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Enhanced Neurotrophin-Induced Axon Growth in Myelinated Portions of the CNS in Mice Lacking the p75 Neurotrophin Receptor

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Axonal growth in the adult mammalian CNS is limited because of inhibitory influences of the glial environment and/or a lack of growth-promoting molecules. Here, we investigate whether supplementation of nerve growth factor (NGF) to the CNS during postnatal development and into adulthood can support the growth of sympathetic axons within myelinated portions of the maturing brain. We have also asked whether $p75^{NTR}$ plays a role in this NGF-induced axon growth. To address these questions we used two lines of transgenic mice overexpressing NGF centrally, with or without functional expression of $p75^{NTR}$ (NGF/$p75^{+/+}$ and NGF/$p75^{-/-}$ mice, respectively). Sympathetic axons invade the myelinated portions of the cerebellum, beginning shortly before the second week of postnatal life, in both lines of NGF transgenic mice. Despite the presence of central myelin, these sympathetic axons continue to sprout and increase in density between postnatal days 14 and 100, resulting in a dense plexus of sympathetic fibers within this myelinated environment. Surprisingly, the growth response of sympathetic fibers into the cerebellar white matter of NGF/$p75^{-/-}$ mice is enhanced, such that both the density and extent of axon ingrowth are increased, compared with age-matched NGF/$p75^{+/+}$ mice. These dissimilar growth responses cannot be attributed to differences in cerebellar levels of NGF protein or sympathetic neuron numbers between NGF/$p75^{+/+}$ and NGF/$p75^{-/-}$ mice. Our data provide evidence demonstrating that growth factors are capable of overcoming the inhibitory influences of central myelin in the adult CNS and that neutralization of the $p75^{NTR}$ may further enhance this growth response.

Key words: nerve growth factor; p75 neurotrophin receptor; myelin; axon growth; sympathetic; cerebellum

One of the greatest obstacles that hampers restoration of neural function after injury is the lack of sustainable axon regrowth in myelinated structures of the adult mammalian CNS. Myelin produced by oligodendrocytes in the brain and spinal cord can inhibit axon growth both *in vitro* and *in vivo* (Caroni and Schwab, 1988a; Crutcher, 1989; Schnell and Schwab, 1990; Schwab, 1990; Bregman et al., 1995; Lozano et al., 1995; Schwab and Brösamle, 1997). Glial scars, which form shortly after the integrity of the brain or spinal cord has been breached, serve as both a physical (Reier and Houle, 1988; Reier et al., 1989) and chemical (McKeon et al., 1991; Fawcett, 1994) barrier to axons attempting to regrow after damage. It has also been suggested that an inadequate supply of growth-promoting molecules may also contribute to a lack of new axon extension in the damaged CNS (Schwartz et al., 1989).

The neurotrophin family of growth factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), has been shown to influence axon sprouting and regeneration in the mammalian CNS. Menesini-Chen et al. (1978) reported that intraparenchymal infusions of NGF in the *neonatal* rat brain promote the directional growth of sympathetic axons toward the site of injection within yet-to-be myelinated tracts. This neurotropism, however, could not be induced through the myelinated tracts of *adult* animals. Sympathetic fibers have been shown to grow into the NGF-rich, denervated hippocampus of adult mammals, but this new growth is confined to the gray matter (Crutcher et al., 1981; Crutcher and Chandler, 1985; Crutcher and Marfurt, 1988). Recent investigations have demonstrated that applications of BDNF and NT-3 can also stimulate the extension of new axonal processes of CNS neurons through damaged areas of the brain and spinal cord, but only over short distances and usually confined to gray matter regions (Logan et al., 1994; Schnell et al., 1994; Mamounas et al., 1995; Xu et al., 1995; Sawai et al., 1996; Bregman et al., 1997; Grill et al., 1997; Ye and Houle, 1997; Schwab and Brösamle, 1997). Even when the inhibitory actions of central myelin are neutralized, new axons growing in response to neurotrophins prefer the gray matter as a more supportive microenvironment (Schwab and Brösamle, 1997).

Biological responses to NGF, including neurite outgrowth and axon elongation, are mediated through binding to two transmembrane receptors. The trkA receptor, of the *trk* family of receptor tyrosine kinases, is critical for initiating these stereotypical actions of NGF (for review, see Klein, 1994; Green and Kaplan, 1995). The second receptor, the p75 neurotrophin receptor (NTR), may be required to enhance trkA function, especially when NGF is in low concentrations (Barker and Shooter, 1994; Hantzopoulos et al., 1994; Verdi et al., 1994; Lachance et al., 1997). Consistent with this, neurons from the trigeminal and superior cervical ganglia of $p75^{NTR}$-deficient mice show a shift to

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the right in the NGF dose–response curve (Davies et al., 1993; Lee et al., 1994b), a finding that may explain the perturbed patterns of sensory and sympathetic innervation in p75 mutant mice (Lee et al., 1992, 1994a; Kawaja, 1998). p75^{NTR} can also initiate its own signal transduction cascades (Dobrowsky et al., 1994; Carter et al., 1996; Casaccia-Bonnefil et al., 1996), suggesting that p75^{NTR} intracellular signals may converge with those generated by trkA to influence neurotrophin responses.

In a previous examination of transgenic mice overexpressing NGF in astrocytes, we reported that postganglionic sympathetic fibers had invaded the postnatal cerebellum (which had 50-fold higher NGF levels than age-matched cerebella) and that this new growth was confined predominantly to the myelinated tracts (Kawaja and Crutcher, 1997). It is not certain whether this ingrowth of sympathetic axons is impeded by the development and presence of myelin in the cerebellum of adult transgenic mice. Thus, the objective of the present study was to provide a quantitative assessment of this growth of sympathetic axons into the developing cerebellum of NGF transgenic mice. To further clarify the role of p75^{NTR} in NGF-induced sympathetic sprouting, we have also examined the postnatal growth of sympathetic axons into the cerebellum of NGF transgenic mice, which have a targeted deletion of the p75^{NTR} gene.

MATERIALS AND METHODS

Animals. Three genotypically distinct strains of mice were used in this investigation: (1) NGF/p75$^{+/+}$ mice, which overexpress NGF centrally in astrocytes under control of the promoter for glial fibrillary acidic protein (GFAP) and possess two normal alleles for the p75^{NTR} gene (Kawaja and Crutcher, 1997); (2) NGF/p75$^{-/-}$ mice, which overexpress NGF in astrocytes and have a null mutation of p75^{NTR} (Coome et al., 1998); and (3) C57Bl/6 mice, which are the background strain for both NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice. These two lines of NGF transgenic mice have been bred to homozygosity. Because all littermates carry the NGF transgene, we chose to use the background strain of mice (i.e., C57Bl/6) as control animals. In the generation of this transgenic strain of mice, we have shown that nontransgenic littermates (like C57Bl/6 mice) display neither elevated levels of NGF in the cerebellum nor an ingrowth of sympathetic axons into this foreign target tissue (Kawaja and Crutcher, 1997). For developmental studies, animals from all three genotypes were killed on postnatal day 14 (P14), P28, P60, and P100. For experimental studies (see below), NGF transgenic lines of mice at P60 and P97 were used, and all were subsequently killed on P100. All animal procedures and surgical protocols were approved by the Queen's University Animal Care Committee.

Surgery. In the first experiment, NGF/p75$^{+/+}$ ($n = 7$) and NGF/p75$^{-/-}$ mice ($n = 10$) at P97 underwent unilateral sympathetic ganglionectomy. Animals were anesthetized with the inhalant Metofane, and under sterile conditions the left superior cervical ganglion (SCG) was surgically excised. After recovering from the anesthesia, animals were assessed for ipsilateral ptosis as confirmation of a successful sympathetic ganglionectomy. Those animals displaying ptosis were allowed to survive for only 3 d after surgery; thus, this surgical procedure was referred to as an *acute ganglionectomy*. On the third postoperative day (i.e., at P100), these animals were deeply anesthetized with sodium pentobarbital (325 mg/kg, i.p.) and killed by transcardial perfusion. In a second experiment, NGF/p75$^{+/+}$ ($n = 5$) and NGF/p75$^{-/-}$ mice ($n = 6$) at P60 underwent unilateral sympathetic ganglionectomy as described above. Those animals displaying ipsilateral ptosis were allowed to survive for 40 d after surgery; thus, this surgical procedure was referred to as a *chronic ganglionectomy*. At P100, these animals were anesthetized with sodium pentobarbital and killed by transcardial perfusion.

Immunohistochemistry. Mice were deeply anesthetized with sodium pentobarbital and then perfused transcardially with a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed, post-fixed for 2 hr in the same fixative, and then immersed for three d in 30% phosphate-buffered sucrose. Brains were sectioned coronally using a freezing microtome (40 μm thickness), and sections were stored in cryoprotectant. To ensure that immunostained tissues could be qualitatively and quantitatively compared between age-matched genotypes, all sections were processed under identical conditions, including buffer concentrations, antibody dilutions, temperature, and incubation and reaction times. Free-floating sections of the hindbrain (with both the cerebellum and brainstem) were initially treated in 0.3% hydrogen peroxide in 0.1 M Tris-buffered saline (TBS), pH 7.4, for 1 hr. Sections were then incubated in 10% bovine serum albumin (BSA) and 0.25% Triton X-100 in TBS for 1 hr. 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 4°C in one of the following primary IgGs: sheep anti-rat tyrosine hydroxylase (TH) IgG (1:1000 dilution; Chemicon, Temecula, CA), rat anti-bovine myelin basic protein (MBP) IgG (1:2000; Chemicon), or rabbit anti-human GFAP IgG (1:1000; Chemicon). Primary antibodies were diluted in a standard solution containing 3% BSA and 0.25% Triton X-100 in TBS. All control sections were processed in the absence of primary IgGs. After a rinse in TBS, the sections were incubated for 2 hr at room temperature in the standard solution containing one of the following biotinylated secondary antibodies (Vector Laboratories): rabbit anti-goat IgG (1:200; for TH immunoreactivity), rabbit anti-rat IgG (1:500; for MBP immunoreactivity), or goat anti-rabbit IgG (1:500; for GFAP immunoreactivity). The sections were then rinsed and incubated in avidin–biotin reaction 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% hydrogen peroxide in 0.1 M TBS. The DAB reaction was terminated by washing the sections in TBS. After this, sections were mounted on chrome alum-gelatin-coated slides, dehydrated through a graded series of ethanols, and coverslipped. All immunostained sections were viewed and photographed under bright-field optics.

