Overexpression of Nerve Growth Factor in Skin Selectively Affects the Survival and Functional Properties of Nociceptors

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Peripheral sensory neurons are over-produced during embryonic development in mammals. Nearly half of these neurons undergo programmed cell death, leaving the appropriate number and functional types in the adult animal. Programmed cell death occurs at the same time that growing sensory axons make functional connections with their peripheral targets in the embryo. The ability of neurons to acquire limited amounts of neurotrophic factors from target tissues regulates the magnitude and specificity of neuronal survival (for review, see Lewin and Barde, 1996).

Studies of mice that lack a specific neurotrophin ligand or receptor have demonstrated that functionally distinct classes of peripheral sensory neurons depend on specific neurotrophic factors for survival (Snider, 1994). Nociceptors, sensory neurons that respond to actual or potentially tissue-damaging stimuli, depend on nerve growth factor (NGF) for survival (Crowley et al., 1994; Smeine et al., 1994). NGF is expressed at high levels in the skin between embryonic day 11 (E11) and E14, after which it falls sharply (Davies et al., 1987). Because the drop in NGF coincides with the death of approximately half of all sensory neurons (Davies and Lumsden, 1984), we hypothesized that enhanced expression of NGF during this critical time in development would selectively rescue nociceptors.

NGF has also been shown to regulate the phenotype of sensory neurons. Sequestration of endogenous NGF induces neurons that would normally become myelinated nociceptors to instead become low-threshold D-hair receptors (Ritter et al., 1991). Injections of neonatal rats with NGF also increases the mechanical sensitivity of myelinated nociceptors (Lewin et al., 1993) and the heat sensitivity of unmyelinated nociceptors (Lewin and Mendell, 1994). Thus, we hypothesized that embryonic overexpression of NGF would also alter the functional properties of nociceptors.

Transgenic mice have been generated that overexpress NGF in skin under the control of the epidermal keratin (K14) gene promoter (Albers et al., 1994). These NGF-overexpressing (NGF-OE) mice begin to express elevated levels of NGF at approximately E11 and continue throughout adulthood, maintaining high levels of NGF in skin. Previous studies have shown that NGF-OE mice have twice the normal number of sensory neurons in trigeminal and dorsal root ganglia and exhibit marked hypertrophy of peripheral axons and cell bodies (Albers et al., 1994; Jhaveri et al., 1996; Mendelson et al., 1996; Davis et al., 1997; Goodness et al., 1997). NGF-OE mice also have increased innervation of the skin and dorsal horn by calcitonin gene-related peptide (CGRP)-positive fibers (Albers et al., 1994; Mendelson et al., 1996; Davis et al., 1997), which are likely to be nociceptors (Lawson, 1992). Behaviorally, NGF-OE mice are hyperalgesic to mechanical (Davis et al., 1993) and thermal (B. M. Davis and K. M. Albers, unpublished data) stimuli, yet none of these studies has directly determined whether nociceptors are selectively affected or whether the enhanced levels of NGF in NGF-OE mice...
change the functional properties of myelinated or unmyelinated nociceptors. Therefore, we used electrophysiological and morphological techniques to directly determine the prevalence and properties of all types of sensory neurons in a purely cutaneous nerve from adult NGF-OE and control mice.

MATERIALS AND METHODS

Transgenic mice overexpressing NGF in the skin under the control of the K14 keratin promoter (Albers et al., 1994) were used. Mice were screened for the presence of the K14-NGF transgene using slot-blot analysis and mRNA isolated from tail samples. The background strain (B6/C3H) in which NGF-OE mice were generated was used as the control strain.

