminal neuron population and those neurons enveloped by TH–IR axons in NGF/p75

\[ \text{mice (Fig. 2). The diameter of trigeminal neurons of C57Bl/6 mice ranged from 10 to 43 μm, with a mean diameter of 20.0 ± 5.0 μm. The diameter of trigeminal neurons of NGF/p75

\[ \text{mice ranged from 11 to 41 μm, with a mean diameter of 23.0 ± 5.5 μm; the mean diameter of trigeminal neurons of NGF/p75

\[ \text{mice is significantly larger than that of C57Bl/6 mice (p < 0.001; Newman–Keuls test), suggesting a neuronal hypertrophy in response to elevated levels of NGF (see below). Neurons exhibiting a TH–IR perineuronal plexus predominantly had large diameters ranging from 22 to 50 μm, with a mean diameter of 35.1 ± 5.4 μm. This increase in the mean diameter of TH-wrapped neurons was found to be statistically significant when compared with the mean diameter of the whole trigeminal neuron population of NGF/p75

\[ \text{mice (p < 0.001). It should be noted that the diameters of TH-wrapped neurons presented here are likely a slight overestimation of the true diameter, because the TH–IR fibers surrounding these neurons obscured the edge of the}} \]
Figure 1. A, B, Sections of trigeminal ganglia from NGF/p75<sup>+/−</sup> mice double-immunostained for trkA (brown reaction product) and TH (blue reaction product). Virtually all (98%) sympathetic perineuronal plexuses are associated with trkA-positive neurons (arrows). Not all trkA-positive somata, however, exhibit a TH-IR perineuronal plexus. C–H, Sections of trigeminal ganglia from NGF/p75<sup>+/−</sup> mice double-immunostained for CGRP (blue reaction product) and TH (brown reaction product). The majority (86%) of TH-IR perineuronal plexuses is associated with CGRP-IR sensory neurons. Some somata displaying a TH-IR perineuronal plexus are intensely immunoreactive for CGRP (arrows), whereas others show weak CGRP immunoreactivity (double arrows). A smaller percentage (14%) of TH-IR perineuronal plexuses is associated with somata displaying no detectable CGRP immunoreactivity (large arrowhead). Scale bars, 50 μm.
small number of TH–IR axons was observed along the ganglionic capsule and associated with blood vessels, forming perivascular networks (Fig. 3A,B). A sparse population of TH–IR neuronal cell bodies was also observed, representing local dopaminergic sensory neurons (Katz et al., 1983; Price and Mudge, 1983). As we have documented previously (Walsh and Kawaja, 1998), numerous TH–IR varicose axons were found throughout the neuronal regions of the ganglia from NGF/p75<sup>+/−</sup> mice (Fig. 3C,E). These immunoreactive fibers, which appeared to enter the ganglionic environment from the trigeminal nerve or by departing from local blood vessels, did not project randomly among the sensory somata. Rather, these fibers appeared to grow specifically toward a subset of trigeminal somata. At high magnification, several TH–IR fibers often converged on a single soma and commenced wrapping around the perimeter of the perikaryon, thereby enveloping the soma in a complete perineuronal plexus of noradrenergic fibers (Fig. 4A,C,E). After the removal of the ipsilateral SCG, all TH–IR plexuses and the vast majority of fibers were no longer evident, thus confirming that these new fibers were sympathetic in origin.

Trigeminal ganglia of NGF/p75<sup>−/−</sup> mice also possessed a robust ingrowth of new TH–IR fibers. Similar to the TH–IR fibers in ganglia of NGF/p75<sup>+/−</sup> mice, TH–IR axons in NGF/p75<sup>−/−</sup> mice were varicose and were again lost after ipsilateral SCG removal. What was most striking about the appearance of TH–IR sympathetic axons in the ganglia of NGF/p75<sup>−/−</sup> mice was an impression of randomness, or disorganization, in the pattern of sprouting (Fig. 3D,F). The directional growth of sympathetic axons toward a subset of trigeminal sensory somata observed in NGF/p75<sup>+/−</sup> animals was not evident in the ganglia of NGF/p75<sup>−/−</sup> animals. Instead, TH–IR axons invading the ganglionic neuropil of NGF/p75<sup>−/−</sup> mice appeared to weave in and around individual somata, rather than becoming associated with one particular neuron cell body. It should be noted that the formation of TH–IR perineuronal plexuses was not fully inhibited, because a few TH–IR sympathetic fibers were observed to envelop somata in the trigeminal ganglia of NGF/p75<sup>−/−</sup> mice (Fig. 4B,D,F). The morphology of these plexuses in NGF/p75<sup>−/−</sup> mice, however, was markedly different from the perineuronal plexuses seen in NGF/p75<sup>+/−</sup> mice: (1) a smaller number of TH–IR fibers appeared to contribute to the perineuronal plexus, and (2) complete envelopment of the sensory somata by TH–IR axons was rarely observed. Consistent with this, fewer sympathetic perineuronal plexuses form in the DRG of p75<sup>−/−</sup> mice after peripheral nerve axotomy (Ramer and Bisby, 1997b).