Electron microscopy. At P60, two NGF/p75$^{+/+}$ mice were deeply anesthetized with sodium pentobarbital and perfused with a solution of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were cut on a vibratome (at 50 μm thickness), and sections of cerebellum were stained immunohistochemically for TH (as described for light microscopy), except that there was no hydrogen peroxide pretreatment, nor was Triton X-100 used in the immunostaining. After the DAB reaction, the sections were rinsed and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 2 hr at room temperature. The sections 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 cerebellum 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.

ELISA. At P100, C57Bl/6 (total $n = 3$), NGF/p75$^{+/+}$ (total $n = 9$), and NGF/p75$^{-/-}$ (total $n = 9$) mice were decapitated, and their cerebella were quickly removed for the determination of NGF protein levels. Cerebella were taken from (1) control, nonganglionectomized mice, (2) acutely ganglionectomized mice (i.e., 3 d survival postoperatively), and (3) chronically ganglionectomized mice (i.e., 40 d survival postoperatively). NGF levels were not measured from ganglionectomized C57Bl/6 mice, because no sympathetic axons are present in the CNS of these animals. The cerebellum of each animal was divided into left (ipsilateral to a ganglionectomy) and right (contralateral to a ganglionectomy) halves, frozen in liquid nitrogen, and stored at −70°C. Coded tissue samples were shipped on dry ice to Dr. Crutcher's laboratory (University of Cincinnati Medical Center). Levels of NGF were determined using a modified two-site ELISA that has previously been shown to be both sensitive and specific for NGF (Saffran and Crutcher, 1990; Crutcher et al., 1993). Because the tissues from NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice required additional dilutions to obtain accurate values for the very high levels of NGF protein, all tissues assayed were identified only as "transgenic" or "nontransgenic." In some cases, samples had to be run again at higher dilution because of the high levels of NGF in the tissue. The final values were calculated from the total dilution to arrive at a concentration per wet weight of the initial sample. No corrections were made for recovery. Results were tested for significance by a Student's t test.

Quantitation of sympathetic sprouting in cerebellum. Coronal sections of cerebellum stained immunohistochemically for TH were used to measure the percent area occupied by TH-immunoreactive (TH-IR) axons in the deep white matter (DWM) of C57Bl/6, NGF/p75$^{+/+}$, and NGF/p75$^{-/-}$ mice. These measurements of axon density are expected to reflect the degree of collateral growth by sympathetic fibers into the cerebella of transgenic mice. Digitized images of brain sections were captured di-

rectly from the light microscope with a 40× objective using a Sony CCD color video camera. The density of TH-IR axons in the cerebellar DWM was quantified using an image analysis software package (Bioquant/TCW, R & M Biometrics). Video thresholding, a feature of the software, was used to outline immunoreactive fibers on screen, and the computer determined the area occupied by immunoreactive fibers. Percent area was calculated by dividing the area of TH-IR axons by the area of the DWM being measured. Measurements were taken from two randomly selected regions of the cerebellar DWM per brain section (two sections per animal). For developmental studies, a two-way ANOVA was used to determine whether sympathetic axon density changed with age and differed between genotypes. A Newman–Keuls multiple comparisons *post hoc* analysis was used to test for differences among ages within each genotype, and a Student's *t* test was used for comparisons between genotypes at each age. For acutely and chronically ganglionectomized animals, sympathetic axon density was measured in both the ipsilateral and contralateral DWM of all animals (as previously described). A two-way ANOVA was used to determine whether sympathetic axon density in either the ipsilateral or contralateral DWM differed between surgical protocols and differed between genotypes. A *post hoc* Student's *t* test was used for comparisons between groups. The data were plotted as mean ± SD.

Morphometric analysis of the superior cervical ganglia. C57Bl/6 ($n = 4$), NGF/p75$^{+/+}$ ($n = 3$), and NGF/p75$^{-/-}$ ($n = 4$) mice were deeply anesthetized with sodium pentobarbital and perfused transcardially with a solution of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The SCGs were removed, post-fixed for 2 hr, and immersed for 2 d in 30% phosphate-buffered sucrose. The ganglia were then embedded in OCT (Miles, Elkhart, IN) and frozen in 2-methylbutane at −20°C. Serial sections through the entire ganglia were cut on a cryostat at 10 μm thickness, thaw-mounted onto chrome alum-gelatin-coated slides, stained for Nissl substance with thionin, dehydrated through a graded series of ethanols, cleared, and coverslipped for viewing under bright-field optics. Under observer-blind conditions, all neuronal profiles displaying a prominent nucleolus (or nucleoli) were counted on every fifth section through each ganglion. The sampling frequency (50 μm) ensures that neurons are not counted twice. Because NGF exposure increases the incidence of split nucleoli in sympathetic neurons (Ruit et al., 1990), total neuron counts were corrected for multiple split nucleoli, according to the calculation of Coggeshall et al. (1984): $N = n \times [N(\text{c.f.})/n(\text{c.f.})]$, where N is the true number of sympathetic neurons, n is the number of neurons displaying a prominent nucleolus (or nucleoli), N(c.f.) is the number of neurons used to estimate the correction factor, and n(c.f.) is the number of individual nucleolar profiles found in these neurons that constitute N(c.f.). To determine the correction factor [N(c.f.)/n(c.f.)], one level from each ganglion was selected, having between 71 and 140 neurons with a nucleolus (or nucleoli). These data were tested for significance using a one-way ANOVA with a *post hoc* Newman–Keuls test for comparisons between groups. The data were represented as the mean ± SD.

mRNA expression of trkA within sympathetic neurons. At P60, C57Bl/6 ($n = 4$), NGF/p75$^{+/+}$ ($n = 4$), and NGF/p75$^{-/-}$ ($n = 4$) mice were decapitated, and the right and left SCGs (plus attached internal carotid arteries) were isolated and frozen in liquid nitrogen. Trizol (Life Technologies, Gaithersburg, MD) was used to isolate total RNA from the pooled tissues from each genotype. One microgram of total RNA from each genotype was used to synthesize cDNA (Superscript II preamplification system, Life Technologies). To be certain that cDNA synthesis and amplification of gene products from all three genotypes were possible, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; a housekeeping gene product) was first assessed, then trkA. Controls consisted of reactions conducted in either the absence of the reverse transcriptase enzyme or the absence of template. DNAs encoding for GAPDH and trkA were amplified with *Taq* polymerase using primer-specific sequences for both gene products. For GAPDH, the primer sequences were 5′-GTTGCCATCAATGACCCCTTCATTG-3′ (5′) and 5′-GCTTCACCACCTTCTTGATGTCATC-3′ (3′). For trkA, the primer sequences were 5′-GGTACCAGCTCTCCAACACTGAGG-3′ (5′) and 5′-CCAGAACGTCCAGGTAACTCGGTG-3′ (3′). All reactions were conducted under identical conditions (e.g., buffers, temperatures, and times) and underwent 38 cycles. The amplified DNA products were separated in a 0.8% agarose gel and photographed.

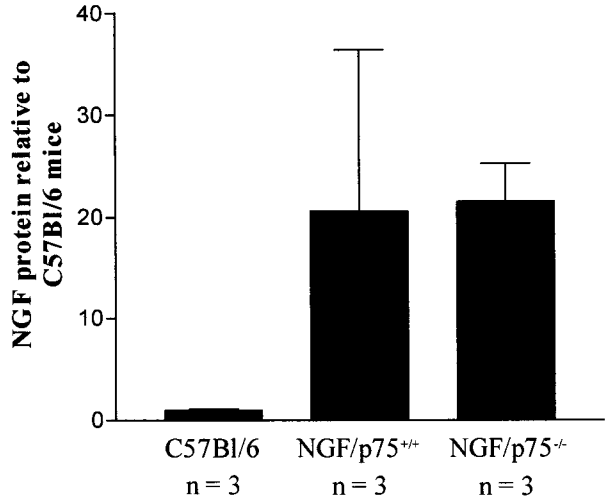

Figure 1. Mean levels of NGF protein in the cerebellum of C57Bl/6, NGF/p75$^{+/+}$, and NGF/p75$^{-/-}$ mice, as measured by a two-site ELISA. The data are represented as fold differences relative to C57Bl/7 mice, and error bars indicate SD. In contrast to the low levels of NGF protein in the cerebellum of C57Bl/6 mice, levels of NGF protein are 21- and 22-fold higher in the cerebellum of age-matched NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice, respectively. No significant difference in cerebellar NGF levels is detected between NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice.

RESULTS

NGF protein in the cerebellum of transgenic mice

In this study, two lines of GFAP-NGF transgenic mice were used, one of which expresses functional p75^{NTR} (NGF/p75$^{+/+}$) and the other of which has a null mutation of the p75^{NTR} gene (NGF/p75$^{-/-}$). Both strains of transgenic mice have been shown to express the NGF transgene within the cerebellum as determined by RT-PCR (Kawaja and Crutcher, 1997; Coome et al., 1998). To confirm that these two strains of NGF transgenic mice possessed equal levels of NGF protein, NGF levels in the cerebellum of C57Bl/6, NGF/p75$^{+/+}$, and NGF/p75$^{-/-}$ mice were measured using a two-site ELISA. In contrast to the low levels of NGF protein found in the cerebellum of C57Bl/6 mice, the mean levels of NGF protein measured in the cerebellum of NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice were 21- and 22-fold higher, respectively (Fig. 1). No significant difference in the mean levels of NGF protein was detected between the cerebellum of NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice. These data are in agreement with previous results (Coome et al., 1998).

