Cutaneous Overexpression of NT-3 Increases Sensory and Sympathetic Neuron Number and Enhances Touch Dome and Hair Follicle Innervation

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Abstract. Target-derived influences of nerve growth factor on neuronal survival and differentiation are well documented, though effects of other neurotrophins are less clear. To examine the influence of NT-3 neurotrophin overexpression in a target tissue of sensory and sympathetic neurons, transgenic mice were isolated that overexpress NT-3 in the epidermis. Overexpression of NT-3 led to a 42% increase in the number of dorsal root ganglia sensory neurons, a 70% increase in the number of trigeminal sensory neurons, and a 32% increase in sympathetic neurons. Elevated NT-3 also caused enlargement of touch dome mechanoreceptor units, sensory end organs innervated by slowly adapting type 1 (SA1) neurons. The enlarged touch dome units of the transgenics had an increased number of associated Merkel cells, cells at which SA1s terminate. An additional alteration of skin innervation in NT-3 transgenics was an increased density of myelinated circular endings associated with the piloneural complex. The enhancement of innervation to the skin was accompanied by a doubling in the number of sensory neurons expressing trkC. In addition, measures of nerve fibers in cross-sectional profiles of cutaneous saphenous nerves of transgenics showed a 60% increase in myelinated fibers. These results indicate that in vivo overexpression of NT-3 by the epidermis enhances the number of sensory and sympathetic neurons and the development of selected sensory endings of the skin.

NEUROTROPHINS are a group of structurally and functionally related growth factors that exert a major influence on the survival and development of sensory and sympathetic neurons. Members of this family include nerve growth factor (NGF)[1] (Levi-Montalcini, 1987), neurotrophin-3 (NT-3) (Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990), brain-derived neurotrophic factor (BDNF) (Leibrock et al., 1989), and neurotrophin 4/5 (NT-4/5) (Berkemeier et al., 1991; Hallbrook et al., 1991; Ip et al., 1992). During development, sensory neuron targets are thought to regulate the number of neurons that survive by secreting limited amounts of neurotrophic compounds (Korsching and Thoenen, 1983; Shelton and Reichardt, 1984). Neurotrophins exert their trophic roles via receptor-mediated uptake that is followed by retrograde transport to the soma (Thoenen et al., 1987; DiStefano et al., 1992; Curtis et al., 1995). Neurotrophin response is facilitated by two types of transmembrane proteins: p75 receptors (Chao et al., 1986) and members of the trk family of receptor tyrosine kinases (Meakin and Shooter, 1992; Barbacid, 1993). Though p75 binds all neurotrophins, biological specificity and signal transduction are mediated through trk receptors with trkA preferentially binding NGF (Hempstead et al., 1991; Kaplan et al., 1991; Klein et al., 1991), trkB binding BDNF and NT-4 (Ip et al., 1993), and trkC binding NT-3 (Lambelle et al., 1991; Ip et al., 1993).

The specificity of neurotrophin proteins in regulating neuron survival has been investigated using cell culture and in vivo approaches (Lindsay and Rohrer, 1985; Davies et al., 1986; Maisonpierre et al., 1990; Rosenthal et al., 1990; Berkemeier et al., 1991; Hallbook et al., 1991; Ruit et al., 1992; Hory-Lee et al., 1993; Crowley et al., 1994; Klein et al., 1994; McMahon et al., 1994). Transgenic mice that overexpress neurotrophin genes (Edwards et al., 1989; Albers et al., 1994) and mice with targeted disruption of genes encoding neurotrophins or their receptors have been generated to examine this specificity (for review see Snider, 1994). Collectively, these studies indicate that neu-

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1. *Abbreviations used in this paper.* BDNF, brain-derived neurotrophic factor; DRG, dorsal root ganglia; NGF, nerve growth factor; NT, neurotrophin; PB, phosphate buffer; SA1, slowly adapting type 1 neurons; SCG, superior cervical ganglion.

rotrophins have overlapping yet distinct patterns of action in supporting neuronal survival. Neurons responsive to NT-3 include subpopulations of the dorsal root ganglia (DRG) (Maisonpierre et al., 1990), nodose ganglia (Rosenthal et al., 1990), trigeminal ganglia (Buchman and Davies, 1993), cochlear ganglia (Farinas et al., 1994), as well as proprioceptive neurons of the trigeminal mesencephalic nucleus (Hohn et al., 1990) and developing muscle sensory neurons (Hory-Lee et al., 1993). Knockout mice lacking the NT-3 or trkC genes exhibit abnormal movements and lack Ia and Ib sensory neurons and the muscle spindles they innervate (Ernfors et al., 1994; Farinas et al., 1994; Klein et al., 1994; Kucera et. al., 1995a,b). Thus, sensory end organ development appears to be dependent on the survival and differentiation of the associated neurons.