To confirm that there were indeed fewer sympathetic axons contributing to the perineuronal plexuses in NGF/p75<sup>−/−</sup> mice, we examined trigeminal ganglia taken from both transgenic lines of mice at the electron microscope level. Sensory neurons, examined in NGF/p75<sup>+/−</sup> and NGF/p75<sup>−/−</sup> mice, were closely apposed by the surrounding satellite cell processes. In the ganglia of NGF/p75<sup>+/−</sup> mice, a small population of somata had bundles of unmyelinated fibers with axonal swellings filled with clear vesicles immediately adjacent to the plasma membrane of the perikaryon (Fig. 5A,B); the ultrastructural appearance of these axons intimately associated with a small number of cell bodies correlates with the observation of TH–IR axons with varicosities surrounding a subset of trigeminal neurons at the light microscope level. These bundles of unmyelinated axonal profiles were evident around the entire surface of the cell body, again resembling that appearance of TH–IR axons enveloping the entire soma. On closer examination, nonmyelinating Schwann cell processes were...
seen ensheathing these axons (Fig. 5A), in a manner reminiscent of that seen in peripheral nerves. These bundles of unmyelinated axons and their glial ensheathment were separate from the outer satellite cell processes; these ultrastructural features of sympathetic axons enveloping sensory somata concur with those reported by Davis et al. (1994) and Chung et al. (1997). In the ganglia of NGF/p75−/− mice, such clusters of unmyelinated axons within the interstitial space between neurons and satellite cells

Figure 3. Sections of trigeminal ganglia from C57Bl/6, BALB/c, NGF/p75+/−, and NGF/p75−/− mice, stained immunohistochemically for TH. The trigeminal ganglia of C57Bl/6 (A) and BALB/c (B) mice possess TH-IR sympathetic fibers forming perivascular plexuses (arrow), as well as a small population of TH-immunopositive dopaminergic sensory neurons. Many TH-IR fibers are seen in ganglia of NGF/p75+/− mice, projecting to a subset of trigeminal somata and enveloping them in a tight perineuronal plexus of fibers (C, E). TH-IR fibers are also present in ganglia of NGF/p75−/− mice, but the pattern of sprouting appears more random and TH-IR plexuses appear less prominent (D, F). Scale bars: A–D, 125 μm; E, F, 50 μm.
were not observed (Fig. 5C,D). Rather, only a few single unmyelinated axons were seen embedded within the processes of satellite cells; axonal swellings were rarely observed. Thus, the observation that TH–IR perineuronal plexuses in NGF/p75$^{-/-}$ mice appeared “less intense” at the light microscope level is a consequence of fewer unmyelinated axons contributing to the formation of these plexuses, and not attributable to reduced levels of tyrosine hydroxylase in p75$^\text{NTR}$-deficient sympathetic axons. Taken together, our findings indicate that the mechanism by which sympathetic axons form perineuronal plexuses in the tri-

Figure 4. Examples of TH–IR perineuronal plexuses in trigeminal ganglia from NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice. In NGF/p75$^{+/+}$ animals (A, C, E), several TH–IR fibers often converge on the soma of individual sensory neurons, enveloping the perimeter in a tight, dense plexus of fibers. In NGF/p75$^{-/-}$ mice (B, D, F), TH–IR fibers randomly grow within the neuropil, weaving around the cell bodies of sensory neurons, sometimes in close contact with the somata. However, intense perineuronal plexuses that tightly envelop the somata of NGF/p75$^{-/-}$ ganglia are rarely seen. Scale bar, 25 μm.
Figure 5. Electron photomicrographs of trigeminal ganglia from NGF/p75