Sympathetic axons invade the cerebellum

As revealed by TH immunohistochemistry, the cerebellum of adult (P100) C57Bl/6 mice has a diffuse network of fine TH-IR fibers extending throughout the gray and white matter portions (Figs. 2*A*, 3*A,B*). In the cerebellar DWM, these TH-IR axons are varicose and often observed coursing perpendicular to the intrinsic cerebellar fibers. These TH-IR axons in control C57Bl/6 mice comprise the normal innervation of the cerebellum by neurons of the locus coeruleus, the principal noradrenergic neurons of the CNS.

In agreement with our previous investigation (Kawaja and Crutcher, 1997), the cerebellum of NGF/p75$^{+/+}$ mice, at P100, possessed a marked increase in the number of TH-IR axons compared with the cerebellum of C57Bl/6 mice (Figs. 2*B*, 3*C,D*). Interestingly, this profusion of new TH-IR axons was localized predominantly to white matter areas of the cerebellum, including

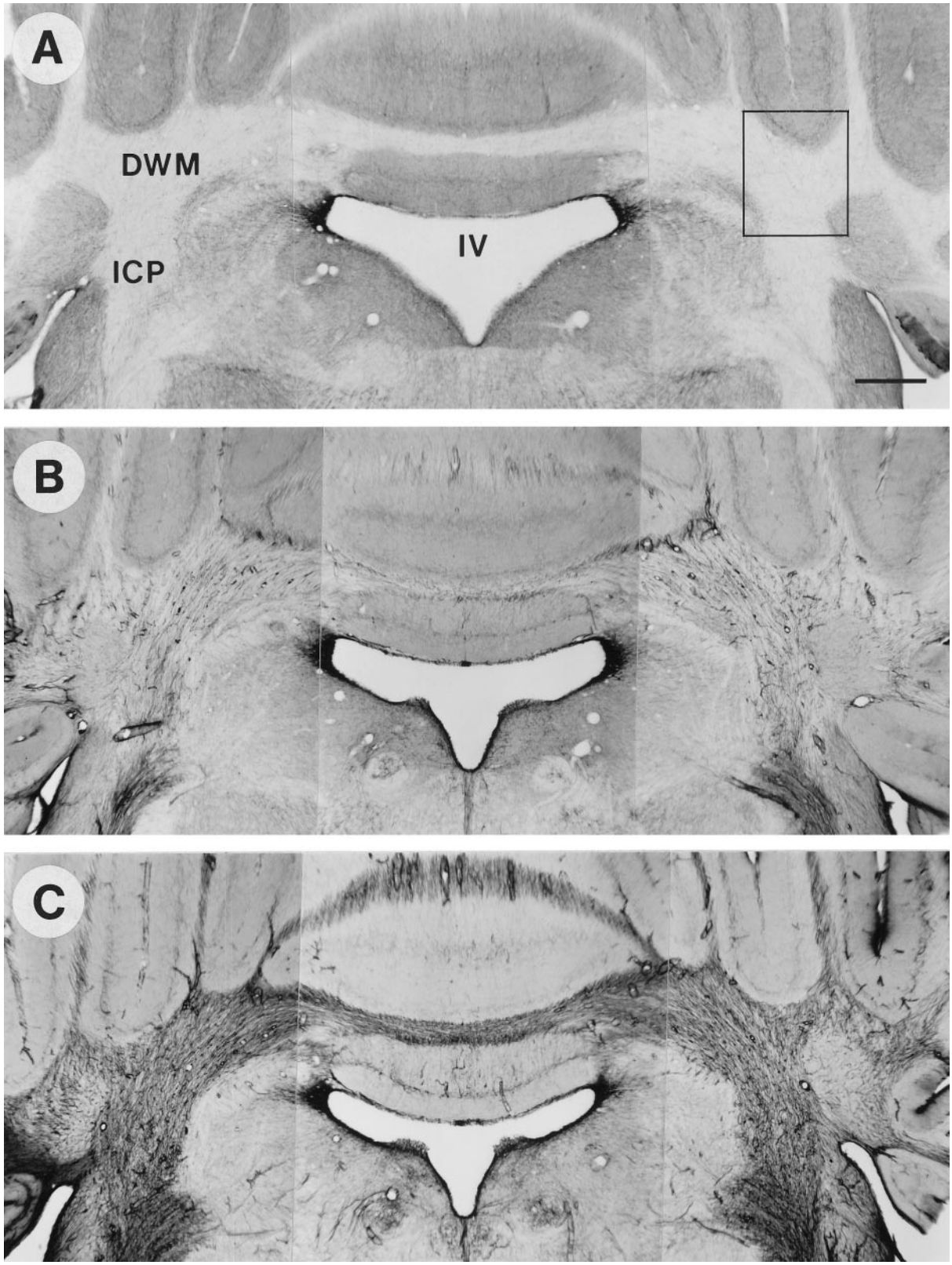

Figure 2. Coronal sections of cerebellum from C57Bl/6, NGF/p75$^{+/+}$, and NGF/p75$^{-/-}$ mice at P100, stained immunohistochemically for TH. In C57Bl/6 mice (*A*), a diffuse network of TH-IR axons is evident in both the cerebellar gray and white matter, representing the local innervation by locus coeruleus neurons. In contrast to these observations, the cerebella of both NGF/p75$^{+/+}$ (*B*) and NGF/p75$^{-/-}$ (*C*) mice display a robust ingrowth of TH-IR axons. These dense plexuses of new TH-IR axons are confined predominantly to the DWM layer and ICPs of the cerebellum and are only occasionally seen extending into the gray matter layers of the cerebellum. Qualitatively, the density of TH-IR axons appears increased in the cerebellum of NGF/p75$^{-/-}$ mice compared with NGF/p75$^{+/+}$ mice. The white matter portions of the cerebellum of these mice, as outlined in the *box* in *A*, are shown at two higher magnifications in Figure 3. Scale bar, 500 μm. *IV*, Fourth ventricle.

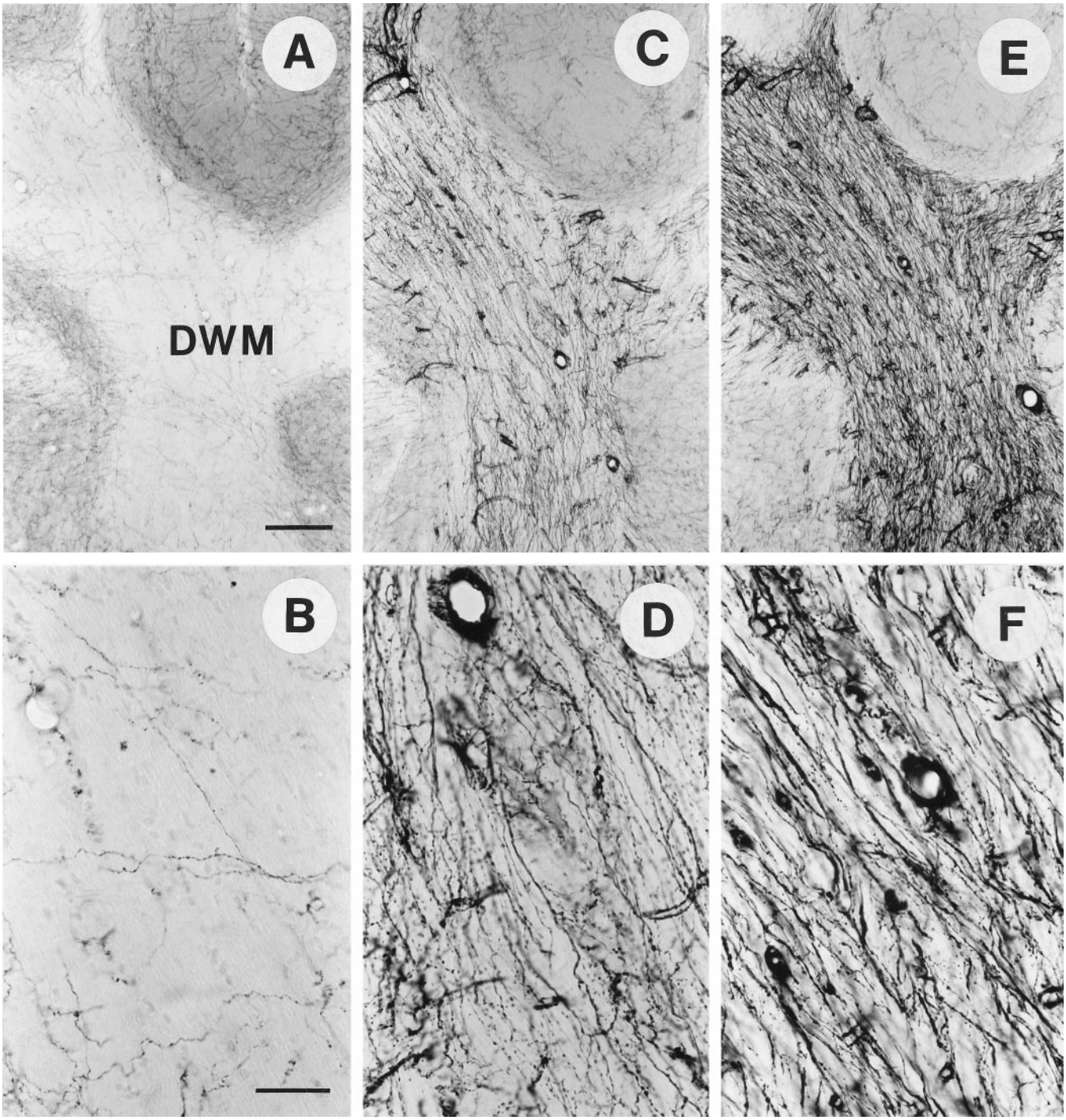

Figure 3. Higher magnifications of the cerebellar DWM of C57Bl/6, NGF/p75 $^{+/+}$, and NGF/p75 $^{-/-}$ mice. Note the fine TH-IR locus coeruleus axons, which often course perpendicular to the DWM fibers, in control C57Bl/6 mice (*A, B*). In contrast, the DWM of both NGF/p75 $^{+/+}$ (*C, D*) and NGF/p75 $^{-/-}$ (*E, F*) mice display a dramatic increase in TH-IR axons, which course approximately in parallel with the intrinsic DWM fibers. Again note that the density of TH-IR axons appears increased in the DWM of NGF/p75 $^{-/-}$ mice relative to NGF/p75 $^{+/+}$ mice. Scale bars: *A, C, E*, 150 μm; *B, D, F*, 50 μm.