Sympathetic neuroblasts are also dependent on NT-3 and express the trkC receptor (Birren et al., 1993; Dechant et al., 1993; DiCicco-Bloom et al., 1993). As sympathetic neurons mature they lose responsiveness to NT-3, switch neurotrophin dependence to NGF, and express trkA (Birren et al., 1993; DiCicco-Bloom et al., 1993). The dual requirement for NT-3 and NGF is demonstrated by the finding that targeted disruption of either the NGF or NT-3 gene causes a 70–90 and 48% loss, respectively, of superior cervical ganglion (SCG) neurons (Crowley et al., 1994; Ernfors et al., 1994; Farinas et al., 1994).

Skin is a target of sensory and sympathetic innervation that produces limited quantities of NGF, NT-3, BDNF, and NT-4 (Buchman and Davies, 1993). Target field production of these proteins is thought to regulate neuronal survival and in so doing influence the pattern and density of innervation. To test this model we have isolated transgenic mice that overexpress the various neurotrophins in the skin. Mice that overexpress NGF (Albers et al., 1994) have a doubling in sensory neuron number, a major increase in skin innervation density, and overall hypertrophy of the peripheral nervous system. Developmental expression of endogenous NT-3 by skin suggests that, similar to NGF, NT-3 functions to support survival of cutaneous neurons and influence innervation density and character. In mouse whisker pad skin, NT-3 mRNA can be detected by embryonic day 9.5 (E9.5), with a peak level at E13, followed by a decline to lower adult levels (Schecterson and Bothwell, 1992; Buchman and Davies, 1993). Arrival of sensory axons in the periphery, which in mouse whisker pad occurs by approximately E11 (Davies and Lumsden, 1984), is thought to allow survival of the subpopulation of neurons that obtain sufficient neurotrophin. Thus, increased levels of target-derived NT-3 should allow increased survival of neurons sensitive to NT-3. To test this hypothesis, transgenic mice were isolated that overexpress NT-3 in basal keratinocytes of the epidermis using promoter and enhancer sequences of the human keratin K14 gene. Keratin K14 mRNA is expressed in basal keratinocytes of the developing mouse whisker pad epidermis as early as E11 (Byrne et al., 1994). Transgene expression should therefore overlap with endogenous NT-3 gene expression and then continue in the adult.

In this report we provide evidence that overexpression of NT-3 selectively increases the NT-3–responsive population of sensory and sympathetic ganglia. The number of neurons was greater in NT-3 transgenics and this increase was accompanied by changes in peripheral innervation patterns. The size and innervation density of touch dome mechanoreceptor complexes (Haarscheiben), sensory end organs innervated by large, slowly adapting type 1 neurons (SA1s) (Smith et al., 1967; Perl and Burgess, 1973), was enhanced. In addition, the number of myelinated fibers within the circular ending component of piloneural complexes were also enhanced. These changes were accompanied by a doubling in the percentage of trkC-expressing neurons and a 60% increase in the number of myelinated fibers.

Materials and Methods

Generation and Characterization of Transgenic Mice

Transgenic mice were generated by linking a PCR-cloned NT-3 cDNA downstream of 2 kbp of the human K14 keratin promoter and enhancer sequence (Vassar and Fuchs, 1991). NT-3 cDNA was cloned by amplification of DNA isolated from tail using primers (5′CCAGCGGGATCC-GTGATGTCCATCTTGTTTTATGTG 3′ and 5′CGGTACGGATCC-GATGCCAATTCATGTTCTTCCG 3′) that amplified the DNA sequence encoding amino acids −140 to +124 according to the numbering of Yancopoulos et al. (1990). This region contains the putative signal peptide (−140 to −1), the mature peptide (+1 to +119), and the NT-3 stop codon (+120). To facilitate cloning, primers were generated to contain a BamH1 recognition sequence (underlined in primer sequence). Sequencing of the cloned NT-3 showed a nucleotide change that resulted in a conservative amino acid change (ala→val) in the signal peptide region at amino acid residue (−9). The K14-NT-3 transgene was gel purified from bacterial sequences, microinjected into pronuclei of B6xC3 F1 hybrid mouse embryos, and implanted into pseudopregnant females as previously described (Albers et al., 1994). Founder and F1 generations were analyzed by Southern blotting to identify positives and verify transgene size. Subsequent generations were screened using slot blot analysis of isolated tail DNA. Copy numbers were estimated by binding 2 μg of DNA to Nytran membrane, hybridizing with a ^{32}P-labeled probe to the NT-3 sequence, and using software (Image 1.4; National Institutes of Health) to measure relative band intensity by densitometry.