\(^{+/+}\) and NGF/p75 \(^{-/-}\) mice. Sensory somata in NGF/p75 \(^{+/+}\) mice (A, B) are surrounded by satellite cell processes closely apposed to their outer cell membrane (S\(_1\)). Some sensory cell bodies (S\(_2\)) have many clusters of small-diameter unmyelinated axons (stars) immediately adjacent to their plasma membrane. Occasionally, axonal swellings filled with clear vesicles (Ax) are also seen among the bundles of axons. These unmyelinated axons are ensheathed by nonmyelinating Schwann cells (Sc). Sensory somata in NGF/p75 \(^{-/-}\) mice (C, D) are likewise surrounded by satellite cell processes closely apposed to their outer cell membrane (S\(_1\) and S\(_2\)). Although small-diameter unmyelinated axons (stars) are also associated with the sensory somata of NGF/p75 \(^{-/-}\) mice, these fibers are fewer in number, do not occur in clusters, and rarely display axonal swellings filled with clear vesicles. Furthermore, these axons are not ensheathed by nonmyelinating Schwann cells but rather appear to be embedded within the processes of satellite cells. Arrowheads indicate intercellular space between S\(_1\) and S\(_2\), which is filled with collagen fibers. Scale bars, 1 \(\mu m\).
gemineral ganglia is perturbed in the absence of p75\textsuperscript{NTR}. Examination of trigeminal ganglia taken from NGF/p75\textsuperscript{-/-} mice, double-immunostained for TH and CGRP, revealed that those few perineuronal plexuses that do form are mostly associated with CGRP-positive trigeminal neurons (data not shown), similar to that seen in NGF/p75\textsuperscript{+/-} mice. These data indicate that the absence of p75\textsuperscript{NTR} does not perturb the specificity of sympathetic axons for a particular subset of trigeminal neurons, but rather reduces the capability of sympathetic axons to locate the appropriate trigeminal cells.

As mentioned previously, the trigeminal ganglia of both lines of NGF transgenic mice have a greater density of TH–IR sympathetic fibers than the ganglia of C57Bl/6 mice. Despite a reduced capability of sympathetic axons projecting toward primary sensory neurons in NGF/p75\textsuperscript{-/-} mice, there was an apparent increase in the density of sympathetic sprouting in the fiber tracts of these animals, in comparison to NGF/p75\textsuperscript{+/-} mice. As seen in the neuropil, only a few TH–IR axons were evident in the fibers tracts of C57Bl/6 mice (Fig. 6A); these likely originated from the intrinsic dopaminergic population of trigeminal neurons. The fiber tract portions of NGF/p75\textsuperscript{+/-} mice displayed a marked increase in the number of TH–IR fibers (Fig. 6B), relative to the ganglia of C57Bl/6 mice. These TH–IR sympathetic fibers were varicose in appearance and coursed in a parallel arrangement to the intrinsic sensory fibers. The density of TH–IR axons was further increased in the fiber tracts of the trigeminal ganglia taken from NGF/p75\textsuperscript{-/-} mice (Fig. 6C). To confirm these increases in sympathetic sprouting, we measured the percentage area occupied by TH–IR axons within the fiber tract portions of trigeminal ganglia (Fig. 6D). This analysis revealed that the mean percentage area occupied by sympathetic axons in the ganglia of NGF/p75\textsuperscript{-/-} mice (9.6 \pm 4.7\%) was significantly higher than that measured in the ganglia of NGF/p75\textsuperscript{+/-} mice (3.8 \pm 2.1\%; \( p < 0.001\), Newman–Keuls test), which in turn was significantly elevated over that seen in the ganglia of wild-type C57Bl/6 mice (0.3 \pm 0.3\%; \( p < 0.001\)).