the DWM, inferior cerebellar peduncle (ICP), and cerebellar folia. These TH-IR axons rarely entered the deep cerebellar nuclei and were only occasionally seen traversing the cerebellar gray matter layers. At high magnification, these TH-IR axons were varicose in nature and coursed mostly parallel to the intrinsic myelinated fibers. There were also perivascular clusters of immunostained axons in the DWM; such arrangements were not seen in P100 C57Bl/6 mice. Our previous investigation with NGF/p75 $^{+/+}$ mice revealed that these new TH-IR axons, which invade the cerebellum, are postganglionic sympathetic fibers, because unilateral removal of the SCG results in a dramatic loss of these axons in the ipsilateral deep white matter (Kawaja and Crutcher, 1997; also see below). The cerebellum of NGF/p75 $^{-/-}$ mice at P100 also possessed a similar dense plexus

of new TH-IR sympathetic axons (Figs. 2*C*, 3*E,F*). There was, however, a striking increase in the density of TH-IR sympathetic axons in the DWM and ICP of NGF/p75$^{-/-}$ mice compared with age-matched NGF/p75$^{+/+}$ mice. There was no obvious increase in TH-IR fibers in the gray matter. Although the reason why sympathetic axons are confined to the white matter and do not extend into the adjacent gray matter remains to be elucidated, possibilities include (1) the presence of inhibitory factors in the gray matter, (2) physical or geometrical constraints of the white matter, and (3) an increased production of transgene NGF in the astrocytes of the white matter relative to those of the gray matter.

In light of the finding that sympathetic axons form a dense plexus within the cerebellum of adult NGF transgenic mice, it was of interest to determine the developmental pattern of sympathetic ingrowth in these animals. We have previously demonstrated that NGF production in the cerebellum of NGF/p75$^{+/+}$ mice begins shortly after the day of birth, increases to 50-fold higher levels until the end of the second postnatal week, and then slowly decreases to 20-fold higher levels in the adult (Kawaja and Crutcher, 1997) (our unpublished data). This pattern of NGF production parallels the ontogeny of GFAP expression in the rodent CNS (Landry et al., 1990). The percent area occupied by TH-IR axons in the cerebellar DWM of NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice was measured at various developmental ages. Statistical analysis of TH-IR axon densities using a two-way ANOVA revealed that there were significant main effects of age ($F_{(3,22)} = 201.6$; $p < 0.001$) and genotype ($F_{(1,22)} = 22.71$; $p < 0.001$), as well as significant interactions between age and genotype ($F_{(3,22)} = 5.060$; $p < 0.01$).

At P14, TH-IR sympathetic axons were already seen invading the cerebellar DWM (Fig. 4*A*). In fact, the percent area occupied by TH-IR axons from the locus coeruleus in the DWM of P100 C57Bl/6 mice was low (4.1 ± 0.3%; $n = 4$), in comparison with that measured in P14 NGF/p75$^{+/+}$ mice (12.8 ± 2.4%; $n = 4$; $p < 0.001$, Student's t test) (Fig. 5). On examination of TH-immunostained sections of the cerebellum from postnatal NGF/p75$^{+/+}$ mice, it appeared as if the density of TH-IR axons in the cerebellar DWM increased with age (compare Figs. 4*A,C,E*, 3*C*). Quantitative analysis confirmed this trend, because the area occupied by TH-IR axons in the DWM at P14 was increased by P28 (46.6 ± 1.5%; $n = 4$; $p < 0.001$, Newman–Keuls test), was similar between P28 and P60 (mean value, 45.9%; $n = 2$), and was increased again between P28 and P100 (65.8 ± 4.3%; $n = 4$; $p < 0.001$) (Fig. 5). These findings demonstrate that TH-IR sympathetic axons begin their growth into the cerebellum of NGF/p75$^{+/+}$ mice shortly before P14 and continue to increase in density into adulthood.

Analysis of NGF/p75$^{-/-}$ mice revealed that, similar to NGF/p75$^{+/+}$ mice, TH-IR sympathetic axons are evident in the DWM by P14, and the density of these TH-IR axons increases throughout the life of the animals (compare Figs. 4*B,D,F*, 3*E*). The percent area occupied by TH-IR axons in the cerebellar DWM of NGF/p75$^{-/-}$ mice increased dramatically between P14 and P28 (26.6 ± 4.4 vs 46.4 ± 6.4%; $n = 4$ for each; $p < 0.001$, Newman–Keuls test), was similar between P28 and P60 (49.7 ± 4.1%; $n = 3$; $p < 0.05$), and was increased again between P60 and P100 (80.1 ± 5.9%; $n = 5$; $p < 0.001$) (Fig. 5). Remarkably, the density of TH-IR axons in the DWM of NGF/p75$^{-/-}$ mice was greater than that measured in NGF/p75$^{+/+}$ mice at both P14 and P100 ($p < 0.01$, Student's t test). Both lines of mice displayed similar densities of TH-IR axons at P28 ($p = 0.9894$, Student's t test), and no statistical comparison could be made for P60. Taken together, our data provide evidence that sympathetic axons, in the absence of p75^{NTR}, are capable of enhanced growth within the NGF-rich cerebellum of transgenic mice during postnatal development and into adulthood.

Axon growth occurs in myelinated portions of the cerebellum

One of the remarkable features of sympathetic growth into the cerebellum of postnatal and adult transgenic mice is the topographical distribution of these aberrant fibers. In both NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice, the majority of TH-IR sympathetic axons remains confined to the DWM and ICP, with few fibers extending into the adjacent gray matter layers. In agreement with other investigations (Foran and Peterson, 1992; Hamano et al., 1996), we were able to detect MBP, a major constituent of central myelin, in the DWM and ICP of the cerebellum using immunohistochemistry. As early as the second week of postnatal life, a comparable intensity of MBP immunostaining was seen in both NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice (data not shown). This strong immunoreactivity was equally evident in the myelinated portions of the cerebellum of P100 NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice. The staining intensity for MBP in both lines of NGF transgenic mice was comparable with that seen in the cerebellum of control C57Bl/6 mice. These data reveal that the collateral growth of TH-IR sympathetic axons into the cerebella of postnatal NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice continues into adulthood, despite the presence of central myelin within the DWM and ICP.

To assess which cellular substrates were used by these TH-IR sympathetic axons, we examined the localization of TH immunoreactivity at the ultrastructural level in P100 NGF/p75$^{+/+}$ mice. Small clusters of unmyelinated axons, some of which had immunoreactivity for TH, were found among the larger myelinated fibers of the cerebellar DWM (Fig. 6). The absence of a glial ensheathment of these unmyelinated TH-IR axons indicates that Schwann cells do not migrate along with the sympathetic axons invading the cerebellum. Rather, these TH-IR unmyelinated axons were seen immediately apposed to nonimmunoreactive unmyelinated axons and myelinated axons alike. Although it is uncertain whether these sympathetic fibers use intrinsic myelinated axons as substrates for growth, it is evident that these peripheral fibers are capable of continued growth within myelinated portions of the adult CNS.

Topography of sympathetic axons within the cerebellar DWM

To assess the extent to which sympathetic axons arising from the right SCG invade both halves of the cerebellum, NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice underwent unilateral removal of the left SCG 3 d before P100. This procedure allowed visualization of only those sympathetic axons arising from the right SCG in both the ipsilateral and contralateral DWM. After acute ganglionectomy in NGF/p75$^{+/+}$ mice, the bulk of TH-IR sympathetic axons from the contralateral SCG did not extend past the cerebellar midline (Fig. 7*A*). In contrast, unilateral SCG removal in NGF/p75$^{-/-}$ mice revealed that many TH-IR sympathetic axons of the contralateral SCG extended up to and beyond the cerebellar midline, some of which continued into the upper portion of the ipsilateral DWM (Fig. 7*B*).