Nerve fiber counts. Mice were anesthetized and perfused transcardially with 4.0% paraformaldehyde and 2.0% glutaraldehyde in 0.1 M phosphate buffer (PB) (NGF-OE, n = 3; control, n = 3). Segments of the saphenous nerve from the mid thigh were post-fixed, washed in PB, dehydrated in graded alcohols, embedded in Spurr’s resin (EM Corp., Chestnut Hill, MA), and cut at 0.7–0.8 nm on an ultramicrotome. Ultrathin sections were stained with lead citrate and uranyl acetate, photographed on an electron microscope (EM) (H7000; Hitachi Ltd., Tokyo, Japan), and the images were assembled into montages. Myelinated and unmyelinated nerve fibers were analyzed with a computer using NIH Image 1.4 software. The entire nerve was analyzed for axon counts and unmyelinated nerve fibers were analyzed with a computer using a template-matching program (Forster and Handwerker, 1990). The sympathetic nerve with its cutaneous innervation territory was removed from the hindlimb, placed corium side up in a tissue bath, and superfused (15 ml/min) with oxygen-saturated, synthetic interstitial fluid for 10 sec, resulting in a minimum skin temperature of 4–8°C. Care was taken to avoid mechanical stimulation during the thermal stimuli. A fiber was considered to be heat- or cold-sensitive if three or more action potentials were evoked during the stimulation.

Statistical analysis. All values are given as mean ± SEM or as median and interquartile range of the 25th and 75th percentile. Parametric and nonparametric statistical tests were performed as appropriate after fulfillment of all necessary prerequisites using the Statistica software package (StatSoft, Tulsa, OK).

RESULTS

Myelinated and unmyelinated sensory axons are increased in the saphenous nerve of NGF-OE mice

Overexpression of NGF in the skin resulted in a 1.8-fold increase in the total number of axons in the purely cutaneous saphenous nerve, corresponding to the twofold increase in the number of sensory neurons reported previously (Albers et al., 1994; Jhaeveri et al., 1996, Mendelson et al., 1996). This indicates that the increase in axon profiles in the saphenous nerve is caused by an increase in cell number and not axonal branching. Interestingly, this increase was attributable to a significant (p < 0.01; t test) 3.3-fold increase in the number of myelinated axons from 508 ± 3 to 1701 ± 168 and a significant (p < 0.001; t test) 1.5-fold increase in the number of unmyelinated sensory fibers from 2482 ± 151 to 3739 ± 340 axons. This means that there is an increase in the percentage of myelinated fibers from 17% in wild-type animals to 31% in NGF-OE mice. In addition, both myelinated and unmyelinated axons were increased in diameter (Fig. 1A,B). The hypertrophy of unmyelinated axons was particularly dramatic, because most of the unmyelinated axons in NGF-OE mice were larger than any of the unmyelinated axons in control mice. This increase in size was further reflected in the mean axonal diameter of transgenic unmyelinated fibers that was almost twice as large as controls (NGF-OE, 0.89 ± 0.01 μm; control, 0.53 ± 0.01 μm; p < 0.001; Kolmogorov–Smirnoff test).

The mean diameter of the myelinated axons (without the myelin sheath) in NGF-OE mice was also increased compared with controls, although to a lesser extent (NGF-OE, 2.83 ± 0.08 μm; control, 2.14 ± 0.08 μm; p < 0.001; Kolmogorov–Smirnoff test). Sym pathetic neurons also depend on NGF for survival (Gorin...
and Johnson, 1980; Ruit et al., 1990), and increased levels of NGF have been shown to increase the density of sympathetic axons in the periphery (Olson, 1967; Davis et al., 1994, 1996). NGF-OE mice have occasional myelinated postganglionic sympathetic axons that are not found in normal animals (Davis, unpublished observation). Therefore, we repeated the myelinated axon counts in NGF-OE mice that had undergone unilateral lumbar sympathectomies (n = 2) to determine whether the increase in myelinated fibers could be attributable to a contribution of sympathetic neurons. Only small decreases on the order of 10% were observed (mouse 1, 1423 myelinated axons contralateral to lesion, 1237 ipsilateral to lesion; mouse 2, 1543 axons contralateral to lesion, 1440 ipsilateral to lesion). These results suggest that myelinated sympathetic axons did not contribute significantly to the number of myelinated axons in NGF-OE mice and that the increase in myelinated fibers was primarily caused by sensory neurons.