Localization and detection of NGF in trigeminal ganglia

To localize NGF among sensory neurons, NGF immunostaining was performed on slides having sections of trigeminal ganglia from each of C57Bl/6, NGF/p75\textsuperscript{+/-}, and NGF/p75\textsuperscript{-/-} mice. In C57Bl/6 mice, few neurons stained immunohistochemically for NGF, and the intensity of immunostaining among these neurons was weak (Fig. 7A). This observation may reflect a difficulty in immunohistochemical detection of the small amount of NGF that is retrogradely transported by normal trigeminal neurons. In contrast, the overexpression of NGF in NGF/p75\textsuperscript{+/-} mice resulted in a dramatic increase in the staining intensity of NGF among a subpopulation of neurons (Fig. 7B). It should be noted that NGF immunoreactivity in these neurons is likely attributable to retrograde transport of NGF from distal sites and not to endogenous production by the neurons themselves (our unpublished data; \textit{in situ} hybridization). In NGF/p75\textsuperscript{-/-} mice, many trigeminal neurons also possessed NGF immunoreactivity, but the intensity of immunostaining was intermediate to that seen in the ganglia of C57Bl/6 and NGF/p75\textsuperscript{+/-} mice (Fig. 7C). It may be possible that the functional expression of p75\textsuperscript{NTR} is necessary for the proper immunolocalization of NGF within neuronal cell bodies; this idea is supported by the fact that we have observed similar reductions in NGF immunostaining among other NGF-responsive neuronal populations (e.g., sympathetic neurons of the superior cervical ganglion and cholinergic neurons of the medial septum; our unpublished observations) in NGF/p75\textsuperscript{-/-} mice.

Previous work has demonstrated a correlation between NGF protein levels and the density of sympathetic sprouting (Campenot, 1982; Isaacon et al., 1997). Therefore we examined the levels of NGF protein in trigeminal ganglia of C57Bl/6, NGF/p75\textsuperscript{+/-}, and NGF/p75\textsuperscript{-/-} mice using a two-site ELISA (Fig. 7D). In contrast to the low levels of NGF protein detected in the trigeminal ganglia of control C57Bl/6 mice (35.4 \pm 5.2 pg NGF/ganglion; \( n = 5\)), levels in the ganglia of NGF/p75\textsuperscript{+/-} mice (269.4 \pm 43.4; \( n = 8\)) were significantly higher (\( p < 0.01\), Newman–Keuls test). Levels of NGF protein in NGF/p75\textsuperscript{-/-} mice (121.9 \pm 59.9; \( n = 8\)) were also increased significantly above that in C57Bl/6 mice (\( p < 0.01\)) but were significantly lower than levels detected in NGF/p75\textsuperscript{+/-} mice (\( p < 0.001\)). It is unlikely that this discrepancy in NGF levels between both transgenic lines of mice is caused by a decreased retrograde transport of NGF among p75-deficient sensory neurons, because Curtis et al. (1995) reported that an absence of p75\textsuperscript{NTR} expression does not affect the transport of NGF among DRG neurons in p75\textsuperscript{-/-} mice. Alternative explanations may include differing rates of degradation of NGF among sensory neurons and/or NGF uptake and retrograde transport away from the ganglia by the greater number of sympathetic axons in the ganglia of transgenic mice lacking p75\textsuperscript{NTR} expression. The most important observation, however, remains that total ganglionic levels of NGF protein of both NGF/p75\textsuperscript{+/-} and NGF/p75\textsuperscript{-/-} mice are higher relative to C57Bl/6 mice.

Numbers of trigeminal ganglionic neurons

To ensure that differences in NGF levels are not attributed to concomitant changes in the numbers of trigeminal ganglionic neurons, we assessed the population sizes of C57Bl/6, NGF/p75\textsuperscript{+/-}, and NGF/p75\textsuperscript{-/-} mice; trigeminal ganglia from BALB/c and p75\textsuperscript{-/-} mice were also assessed as additional controls (Table 1). Because the average nuclear diameter of trigeminal neurons from all five genotypes of mice did not exceed 16 \( \mu m\), our sampling frequency of sections every 50 \( \mu m\) ensured that neurons were not counted twice, and thus no corrections of neuron numbers were made. Both C57Bl/6 and NGF/p75\textsuperscript{+/-} mice (as well as BALB/c mice) had similar numbers of trigeminal neurons. NGF/p75\textsuperscript{-/-} mice, however, possessed a 15\% reduction in the number of trigeminal neurons, in comparison to the three aforementioned genotypes (\( p < 0.05\), Newman–Keuls test). A greater decrease in neuron number was observed among p75\textsuperscript{-/-} mice, with a 30\% reduction in comparison to BALB/c (control) mice (\( p < 0.001\)). These results are in agreement with other investigations that have suggested that p75\textsuperscript{-/-} mice exhibit a dramatic loss of dorsal root sensory neurons (Lee et al., 1992; Bergmann et al., 1997; Stucky and Koltzenburg, 1997). From these quantitative data it is evident that NGF/p75\textsuperscript{-/-} mice display only a modest reduction in neuron number, as compared with NGF/p75\textsuperscript{+/-} mice, and this loss cannot solely account for the discrepancies in ganglionic levels of NGF between the two transgenic genotypes. Using unbiased quantitative methods, NGF/p75\textsuperscript{+/-} and NGF/p75\textsuperscript{-/-} mice display similar numbers of SCG neurons, both of which are increased in comparison to that determined from C57Bl/6 mice (our unpublished data). Thus, the discrepancies observed in the sprouting responses by sympathetic axons in the trigeminal ganglia of NGF/p75\textsuperscript{+/-} and NGF/p75\textsuperscript{-/-} mice cannot be attributed to differences in neuron numbers of the SCG.
Neurochemical organization of the trigeminal ganglia