We next made a detailed examination of sympathetic fiber density in the ipsilateral and contralateral DWM of animals that had undergone either an acute ganglionectomy or a chronic

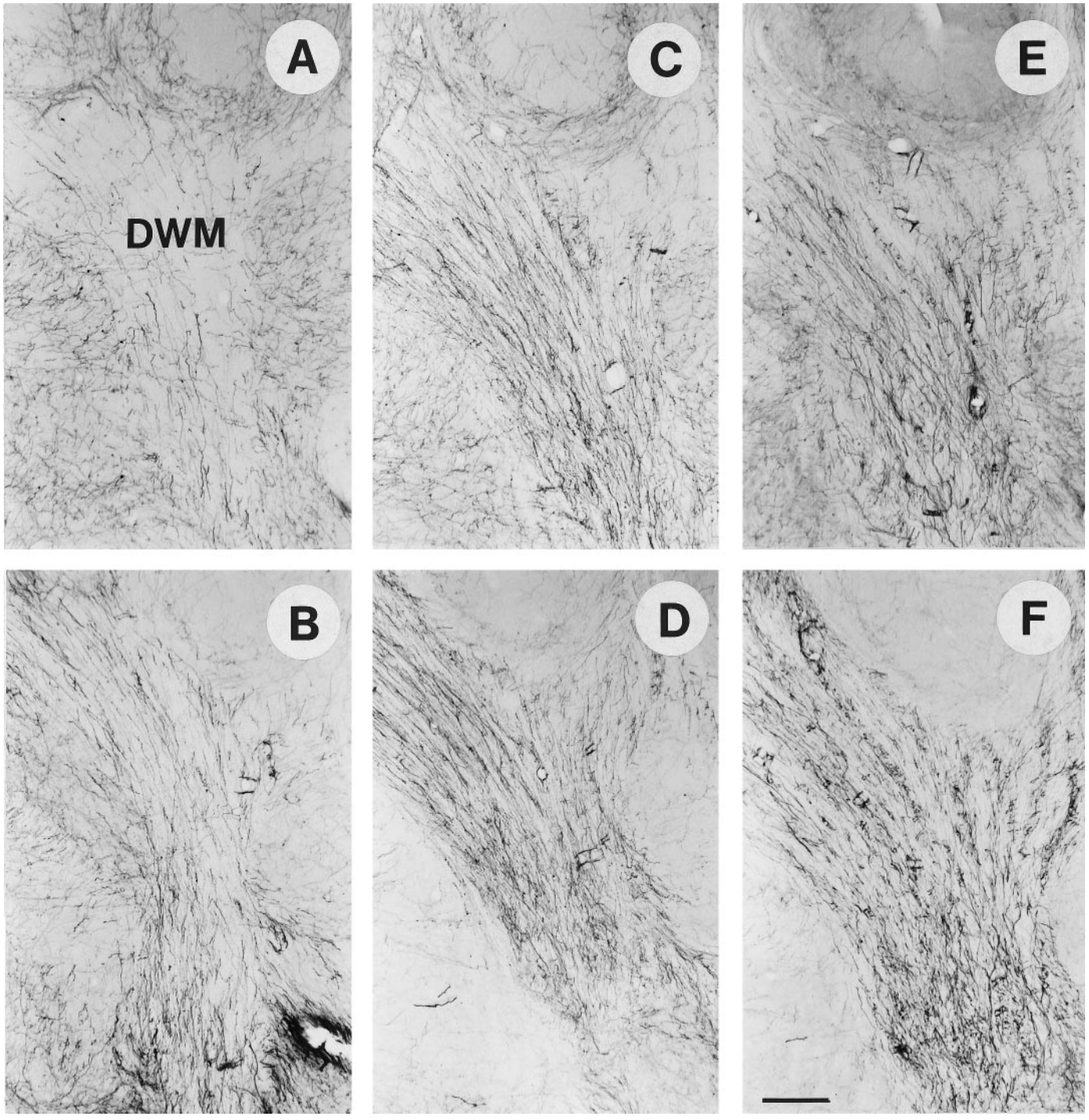

Figure 4. Coronal sections of cerebellum from NGF/p75$^{+/+}$ (*top row*) and NGF/p75$^{-/-}$ (*bottom row*) mice at P14 (*A, B*), P28 (*C, D*), and P60 (*E, F*), stained immunohistochemically for TH. TH-IR sympathetic axons are already observed growing into the cerebellar DWM by P14 in both NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice. In both NGF transgenic lines of mice, the density of TH-IR axons in the cerebellar DWM appears to increase with age. It is also apparent that the density of TH-IR axons is greater in the DWM of NGF/p75$^{-/-}$ mice compared with NGF/p75$^{+/+}$ mice. Scale bar, 150 μm.

ganglionectomy. This was done to determine (1) the degree to which sympathetic axons of the contralateral SCG extend into the ipsilateral DWM and (2) whether this unilateral sympathetic "denervation" of the cerebellum is followed by collateral sprouting from the intact contralateral fibers. Quantitation of sympathetic fiber density is shown in Figure 8. For all animals, with either an acute or chronic ganglionectomy, the percent area occupied by TH-IR axons was dramatically reduced in the ipsilateral DWM compared with the contralateral DWM of the same animal ($p < 0.001$, Student's t test). Regardless of acute or chronic ganglionectomy, the percent area occupied by TH-IR axons in the contralateral (intact) DWM was higher in NGF/p75$^{-/-}$ mice relative to NGF/p75$^{+/+}$ mice ($p < 0.001$, two-way ANOVA; $F_{(1,24)} = 148.6$), consistent with our observations of sympathetic axon density in nonganglionectomized animals. Moreover, sympathetic axon density in the contralateral DWM

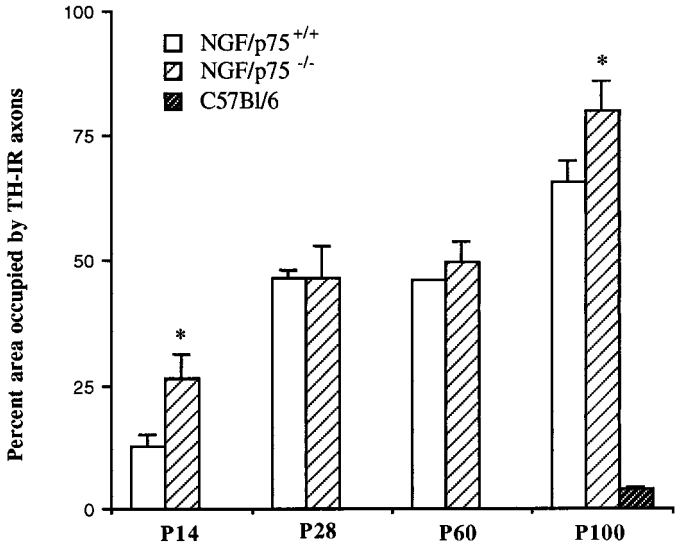

Figure 5. Quantitation of TH-IR axon density in the cerebellar DWM of C57Bl/6, NGF/p75 $^{+/+}$, and NGF/p75 $^{-/-}$ mice at developmental time points. There are significant increases in the percent area occupied by TH-IR axons in the DWM of NGF/p75 $^{-/-}$ mice relative to NGF/p75 $^{+/+}$ mice at both P14 and P100 ($*p < 0.001$). The density of TH-IR axons is lowest in the DWM of P100 C57Bl/6 mice.

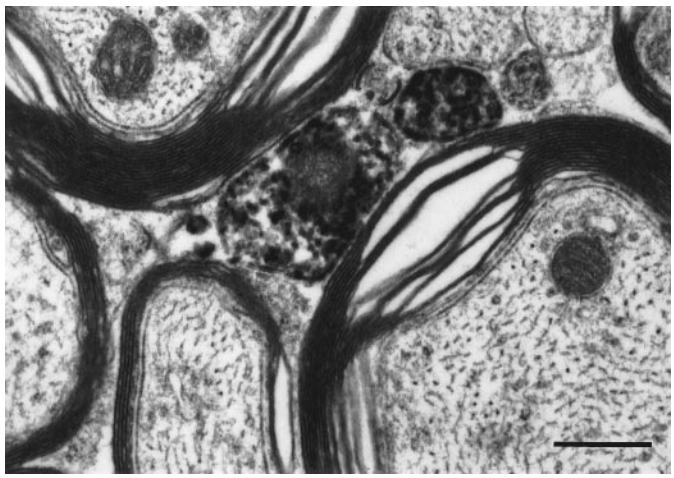

Figure 6. Electron photomicrograph showing the localization of TH immunoreactivity in the cerebellar deep white matter of adult NGF/p75 $^{+/+}$ mice. TH-IR unmyelinated axons are seen coursing among myelinated fibers of the cerebellum, often as part of a small cluster of unmyelinated axons. TH-IR unmyelinated axons lack any glial support and are often observed in close apposition to the surface of myelinated axons and unmyelinated axons alike. Scale bar, 0.5 μm.

did not differ between acute and chronic ganglionectomy in either of the NGF transgenic strains. Examination of the ipsilateral DWM revealed that the density of sympathetic fibers was again higher in NGF/p75 $^{-/-}$ mice relative to NGF/p75 $^{+/+}$ mice ($p < 0.001$, two-way ANOVA; $F_{(1,24)} = 37.82$), regardless of the type of ganglionectomy. The finding that a small number of TH-IR axons persisted in the ipsilateral DWM after acute ganglionectomy suggests that a minor proportion of contralateral (intact) SCG axons normally project past the cerebellar midline and into the ipsilateral DWM of both NGF transgenic strains. This proportion, however, is greater in NGF/p75 $^{-/-}$ mice. Our data also indicate that the effect of chronic ganglionectomy was not the same for NGF/p75 $^{+/+}$ and NGF/p75 $^{-/-}$ mice. Whereas no significant change in sympathetic fiber density was observed in the ipsilateral DWM of NGF/p75 $^{+/+}$ mice between acute and chronic ganglionectomy, sympathetic fiber density in the ipsilateral DWM of NGF/p75 $^{-/-}$ mice was increased after chronic ganglionectomy compared with acute ganglionectomy ($p < 0.01$, two-way ANOVA; $F_{(1,24)} = 9.185$). These findings suggest that unilateral removal of the SCG results in the collateral sprouting of sympathetic axons of the contralateral (intact) SCG within the myelinated portions of the adult cerebellum in NGF/p75 $^{-/-}$ mice but not NGF/p75 $^{+/+}$ mice.