Conduction velocity of sensory neurons remains unaltered in NGF-OE mice

Despite the hypertrophy of axons in NGF-OE mice, the distribution of the conduction velocities of both myelinated (NGF-OE, 9.6 ± 0.6 m/sec; control, 10.2 ± 1.1 m/sec; p > 0.5; t test) and unmyelinated (NGF-OE, 0.63 ± 0.04 m/sec; control, 0.67 ± 0.03; p > 0.2; t test) axons was not significantly different (Fig. 2). Measurement of myelinated conduction velocities for both control and NGF-OE mice (Fig. 2A) appear to be bimodal, with a minimum occurring (between the two peak values) at 10–12 m/sec, as reported previously (Koltzenburg et al., 1997). The similarity in the overall distribution of conduction velocities for both strains suggests that the cutoff point (10 m/sec) for distinguishing Aδ from Aβ fibers is valid.

For myelinated axons, both axon diameter and myelin thickness correlates positively with conduction velocity (Waxman, 1980). Therefore, we also examined myelin thickness in control and NGF-OE axons (Fig. 3). Although there was a significant linear correlation between the thickness of myelin and the axon diameter (control, r = 0.76; p < 0.01; NGF-OE, r = 0.83; p < 0.001) for both genotypes, NGF-OE axons had consistently thinner myelin for a given axonal diameter. This fact is also reflected by calculation of the G-ratio (which equals the axon diameter/total diameter of axon plus myelin sheath). The mean G-ratio for control mice was 0.67 ± .005, whereas it was 0.75 ± 0.005 for NGF-OE mice (p < 0.001; t test). Thus, in NGF-OE mice, the
increase in the diameter of the axon was not accompanied by the same proportional increase in the diameter of the myelin thickness that was observed in control mice.

**Non-nociceptive mechanoreceptors are unchanged in NGF-OE mice**

Because the counts of axons in the saphenous nerve showed that myelinated and unmyelinated axons were increased in NGF-OE mice, electrophysiological techniques were used to directly determine which functional subtypes of afferent neurons were affected. Among the large myelinated, low-threshold Aβ fibers, there was no change in the proportion of SA or RA fibers encountered compared with controls (Fig. 4A). There was also no significant difference in the conduction velocity of SA fibers (NGF-OE, 15.9 ± 0.8 m/sec; control, 15.2 ± 1.9 m/sec; p > 0.5; t test) or RA fibers (NGF-OE, 16.6 ± 0.8 m/sec; control, 16.0 ± 0.6 m/sec; p > 0.5; t test). These data suggest that the increase in the number of myelinated axons in NGF-OE saphenous nerve is not attributable to an increase in SA or RA fibers and that the receptive properties of these units is not influenced by the excess amount of NGF.

**Myelinated nociceptors increase in number**

In contrast to the Aβ fibers, both Aδ and C fiber nociceptors were clearly affected in NGF-OE mice. First, the percentage of mechanosensitive Aδ (AM) nociceptors encountered during neurophysiological recordings was significantly increased. In control mice used in the present study, AM fibers constituted 65% of all Aδ fibers. This percentage of AM fibers was identical to that recorded in two other strains of control mice (Airaksinen et al., 1996; Stucky et al., 1998). In NGF-OE mice, however, AM nociceptors accounted for 97% of all Aδ fibers encountered (p < 0.001; χ²-test) (Fig. 5A). These results are consistent with the notion that the total increase of myelinated axons were caused by a selective survival of AM nociceptors.