There are several lines of evidence that suggest that the trkA-expressing subpopulation of sensory neurons plays a critical role in the development of sympathosensory sprouting. First, this sprouting response is correlated with a rise in NGF levels within sensory ganglia, which, as our data suggests, occurs as a result of an increased retrograde transport of NGF among trkA-expressing neurons. Second, sympathetic axons selectively target the trkA-/CGRP-IR population of sensory neurons. Third, in NGF/p75<sup>−/−</sup> mice, few if any sympathetic perineuronal plexuses form in the

Figure 6. Sections of trigeminal ganglia from C57Bl/6, NGF/p75<sup>+/+</sup>, and NGF/p75<sup>−/−</sup> mice, stained immunohistochemically for TH, and a bar graph depicting the density of TH-IR axons in the ganglia of these three mouse genotypes. In the trigeminal fiber tracts of C57Bl/6 mice (A), few if any TH-IR axons are evident. In marked contrast, the trigeminal fiber tracts of both NGF/p75<sup>+/+</sup> mice (B) and NGF/p75<sup>−/−</sup> mice (C) possess numerous TH-IR axons coursing in parallel arrangement with the intrinsic sensory fibers. Note the apparent increase in the number of TH-IR axons in fiber tracts of NGF/p75<sup>−/−</sup> mice relative to NGF/p75<sup>+/+</sup> mice. Scale bar, 100 μm. Quantitation of the percentage area occupied by TH-IR axons in trigeminal fiber tracts (D) confirms an increase in the density of TH-IR axons in the fiber tracts of both NGF/p75<sup>+/+</sup> and NGF/p75<sup>−/−</sup> mice, relative to C57Bl/6 mice (*p < 0.001). The density of TH-IR axons in the fiber tracts of NGF/p75<sup>−/−</sup> mice is also significantly higher than that in NGF/p75<sup>+/+</sup> mice (**p < 0.001). Error bars represent SDs.
nodose ganglia (our unpublished data), which contains only a small number of trkA-expressing visceral somata (Wetmore and Olson, 1995). To exclude the possibility that the perturbed pattern of sympathetic sprouting seen in NGF/p75^{−/−} mice is caused by a selective loss of the trkA- and CGRP-expressing population of sensory neurons, we examined trkA and CGRP immunohistochemistry in trigeminal ganglia of C57Bl/6, NGF/p75^{+/+}, and NGF/p75^{−/−} mice. Many trkA-IR neurons of various sizes were clearly evident in trigeminal ganglia of all mouse genotypes (Fig. 8A,C,E). Likewise, CGRP-IR trigeminal neurons were seen in