Levels of NGF protein and reactive cerebellar astrocytes

Could the collateral sprouting of sympathetic axons observed in the ipsilateral DWM of chronically ganglionectomized NGF/p75 $^{-/-}$ mice be attributed to a selective upregulation of NGF protein in the cerebella of NGF/p75 $^{-/-}$ mice only? To address this possibility, NGF levels were measured in the ipsilateral and contralateral halves of the cerebellum of NGF/p75 $^{+/+}$ and NGF/p75 $^{-/-}$ mice 3 and 40 d after unilateral sympathetic ganglionectomy. For each animal, the level of NGF protein in the ipsilateral cerebellum was plotted as the fold difference of NGF levels measured in the contralateral half of the same animal (Fig. 9). In NGF/p75 $^{+/+}$ mice, the mean levels of NGF protein in the ipsilateral cerebellum were increased relative to the contralateral cerebellum both 3 and 40 d after ganglionectomy, but these increases were not statistically significant. In contrast, the mean level of NGF protein in the ipsilateral cerebellum of NGF/p75 $^{-/-}$ mice 3 d after ganglionectomy was dramatically increased above that measured in the contralateral cerebellum ($p < 0.001$, Student's t test). This upregulation in NGF protein in the ipsilateral cerebellum appeared to be short-lived, because at 40 d after ganglionectomy, the mean level of NGF protein in the ipsilateral cerebellum was again not statistically different from that measured in the contralateral cerebellum of NGF/p75 $^{-/-}$ mice.

What could account for this marked increase in NGF protein ipsilateral to the SCG removal in acutely ganglionectomized NGF/p75 $^{-/-}$ mice? Because the NGF transgene is driven by the promoter for GFAP, we examined sections of cerebella stained immunohistochemically for GFAP. Cerebellar sections from nonganglionectomized C57Bl/6, NGF/p75 $^{+/+}$, and NGF/p75 $^{-/-}$ mice all possessed numerous GFAP-positive astrocytes in the cerebellar DWM but fewer in the adjacent gray matter layers. The staining intensity of GFAP immunoreactivity appeared homogeneous throughout these structures and comparable among genotypes (data not shown). In chronically ganglionectomized NGF/p75 $^{+/+}$ and NGF/p75 $^{-/-}$ mice, as well as acutely ganglionectomized NGF/p75 $^{+/+}$ mice, a modest increase in GFAP immunostaining was evident in the cerebellar white and gray matter areas ipsilateral to the SCG removal. The ipsilateral cerebellum of acutely ganglionectomized NGF/p75 $^{-/-}$ mice, however, displayed the greatest increase in GFAP immunostaining, which was evident in astrocytes of both cerebellar white and gray matter areas. These observations provide an anatomical correlate of the increased transgene production of NGF in these animals only. We speculate that a greater gliotic reaction (which includes GFAP upregulation) is triggered in the cerebella of NGF/p75 $^{-/-}$ mice by the degeneration of relatively more sympathetic fibers and, hence, upregulation of the NGF transgene. Taken together, our data indicate that, at least in NGF/p75 $^{-/-}$ mice, collateral sprouting of intact (contralateral) sympathetic axons within my-

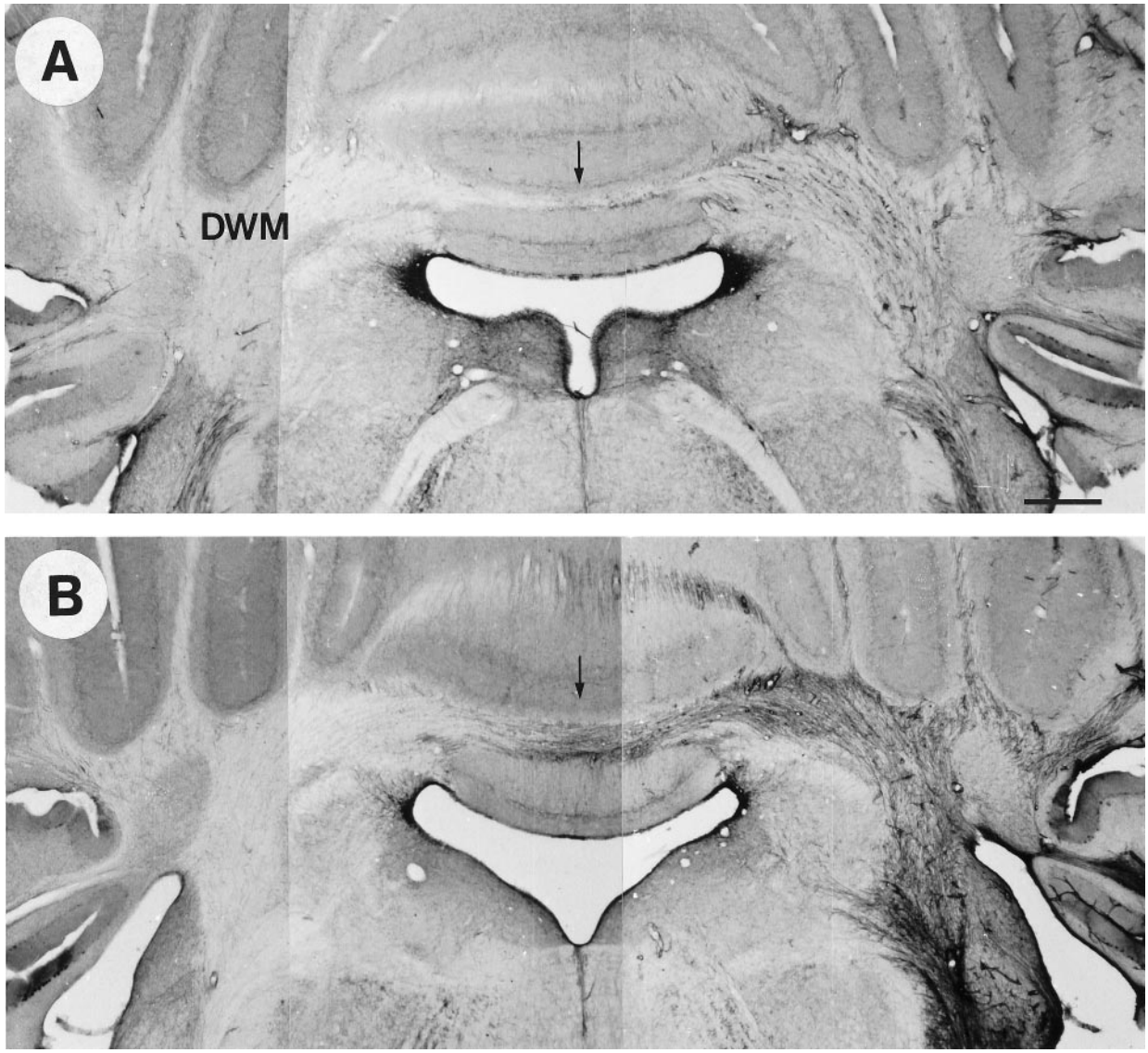

Figure 7. Coronal sections of cerebellum from acutely ganglionectomized NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice, stained immunohistochemically for TH. The unilateral removal of the SCG allows visualization of those sympathetic fibers from the contralateral ganglion. In NGF/p75$^{+/+}$ mice (*A*), few TH-IR axons from the contralateral SCG extend as far as the cerebellar midline (*arrow*). In contrast, numerous contralateral sympathetic axons in NGF/p75$^{-/-}$ mice (*B*) extend past the cerebellar midline (*arrow*) and into the upper portions of the ipsilateral DWM. Scale bar, 500 μm.

elinated portions of the mature CNS can occur and is likely stimulated by a short-lived upregulation of NGF protein. Because ganglionectomy failed to cause an upregulation of NGF protein in NGF/p75$^{+/+}$ mice, we cannot exclude the possibility that sympathetic axons are capable of collateral sprouting in the CNS of these mice.

Sympathetic neuron number

To exclude the possibility that this increased sympathetic sprouting into the cerebellum was directly linked to increased sympathetic neuron survival in NGF/p75$^{-/-}$ mice, we determined the numbers of SCG neurons in adult animals. Specifically, the SCGs from C57Bl/6, NGF/p75$^{+/+}$, and NGF/p75$^{-/-}$ mice were serially sectioned at 10 μm, and the number of neurons with a prominent nucleolus (or nucleoli) were counted on every fifth section. Exposure to high levels of NGF is known to increase the incidence of multiple nucleoli among postganglionic sympathetic neurons (Ruit et al., 1990). Qualitative assessment of the SCG revealed more sympathetic neurons with two to four nucleolar fragments in NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice than in C57Bl/6 mice (Fig. 10*A*). Quantitation of the incidence of multiple nucleoli confirmed a significant increase in the ratio of total nucleoli to SCG neurons counted among NGF/p75$^{+/+}$ mice (2.95 $\pm$ 0.13) and NGF/p75$^{-/-}$ mice (2.97 $\pm$ 0.12) relative to C57Bl/6 mice (2.28 $\pm$ 0.06). In light of these observations, the total SCG neuron counts were corrected for the presence of multiple nucleoli, according to the method of Coggeshall et al. (1984). This analysis demonstrated a statistically significant increase in the numbers of sympathetic neurons of both NGF/p75$^{+/+}$ mice (1789 $\pm$ 32; n = 3) and NGF/p75$^{-/-}$ mice (2045 $\pm$ 338; n = 4) relative to control C57Bl/6 mice (1112 $\pm$ 168; n = 4; $p < 0.05$, Newman–Keuls test) (Fig. 10*B*). No statistically significant difference in the number of SCG neurons was detected between NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice. Thus, these finding indicate that the enhanced sprouting of sympathetic axons in the cerebellum in NGF/p75$^{-/-}$