**Sensitivity for mechanical, but not for heat, stimuli increases in myelinated nociceptors**

Not only were AM nociceptors increased in survival but individual AM fibers also showed significantly increased mechanical responsiveness (Fig. 5B) that became particularly evident for suprathreshold stimuli. Application of 100–300 mN sustained force evoked discharge rates in NGF-OE AM fibers that were twice as high as those in controls. However, the NGF-induced enhanced responsiveness was not a general attribute for all noxious modalities because the heat sensitivity of AM fibers in NGF-OE mice was unchanged compared with controls (Fig. 5C). There was no significant difference in the percent of AM fibers responding to heat, the threshold for a response, or in the mean spikes per heat stimulus (Table 1). Interestingly, there was a significant increase in the number of AM fibers in NGF-OE mice responding to a noxious cold stimulus, indicating that the cellular elements that mediate thermal transduction are differentially regulated for cold and heat in Aδ nociceptors.

A small number of AM fibers in NGF-OE mice had ongoing
Sensitivity for heat increases and mechanical stimuli decreases in unmyelinated nociceptors

C fiber nociceptors were also affected in NGF-OE mice. In addition to a 50% increase in the total number of C fibers in the saphenous nerve, nearly every C fiber (96%) in NGF-OE mice responded to heat (Table 1). Furthermore, the number of heat-evoked action potentials per C fiber was increased fourfold in NGF-OE mice (Table 1, Fig. 6B). In addition, C fibers in NGF-OE mice exhibited spontaneous activity that was much higher than that in controls. In fact, 60% of C fibers in NGF-OE (16 of 27) had ongoing activity compared with 6.5% of those in control mice (2 of 31; \( p < 0.001 \); \( \chi^2 \)-test). In direct contrast to AM nociceptors, which were increased in mechanical responsiveness, the mean firing frequency of NGF-OE C fibers to strong mechanical stimuli was reduced by half (Fig. 6A), providing another example of the dissociation between mechanical and thermal responsiveness in nociceptors. Also, in contrast to the finding of increased cold sensitivity in Aδ fibers, cold sensitivity was unaltered in C fibers. Together, the differential regulation of mechanical, heat and cold sensitivity, as well as the different prevalence of ongoing activity, suggests that increased availability of NGF in mice regulates the receptive properties of C and Aδ fibers differentially.

DISCUSSION

The principal finding of our study is that increased expression of NGF in the skin from the period of programmed cell death through adulthood selectively rescues myelinated and unmyelinated nociceptors from cell death. Furthermore, NGF specifically alters the receptive properties of nociceptors, and responsiveness to mechanical and thermal stimuli is differentially regulated in subpopulations of thin myelinated and unmyelinated nociceptors.

NGF leads to an increase in the number of nociceptors that affects thin myelinated and unmyelinated fibers differently

Ultrastructural analysis of the saphenous nerve in this study revealed a 1.8-fold increase in axon number, which is consistent with the twofold increase in the total number of sensory neurons in the dorsal root and trigeminal ganglia (Albers et al., 1994; Jhaveri et al., 1996; Mendelson et al., 1996), indicating that the increase in the number of axon profiles is not the consequence of sprouting. Combining results from nerve counts and electrophysiological recordings, we can conclude that mechanosensitive afferents with large myelinated neurons were unaffected by the increased availability of NGF. The lack of effect on large myelinated neurons is in agreement with previous examination of NGF-OE mice that found no change in the Merkel cells (Davis et al., 1997), which depend on their postnatal survival on the innervation by SA fibers and NT-3 (Airaksinen et al., 1996). Whereas the properties and prevalence of slowly and rapidly adapting neurons was unaltered, the increase in cutaneous nociceptors observed in this study is consistent with the fivefold increase observed in trkA-positive sensory neurons in NGF-OE mice (Goodness et al., 1997). NGF-OE mice also have increased innervation of the skin and dorsal horn by CGRP-positive fibers (Albers et al., 1994; Mendelson et al., 1996; Davis et al., 1997), which are likely to be nociceptors (Lawson, 1992).