Figure 7. Sections of trigeminal ganglia from C57Bl/6, NGF/p75^{+/+}, and NGF/p75^{−/−} mice, stained immunohistochemically for NGF, and a bar graph depicting the levels of NGF protein in the ganglia of these three mouse genotypes. In C57Bl/6 mice (A), few NGF-positive somata are detected. In contrast, many somata in trigeminal ganglia of NGF/p75^{+/+} mice (B) display strong immunostaining for NGF. Numerous somata in the trigeminal ganglia of NGF/p75^{−/−} mice (C) also display NGF immunoreactivity, but the intensity of immunostaining is moderate as compared with that in C57Bl/6 and NGF/p75^{+/+} mice. Scale bar, 50 μm. Mean levels of NGF protein in the trigeminal ganglia of C57Bl/6 (n = 5), NGF/p75^{+/+} (n = 8), and NGF/p75^{−/−} (n = 8) mice were measured by a two-site ELISA (D). This analysis reveals that trigeminal ganglia of NGF/p75^{+/+} and NGF/p75^{−/−} mice both possess significantly higher levels of NGF protein than trigeminal ganglia of age-matched C57Bl/6 mice (∗p < 0.01). The amount of NGF protein in trigeminal ganglia of NGF/p75^{−/−} mice is significantly reduced from that of NGF/p75^{+/+} mice (∗∗p < 0.001). Error bars represent SDs.
ganglia of all mouse genotypes, with a neuronal distribution similar to that of trkA (Fig. 8B,D,F). Qualitatively, the staining intensity for both trkA and CGRP among trigeminal neurons appeared comparable in NGF/p75+/+ and NGF/p75−/− mice. Thus, a lack of p75NTR expression markedly affects the pattern and distribution of sympathetic sprouting, in the absence of a selective loss of trkA- and CGRP-expressing neurons, that population of trigeminal sensory neurons specifically targeted by the invading sympathetic axons.

DISCUSSION

Specificity of sympathosensory projections

The occurrence of sympathosensory sprouting has now been reported in the spinal ganglia after peripheral nerve injury (McLachlan et al., 1993; Chung et al., 1996; Zhou et al., 1996; Ramer and Bisby, 1997a) and in the trigeminal ganglia as a consequence of transgenic overexpression of NGF in the target tissues of sensory neurons (Davis et al., 1994; Walsh and Kawaja, 1998). The presence of elevated NGF levels in the spinal ganglia after sciatic nerve injury (Herzberg et al., 1997) and in the undamaged trigeminal ganglia of NGF transgenic mice (Davis et al., 1994; Walsh and Kawaja, 1998) suggests that sympathosensory sprouting is an NGF-dependent phenomenon. In further support of this idea, we have reported previously that sympathetic axons invading the trigeminal ganglia of NGF/p75+/+ mice preferentially associate with a small number of sensory neurons displaying NGF immunoreactivity (Walsh and Kawaja, 1998). This finding also indicates that sympathetic axons specifically target that subgroup of neurons that retrogradely transport and accumulate NGF: the trkA-expressing neurons. To confirm this hypothesis, we determined that virtually all (98%) perineuronal plexuses of sympathetic fibers are found surrounding trkA-IR sensory somata in NGF/p75+/+ mice; these findings are in agreement with those of Davis et al. (1998). The observation that sympathetic axons are attracted exclusively toward trkA-expressing somata implies a central role for these neurons in the mechanism underlying sympathosensory sprouting. We speculate that trkA-expressing sensory neurons, which bind and retrogradely transport target-derived NGF, are responsible for delivering NGF to the ganglionic environment, which in turn initiates the directional ingrowth of sympathetic axons.

Our results further showed that the majority (86%) of sympathetic plexuses in NGF/p75+/+ mice form around trigeminal somata possessing CGRP immunoreactivity. The co-occurrence of trkA and CGRP in most TH-wrapped neurons is not unexpected because these two neural antigens are known to colocalize within the same subset of sensory neurons (Verge et al., 1992; Averill et al., 1995), which are typically small-diameter cells with unmyelinated axons, subserving nociception (Lawson, 1992; Snider and McMahon, 1998). Because trkA- and CGRP-expressing neurons are normally small in size, it is surprising that those trigeminal neurons displaying sympathetic plexuses have predominantly large diameters. As well, studies of nerve-injured rats have demonstrated that sympathetic axons preferentially wrap large-diameter DRG neurons (McLachlan et al., 1993; Chung et al., 1996). One explanation that reconciles these data is that large-diameter sensory neurons, which do not normally express trkA, begin to express this receptor de novo, as has been proposed to occur in adjuvant-induced models of inflammation (Woolf, 1996). Alternatively, small-diameter sensory neurons, which normally express trkA (McMahon et al., 1994), hypertrophy in response to the high levels of NGF in NGF/p75+/+ mice. Support for this idea comes from studies showing that NGF causes an increase in soma size among trkA-expressing neuronal populations, both peripherally and centrally (Gage et al., 1989; Higgins et al., 1989; Ruit et al., 1990; Goodness et al., 1997). Furthermore, Chung et al. (1996) reported that shortly after spinal nerve ligation, sympathetic axons associate with small- and large-diameter somata, whereas at longer postoperative periods, sympathetic fibers preferentially associate with only large-diameter somata. These authors also proposed neuronal hypertrophy as a possible explanation for their results. It seems likely, therefore, that NGF-induced neuronal hypertrophy of small-diameter neurons, rather than a phenotypic alteration, explains the presence of trkA (and CGRP) within large-diameter sensory somata exhibiting a perineuronal plexus of sympathetic fibers. Taken together, sympathetic axons are attracted to sensory neurons, which all display a common responsiveness to the neurotrophin NGF, and the majority produce the neuropeptide CGRP.