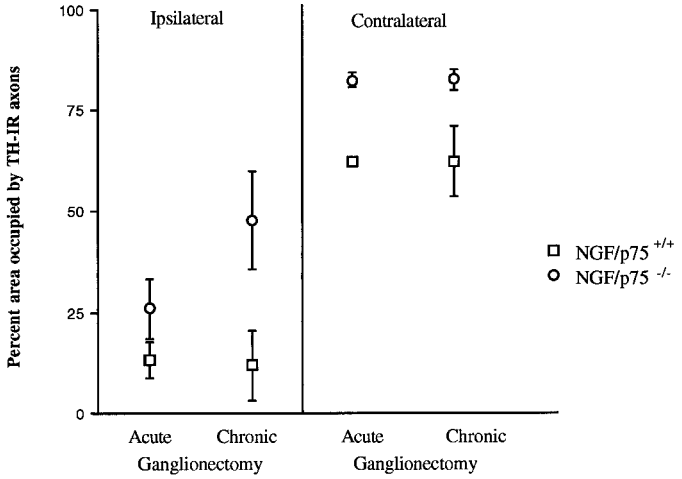

Figure 8. Quantitation of sympathetic axon density in the ipsilateral and contralateral DWM of both lines of transgenic mice after acute and chronic ganglionectomy. Data for NGF/p75 $^{+/+}$ mice (n = 7 acute and 5 chronic ganglionectomized animals) are represented by *boxes*, and data for NGF/p75 $^{-/-}$ mice (n = 10 acute and 6 chronic ganglionectomized animals) are represented by *circles*. In all acutely and chronically ganglionectomized animals, there is a significant decrease in the percent area occupied by TH-IR axons in the ipsilateral DWM relative to the contralateral DWM within the same experimental group ($p < 0.001$). In the contralateral DWM, the density of sympathetic axons is higher in NGF/p75 $^{-/-}$ mice relative to NGF/p75 $^{+/+}$ mice ($p < 0.001$), and these densities are not affected by chronic ganglionectomy. Note that the density of sympathetic axons that persist in the ipsilateral DWM, after either acute and chronic ganglionectomy, is also higher in NGF/p75 $^{-/-}$ mice relative to NGF/p75 $^{+/+}$ mice ($p < 0.001$). Importantly, sympathetic fiber density increases in the ipsilateral DWM between acutely and chronically ganglionectomized NGF/p75 $^{-/-}$ mice but not between acutely and chronically ganglionectomized NGF/p75 $^{+/+}$ mice ($p < 0.01$). All data are represented as mean ± SD.

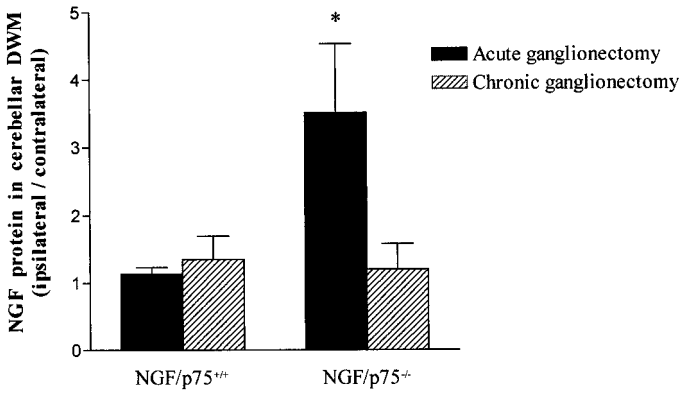

Figure 9. Levels of NGF protein in the cerebellum of acutely and chronically ganglionectomized NGF/p75 $^{+/+}$ and NGF/p75 $^{-/-}$ mice (n = 3 animals per experimental group), as measured by a two-site ELISA. Levels of NGF in the ipsilateral half of the cerebellum are presented as fold differences relative to the contralateral half from the same animal, and error bars indicate SD. This analysis reveals a significant increase in NGF levels only within the ipsilateral cerebellum of NGF/p75 $^{-/-}$ mice after acute ganglionectomy ($^{*}p < 0.001$). In all other cases, no significant differences in NGF levels are detected between ipsilateral and contralateral cerebellar halves.

mice is not a reflection of increased survival of sympathetic neurons compared with NGF/p75 $^{+/+}$ mice.

trkA expression among sympathetic neurons

To exclude the possibility that differences in the degree of sympathetic sprouting were attributable to a perturbed expression for trkA mRNA, we used RT-PCR to ensure continued mRNA expression for trkA in sympathetic neurons of the SCG (Fig. 10*C*). All three genotypes had detectable levels of the DNA products for trkA (and GAPDH), thereby revealing that the absence of p75 ^{NTR} expression did not affect the detection of trkA mRNA from the SCG of NGF/p75 $^{-/-}$ mice; these data provide only a qualitative assessment of the presence or absence of mRNA expression.

DISCUSSION

In the present study, elevated levels of NGF protein in the developing CNS of transgenic mice coincides with the growth of sympathetic axons into the deep white matter portions of the cerebellum during the period of myelination. Despite the presence of central myelin, sympathetic axons continue to grow and increase in density within the cerebellar DWM as the animals mature into adulthood. Surprisingly, the growth of sympathetic axons within the myelinated portions of the NGF-rich cerebellum was greater in NGF transgenic mice lacking expression of the p75 ^{NTR}.

Axon growth within myelinated structures

It is generally accepted that the adult mammalian CNS is unable to support axon growth over long distances. One of the factors influencing the limited growth of axons is the presence of active growth-inhibitory factors associated with oligodendrocytes and CNS myelin (Caroni and Schwab, 1988a,b; Schwab and Caroni, 1988; Crutcher, 1989; Savio and Schwab, 1990; for review, see Schwab et al., 1993). In support of this notion, neutralization of the inhibitory properties of central myelin allows both the regeneration and collateral sprouting of corticospinal fibers (Savio and Schwab, 1990; Schnell and Schwab, 1990; Bregman et al., 1995; Vanek et al., 1998). Another factor that may contribute to the loss of plasticity in the CNS is the lack of appropriate growth-promoting molecules (Schwartz et al., 1989). Menesini-Chen et al. (1978) were the first to show that neurotrophins could promote axon growth within the mammalian CNS. Infusions of NGF into the brainstem of neonatal rats resulted in the ingrowth of sympathetic axons within yet-to-be myelinated tracts of the CNS (Menesini-Chen et al., 1978). However, similar infusions of NGF into the brainstem of adult animals failed to elicit such ingrowth. Since this time, a number of studies have demonstrated that local application of neurotrophins can enhance sprouting responses of various CNS axons (Schnell et al., 1994; Tuszynski et al., 1994; Xu et al., 1995; Sawai et al., 1996; Bregman et al., 1997; Grill et al., 1997; Schwab and Brösamle, 1997; Ye and Houle, 1997). In these experimental paradigms, however, neurotrophin-induced axon sprouting remains primarily confined to peripheral nerve grafts, transplants, and nonmyelinated portions of the mature CNS. Our observation that NGF overexpression in the cerebellum of postnatal transgenic mice elicits the growth of sympathetic axons into the cerebellar white matter complements the findings of Menesini-Chen et al. (1978). Our data further reveal that if NGF levels remain elevated in the CNS as the animals mature, the growth of sympathetic axons within myelinated tracts continues unabated into adulthood. Importantly, Davies et al. (1997) recently showed that adult neurons implanted into the adult CNS white matter are capable of long-distance axonal growth. Using a microtransplantation technique that minimizes glial scar formation, these authors demonstrated that grafted neurons are capable of rapid and extensive growth of new axons through this myelinated environment. Taken together, our study and that of Davies