In absolute numbers, there was an approximately equal increase of 1200 axons for both myelinated and unmyelinated fibers, and, as evidenced by the results of the sympathectomy, the vast majority of these fibers are afferents. However, overexpression of NGF has a differential effect on unmyelinated and thinly myelinated fibers. There are ~300 Aδ axons in the saphenous nerve of wild-type mice. The total number of AM nociceptors has been calculated to be 200 (65% of all Aδ fibers), and the total number of D-hair receptors is ~100 (35% of Aδ fibers) (Koltzenburg et al., 1997). Assuming that the surplus of myelinated axons in...
NGF-OE mice is the consequence of a selective survival of AM nociceptors, there would be 1400 AM fibers and 100 D-hairs. This would result in an AM fiber/D-hair ratio of 93%, which is close to the observed ratio of 97%. Although this altered ratio of AM fibers/D-hairs could be attributable to phenotypic switching, previous experiments suggest that this is unlikely; injection of neonatal rats with NGF does not induce D-hair receptors, which depend on NT-3 (Airaksinen et al., 1996) and NT-4 (Stucky et al., 1998) for survival to undergo phenotypic switching and become AM nociceptors (Lewin et al., 1993).

Among the unmyelinated fibers projecting into a cutaneous nerve in rodents, only 20% are sympathetic fibers (Baron et al., 1988), and this means that there are ~2000 unmyelinated afferent fibers in the saphenous nerve of wild-type mice. The increase of 1200 unmyelinated axons in NGF-OE mice, therefore, translates into an increase of only 60%. This suggests that the survival-promoting effects of NGF are much higher for neurons with thin myelinated axons (which exhibited a sevenfold increase) than for unmyelinated nociceptors. However, unlike the increase in AM nociceptors, the increased percentage of heat-sensitive C fibers cannot be explained by a selective survival effect. In control saphenous nerve, ~800 units (40% of 2000 neurons) are mechanoheat-sensitive nociceptors. In NGF-OE mice, even if the entire increase in C fibers were caused by a selective increase in heat-sensitive fibers, this would result only in 62.5% (2000 of 3200) of all C fibers being heat-sensitive. This percent is much lower than the observed percentage of 96%, implying that NGF is capable of inducing a novel heat responsiveness in C fibers that would normally be heat-insensitive.

Conduction velocity remains unchanged despite the change in axon diameter

Axonal hypertrophy was most pronounced for the unmyelinated NGF-OE fibers in that their size distribution overlapped more with myelinated control fibers than with unmyelinated control fibers. Such hypertrophy was expected because a previous study showed that trkA-positive sensory neurons were markedly increased in somal size compared with cells expressing other neurochemical markers in NGF-OE mice (Goodness et al., 1997). Both control and NGF-OE mice exhibited significant correlation between axonal diameter and the thickness of the myelin sheath, confirming that size plays an important role in determining which axons become myelinated. This is in agreement with previous

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** indicates that percent of fibers in NGF-OE animals is significantly different from that in controls (p < 0.01; χ² test).
### indicates that the mean number of spikes for fibers in NGF-OE animals is significantly different from that in controls (p < 0.001; t test). All other measures do not differ significantly between wild type (WT) and NGF-OE.

Figure 6. A, Mechanical stimulus-response functions for C fibers from control and NGF-OE mice. C fibers in NGF-OE mice were significantly reduced in heat responsiveness compared with control mice (F₁₁₇₀ = 21.8; p < 0.001; ANOVA). B, Average action potentials per second during 15 sec heat ramp stimulus for all C fibers tested with heat stimuli.
studies that have shown that normally unmyelinated axons can induce novel myelination in response to axonal hypertrophy (Voyodic 1989; B. M. Davis, unpublished results). For both NGF-OE and control mice, the apparent threshold for myelination of saphenous axons was ~1 µm because no myelinated axons had diameters <1 µm (Fig. 1A). Because conduction velocity is positively correlated with axon diameter (Arbuthnott et al., 1980; Hoffmeister et al., 1991) the lack of change in the present study was surprising. Because conduction velocity is also correlated to myelin thickness (Waxman, 1980), it is possible that the uniformly thinner myelin sheath in NGF-OE axons relative to controls negates an increase in conduction velocity expected from the increase in axon diameter. In addition the internodal length, as well as the density and efficacy of ion channels, pumps and exchangers at the node of Ranvier have been shown to alter the conduction velocity of an axon (Waxman and Ritchie, 1993), and NGF may alter some of these properties (Lesser et al., 1997).