Our results also show that a small but significant number (~14%) of sympathetic plexuses are associated with sensory neurons lacking detectable CGRP immunoreactivity. This raises the possibility that the second population of trkA-IR neurons that attract sympathetic axons are those that are normally referred to as “large light,” having myelinated axons presumably connected to mechanosensitive endings (Lawson, 1992). In support of this notion, a subgroup of large-diameter sensory neurons in the undamaged DRG of rats (5% of the total population) display high-affinity binding sites for NGF but no CGRP immunoreactivity (Verge et al., 1989).

Role of p75NTR in sympathosensory sprouting

It is well documented that sympathetic axons sprout into tissues displaying high concentrations of NGF, as a result of site-directed expression (Edwards et al., 1989; Albers et al., 1994; Hassankhani et al., 1995; Kawaja and Crutcher, 1997) and damage- or disease-induced production (Crutcher, 1987; Aloe et al., 1992b; Zettler and Rush, 1993). Sympathetic axons ramify throughout these NGF-rich tissues giving the appearance of a diffuse network of fibers. Sympathetic axons also invade the NGF-rich trigeminal ganglia of transgenic mice overexpressing NGF, but unlike the random pattern of growth seen in other NGF-rich tissues, sympathetic axons display a cellular specificity for certain neurons within sensory ganglia. In the absence of p75NTR expression, we have shown that the bulk growth of sympathetic axons can still occur into the NGF-rich trigeminal ganglia of NGF transgenic mice, but the ability of sympathetic axon collaterals to wrap individual neurons is perturbed. How does the loss of p75NTR result in an alteration of NGF-induced sympathosensory sprouting?
Because trkA-expressing sensory neurons have a central role in sympathetic sprouting, the alteration of sympathetic sprouting observed in NGF/p75<sup>−/−</sup> mice could be an indirect consequence of the role of p75 NTR in the survival of trkA-expressing neurons. Recent experiments with p75 NTR-deficient mice have suggested a dramatic loss of sensory DRG neurons (Lee et al., 1992; Stucky and Koltzenburg, 1997). This cell loss, however, is apparently nonselective for trkA-expressing sensory neurons (Bergmann et al., 1997). Quantitative data from this investigation demonstrate that NGF/p75<sup>−/−</sup> mice have only a 15% reduction in the number of trigeminal neurons relative to NGF/p75<sup>+/+</sup> mice, suggesting that the overexpression of NGF can ameliorate the survival of sensory neurons in mice lacking functional expression of p75<sup>NTR</sup>. Furthermore, our results reveal a similar distribution and staining intensity of trkA-immunoreactive neurons in the trigeminal ganglia of NGF/p75<sup>+/+</sup> and NGF/p75<sup>−/−</sup> mice. Thus, the loss of sensory neurons attributable to the absence of p75<sup>NTR</sup> expression is minimized in NGF/p75<sup>−/−</sup> mice and is likely not a determinant of the alteration in the sympathetic sprouting response.

Detection of NGF protein, using ELISA, reveals half the total level of NGF in ganglia of NGF/p75<sup>−/−</sup> mice as compared with NGF/p75<sup>+/+</sup> mice. Does a lack of p75<sup>NTR</sup> affect the delivery of NGF to the ganglionic environment by trkA-expressing trigeminal neurons? p75<sup>NTR</sup> can increase the amount of NGF that becomes bound to the trkA receptor, particularly at low ligand concentrations.
concentrations (Barker and Shooter, 1994; Mahadeo et al., 1994), a function that may enhance the initial binding and subsequent internalization of NGF at distal sensory axons in normal target tissues. Such a role for \(p75^{NTR}\) on sensory axons in NGF transgenic mice may be irrelevant because NGF levels are expected to be high near distal axons of trkA-expressing sensory neurons [trigeminal axons invade the cerebellum of NGF/p75\(^{+/+}\) and NGF/p75\(^{-/-}\) mice, which both have 20-fold higher NGF levels (Kawaja et al., 1997; Coome et al., 1998)]. Furthermore, the retrograde transport of \(^{125}\)I-NGF in sensory neurons is not affected in p75\(^{NTR}\)-deficient mice (Curtis et al., 1995). Thus, it is doubtful that the perturbed patterns of sympathosensory sprouting in NGF/p75\(^{-/-}\) mice is a consequence of an altered ability for the retrograde transport of NGF in p75\(^{NTR}\)-deficient sensory neurons.