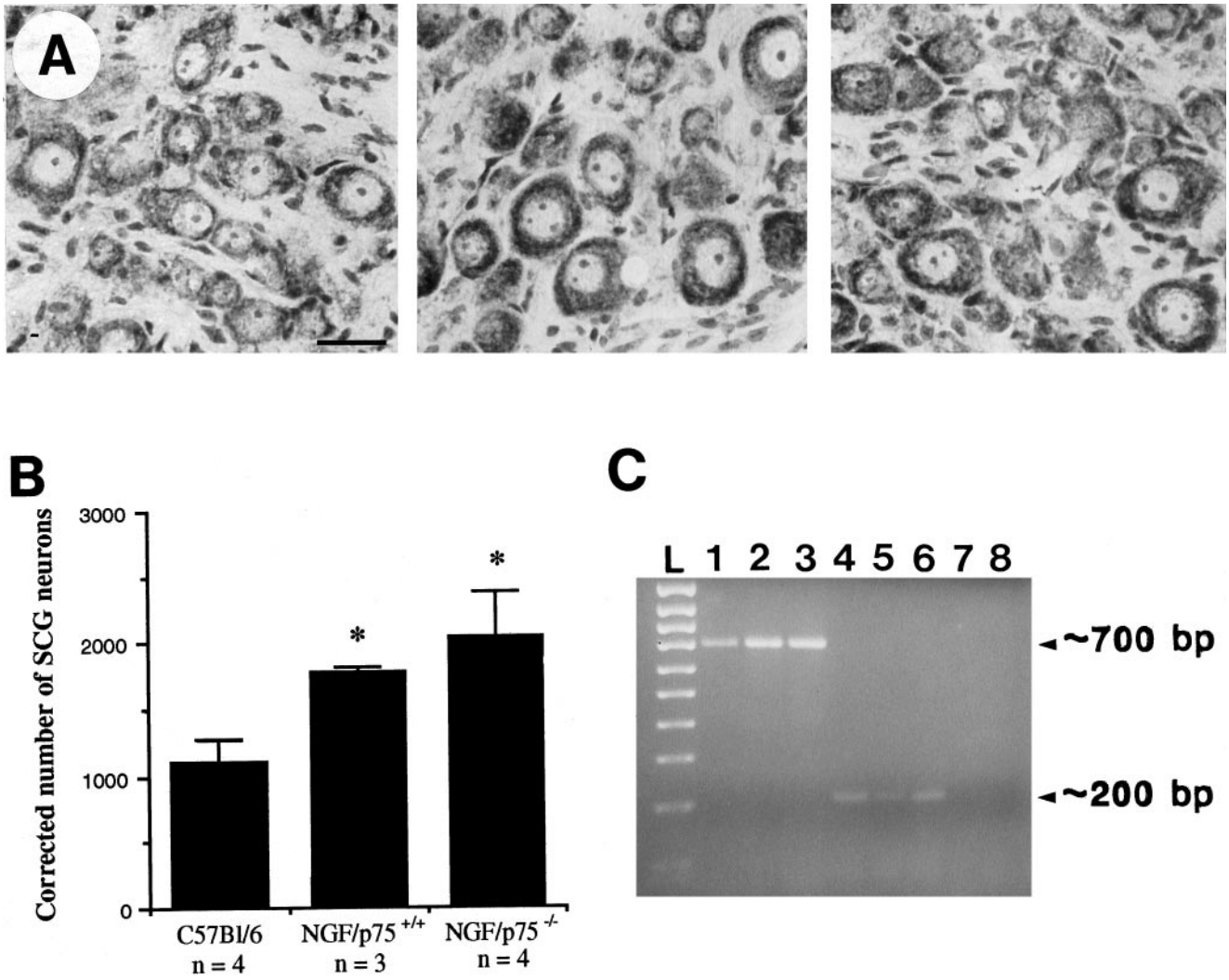

Figure 10. Morphological and neurochemical characteristics of SCG from P60 C57Bl/6, NGF/p75 $^{+/+}$, and NGF/p75 $^{-/-}$ mice. *A*, Nissl-stained sections of SCG from C57Bl/6 (*left*), NGF/p75 $^{+/+}$ (*center*), and NGF/p75 $^{-/-}$ (*right*) mice reveal a greater incidence of multiple nucleoli among somata of both NGF transgenic lines of mice. *B*, Counts of neuronal profiles in SCG of adult animals show a significant increase in the number of SCG neurons in both NGF/p75 $^{+/+}$ and NGF/p75 $^{-/-}$ mice compared with C57Bl/6 mice ($*p < 0.05$). No significant difference was detected in the number of SCG neurons between NGF/p75 $^{+/+}$ and NGF/p75 $^{-/-}$ mice. *C*, RT-PCR amplification of GAPDH and trkA in SCG taken from P60 C57Bl/6, NGF/p75 $^{+/+}$, and NGF/p75 $^{-/-}$ mice. GAPDH cDNA product (~700 bp) is detectable in SCG from C57Bl/6 (*lane 1*), NGF/p75 $^{+/+}$ (*lane 2*), and NGF/p75 $^{-/-}$ (*lane 3*) mice. Likewise, trkA cDNA product (~200 bp) is detectable in SCG from C57Bl/6 (*lane 4*), NGF/p75 $^{+/+}$ (*lane 5*), and NGF/p75 $^{-/-}$ (*lane 6*) mice. No trkA cDNA product is detectable in the absence of RNA template (*lane 7*), and GAPDH cDNA product is not detectable in the absence of the reverse transcriptase enzyme (*lane 8*). *L*, 100 bp ladder.

et al. (1997) provide evidence supporting the notion that axonal growth in myelinated portions of the adult CNS is possible, provided that there is an adequate supply of the appropriate neurotrophin.

An important issue is how sympathetic axons are able to increase in density in the cerebellar DWM of NGF transgenic mice throughout life, despite the presence of central myelin. At the ultrastructural level, TH-IR axons are observed coursing through the cerebellar white matter as part of a small cluster of unmyelinated axons. Thus, although TH-IR unmyelinated axons are seen in contact with intrinsic myelinated fibers, they are also in close apposition to other unmyelinated axons. Our previous work with NGF/p75 $^{+/+}$ and NGF/p75 $^{-/-}$ mice has demonstrated that unmyelinated sensory axons, immunoreactive for the neuropeptide calcitonin gene-related peptide, also invade the cerebellar DWM by the second postnatal week (Kawaja et al., 1997; Coome et al., 1998). Moreover, in both lines of NGF transgenic mice, these sensory axons are immunoreactive for the glycoprotein L1 (Walsh et al., 1998), a cell adhesion molecule that plays an important role in promoting axon fasciculation (Fischer et al., 1986). On the basis of these findings, we propose that sympathetic axons are capable of continued growth within a myelinated environment by using other unmyelinated axons as a substrate for growth. Such a postulate would predict that the expression of cell surface molecules that promote axon–axon fasciculation, such as L1, would be extremely important in allowing neurotrophin-induced growth within myelinated environments of the mature CNS.

Role of the p75NTR in the growth of sympathetic axons

In the present study, we also used a hybrid line of mice, which overexpresses NGF in astrocytes and has a null mutation in

p75^{NTR} (NGF/p75$^{-/-}$ mice), to further clarify the role of p75^{NTR} in neurotrophin-induced axon growth responses. Sympathetic axons invade the myelinated cerebellar tracts of NGF/p75$^{+/+}$ and NGF/p75$^{-/-}$ mice alike, both of which display comparable levels of NGF protein. The most striking observation in NGF/p75$^{-/-}$ mice is that the magnitude of the growth response by sympathetic axons within the cerebellum is greater than that of NGF transgenic mice expressing functional p75^{NTR}. More specifically, the density of sympathetic axons within the cerebellar DWM is increased, and sympathetic axons extend further into the cerebellum of NGF/p75$^{-/-}$ mice compared with NGF/p75$^{+/+}$ mice.

How might the presence of p75^{NTR} limit the growth of sympathetic axons in an NGF-rich target? Because p75^{NTR} is not expressed by glial cells in the cerebellar DWM, attenuation of axon growth must be a consequence of the presence of p75^{NTR} on sympathetic terminals. The levels of p75^{NTR} and trkA in sympathetic neurons usually exist in a ratio of ~10:1 (Chao and Hempstead, 1995), despite the fact that the trkA receptor is necessary for stereotypical NGF responses, including neurite outgrowth (Loeb et al., 1991; Loeb and Greene, 1993). Moreover, sympathetic neurons undergoing collateral sprouting, in response to terminally derived NGF, upregulate only their expression of p75^{NTR} and not trkA (Miller et al., 1994). Consistent with this, sympathetic neurons of the NGF/p75$^{+/+}$ mice have increased levels of expression for p75^{NTR} but not trkA (Coome and Kawaja, 1999). It has been suggested that a selective upregulation in p75^{NTR} may represent an inhibitory feedback loop, whereby the presence of more p75^{NTR} at terminal axons sequesters NGF away from the high-affinity receptor complex and thus attenuates trkA-mediated NGF signaling (Miller et al., 1994). The idea that p75^{NTR} plays a role in the sequestration of NGF on sympathetic terminals is supported by two lines of evidence. First, examining the dissociation of ^{125}I-NGF from distal axons of sympathetic neurons in compartmented cultures, Ure and Campenot (1997) reported that ~85% of axon-associated NGF is surface-bound, only a portion of which is associated with very slow dissociating (high affinity) sites. Second, sympathetic axons that sprout into an NGF-rich tissue display NGF immunoreactivity (Yu and Crutcher, 1995; Coome et al., 1998). This NGF immunostaining at sympathetic terminal axons is dramatically reduced in the absence of p75^{NTR} (Coome et al., 1998). Another possible outcome of p75^{NTR}-mediated sequestration of NGF on distal axons is that p75^{NTR}–NGF receptor–ligand complexes sterically interfere with axon–axon interactions mediated by cell adhesion molecules. Although it is conceivable that factors that inhibit fasciculation could reduce axonal growth responses, there is little evidence supporting such a role for p75^{NTR}.

Finally, the possibility that p75^{NTR} modulates NGF signaling and collateral sprouting responses by directly activating an intracellular pathway should not be ruled out. Recent studies, using cell lines expressing p75^{NTR} in the absence of detectable levels of trkA, have demonstrated that ligand binding of p75^{NTR} can result in the activation and nuclear translocation of the transcription factor NF-κB (Carter et al., 1996), enhancement of jun kinase activity (Casaccia-Bonnefil et al., 1996), and generation of ceramide (Dobrowsky et al., 1994, 1995; Casaccia-Bonnefil et al., 1996). In fact, increased levels of ceramide in the distal neurites of sympathetic neurons perturbs new growth of these fibers (de Chaves et al., 1997). Furthermore, BDNF-mediated activation of p75^{NTR} signaling leads to apoptosis during the period of naturally occurring cell death among sympathetic neurons (Bamji et al., 1998). That such a mechanism could account for our observations of enhanced sympathetic sprouting in the absence of p75^{NTR} remains to be elucidated. Interestingly, addition of BDNF has been shown to inhibit NGF-induced growth of sensory axons (Kimpinski et al., 1997). This study, however, did not examine whether this inhibition was a direct result of p75^{NTR}-mediated signaling. In addition, loading PC12 cells and embryonic sensory neurons with a peptide, identical to a region of the cytoplasmic domain of p75^{NTR}, modulates NGF-induced neurite outgrowth in a manner that appears to be mediated downstream of the ligand–receptor level (Dostaler et al., 1996).

In sum, our data provide evidence demonstrating that supplementation of NGF can elicit the growth of sympathetic axons within myelinated environments of the adult mammalian brain. Furthermore, our results indicate that the growth of sympathetic axons in response to elevated levels of NGF is enhanced when the function of the p75^{NTR} is neutralized. These findings of NGF-induced growth of peripheral axons provide encouragement that similar elongation of CNS axons in myelinated environments of the adult brain is possible in response to other neurotrophins, such as BDNF and NT-3, especially when p75^{NTR} function is perturbed.

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