Differential regulation of the sensitivity of nociceptors to heat and mechanical stimuli

One of the striking observations of this study was the marked change of the receptive properties of nociceptors, which cannot be attributed to survival effects. The increase of sensitivity was modality-specific in the myelinated and unmyelinated subpopulation. Whereas there was a profound increase in the mechanical sensitivity of myelinated nociceptors, the heat responsiveness remained unchanged. The reverse was found for the unmyelinated nociceptors, which showed a striking increase in their heat sensitivity and a decrease in their mechanical sensitivity. This finding is consistent with the possibility that the mechanisms underlying the heat sensitivity in AM and C fiber nociceptors are differently regulated by the increase of endogenous NGF and that the changes are not the consequence of a general increase of excitability in each fiber population. A differential regulation of the thermal and mechanical sensitivity is also apparent in animals lacking the neurotrophin receptor p75 in which AM fibers show a reduced heat sensitivity, whereas that of C fibers is normal (Stucky and Koltzenburg, 1997).

AM fibers

The increased mechanical responsiveness of AM fibers in NGF-OE mice is in agreement with the finding that treatment of neonatal rats with NGF results in increased sensitivity of AM fibers to mechanical stimuli (Lewin et al., 1993) and could explain, at least in part, the mechanical hyperalgesia that NGF-OE mice display. These results are, however, notably different from the effects of an acute application of NGF, which can induce heat sensitivity in previously insensitive fibers without sensitizing the neurons to mechanical stimuli (Rueff and Mendell, 1996). This indicates that the changes observed in this study cannot easily be explained by a maintained high level of NGF expression in NGF-OE mice.

C fibers

Functional analysis of C fibers further confirmed that NGF has an effect on the phenotype of sensory neurons. Virtually every C fiber was heat-sensitive compared with only 42% in control mice, and the response magnitude to heat was fourfold greater than in controls. The observation that sensitivity of the same group of fibers to mechanical stimuli was decreased speaks against a global increase of neuronal excitability. These findings are consistent with the observation that postnatal treatment of neonatal rats with NGF increases the proportion of unmyelinated units that responds to heat (Lewin and Mendell, 1994). One possibility why heat sensitivity is differentially regulated by NGF is the fact that the currently known heat transducers in mammals, i.e., vanilloid receptor 1 (VR1) (Caterina et al., 1997) and VR1-like receptor (Caterina et al., 1999), are differentially expressed on small and large neurons (Caterina et al., 1999).

Ongoing activity

Another striking observation was the finding that 60% of C fibers and 29% of the AM fibers in NGF-OE mice showed ongoing activity. One reason for this may be acute effects of NGF (Koltzenburg et al., 1999). An alternative explanation is that these neurons were sufficiently sensitized to heat to be capable of being activated at 32°C (the temperature of the recording bath). However, this is less likely because many heat-insensitive AM fibers also displayed ongoing activity.

In conclusion, the studies presented here confirm for the first time in vivo that target-derived NGF selectively rescues both myelinated and unmyelinated nociceptive neurons but does not rescue low-threshold non-nociceptive sensory neurons. Moreover, the level of endogenous NGF appears to selectively determine the receptive properties of nociceptors and does this differentially for mechanical and thermal sensitivity of neurons with myelinated and unmyelinated fibers.

REFERENCES