It is most likely that p75\(^{NTR}\) exerts its effects on sympathosensory sprouting at the level of the sympathetic growth cone. Sympathetic axons invading the ganglionic environment turn toward NGF-IR sensory somata (Walsh and Kawaja, 1998); this pattern of sprouting is reminiscent of growth cone turning responses to substratum-bound NGF in vitro (Letourneau, 1978; Gallo et al., 1997). Our finding that sympathetic pericellular plexuses form less often in NGF/p75\(^{-/-}\) mice parallels the observation that p75\(^{NTR}\) function-blocking antibodies reduce, but do not inhibit, the turning response of growth cones to NGF-coated beads. Inhibitors of trkA signaling, however, totally block this response (Gallo et al., 1997). Thus, p75\(^{NTR}\) appears to enhance NGF-induced local guidance of elongating axons. The mechanism by which p75\(^{NTR}\) acts to positively modulate NGF-induced axonal morphogenesis most likely involves altering the conformation of trkA into a high-affinity state through direct receptor interactions (Mahadeo et al., 1994; Ross et al., 1996, 1998), thereby enhancing trkA-mediated growth cone turning. The ability of p75\(^{NTR}\) to increase the NGF/trkA association rate (Mahadeo et al., 1994) may be particularly important in allowing growth cones to discriminate gradients of NGF, as may be present in the trigeminal ganglia of NGF transgenic mice. p75\(^{NTR}\) expression on glial cells may also serve to enhance NGF gradients near the site of NGF release, namely trkA-expressing sensory neurons. Recently, Zhou et al. (1996) reported that sympathetic pericellular plexuses in the DRG of nerve-injured rats were associated with p75\(^{NTR}\)-IR glial cells. These authors proposed that p75\(^{NTR}\) may act as a presenting molecule for NGF to nearby sympathetic sprouts (also see Taniuchi et al., 1986; Johnson et al., 1988).

Last, our data show that despite lower levels of ganglionic NGF, NGF/p75\(^{-/-}\) mice have a greater density of sympathetic axons invading their trigeminal ganglia, as compared with NGF/p75\(^{+/+}\) mice. These findings are not consistent with previous investigations correlating an increased density of sympathetic fibers with increasing concentrations of NGF (Korschning and Thoenen, 1977; Campenot, 1982; Sheltton and Reichardt, 1984; Isaacson et al., 1997). The reason a greater density of sympathetic axons is observed in NGF/p75\(^{-/-}\) mice is not clear. It is unlikely that this increase in sympathetic sprouting is related to an enhanced number of sympathetic neurons in NGF/p75\(^{-/-}\) mice, because unbiased neuron counting reveals comparable numbers of SCG neurons in both NGF/p75\(^{+/+}\) and NGF/p75\(^{-/-}\) mice (our unpublished observations). Rather, the coexpression of trkA and p75\(^{NTR}\) may attenuate NGF-induced, trkA-mediated axon elongation by sympathetic neurons. In support of this, brain-derived neurotrophic factor (BDNF) activation of p75\(^{NTR}\) reduces NGF-induced trkA tyrosine phosphorylation (MacPhee and Barker, 1997) and sympathetic neuron survival (Bamji et al., 1998) through signaling pathways that involve ceramide production and c-jun phosphorylation, respectively. In both experiments, however, BDNF activation of p75\(^{NTR}\) negatively modulated trkA function only at low NGF concentrations, and hence, it is unclear whether such a mechanism could account for the increased sprouting of sympathetic axons in NGF-overexpressing mice that lack p75\(^{NTR}\). It will be interesting to test this hypothesis in other models of NGF-induced sprouting of sympathetic axons.

REFERENCES