RNA was purified using Trizol reagent (GIBCO-BRL, Gaithersburg, MD) according to the manufacturers protocol. 10 μg of RNA was resolved on a 1.2% agarose formaldehyde denaturing gel, transferred to Nytran, baked at 80°C, and hybridized at 65°C in the presence of 50% formamide to a [^{32}P]CTP-labeled riboprobe made to the full-length NT-3 sequence. Membranes were hybridized overnight, washed, and exposed to x-ray film.

Histology and Immunohistochemistry

Immunohistochemistry. Skin from 4–8-mo-old age-matched mice was analyzed. Mice were deeply anesthetized with Avertin (Aldrich Chemical Co., Milwaukee, WI) and transcardially perfused with 4% paraformaldehyde in phosphate buffer (PB). Tissue was removed and immersion fixed for at least 4 h, placed in 25% sucrose overnight, embedded in gelatin and cut at 40-μm thickness on a sliding microtome. Sections were blocked 1–2 h in 5% normal goat serum (NGS), 2% BSA, and 0.25% Triton X-100 made in TBS (100 mM Tris, 5 mM NaCl, pH 7.4), incubated overnight at room temperature in primary antibody (anti-PGP 9.5, 1:5,000; anti-NF150, 1:3,000; dilutions made in 5% NGS and 0.25% Triton), washed, and incubated 1 h in a 1:500 dilution of biotinylated goat anti–rabbit secondary followed by a strep-avidin complex incubation (Vector Laboratories, Inc., Burlingame, CA). Antibody binding was visualized using a nickel cobalt-enhanced diaminobenzidine reaction. Sections were washed, mounted on slides, and counterstained with methyl green.

Merkel Cell and Touch Dome Counting. Touch domes and Merkel cells associated with touch domes were identified on flank skin by depilating skin and 24 h later injecting quinacrine (Sigma Chemical Co., St. Louis, MO) (15 mg/kg; made in PBS) intraperitoneally. Quinacrine is a fluorescent compound that concentrates in neuroendocrine cell types (Nurse and Diamond, 1984). After 12–20 h mice were deeply anesthetized, killed by cervical dislocation, and flank skin removed. Skin was trimmed of connective tissue, mounted on a glass slide with a coverslip using glycerol, and

Merkel cells associated with touch dome units counted using FITC fluorescent optics. The number of touch domes per cm² of flank skin was determined in a similar manner.

In Situ Hybridization

In situ hybridization was carried out on tissues from age-matched mice between 7 and 63 wks old using ³⁵S-labeled probes as previously described (Albers et al., 1994). The trkC probe encompassed nucleotides 1,119–1,493 of rat trkC cDNA (Merlio et al., 1992; Dixon and McKinnon, 1994) and recognized full-length and truncated receptor forms. Sense transcript controls showed no specific hybridization.

Nerve Fiber Count Analysis

Age-matched adult mice were deeply anesthetized and perfused transcardially with 2.0% paraformaldehyde, 2.0% glutaraldehyde, and 1.0% acrolein in 0.1 M PB. Saphenous nerve segments from mid-thigh level were postfixed overnight, 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 (H7000; Hitachi Ltd., Tokyo, Japan), and the images assembled into montages. Myelinated and unmyelinated nerve fibers were counted and analyzed with the aid of a computer using Image 1.4 software. Nerve counts and Merkel cell counts were analyzed from the same mice. Differences were tested using a two-tailed t-test.

Cell Counting Methodology

Counts of L4/L5 neurons and sympathetic neurons were made by determining the number of neuronal nucleoli (Coggeshall et al., 1984, 1990). Age-matched adult mice were deeply anesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 M PB, pH 7.4. Ganglia were removed, immersion fixed in 4% paraformaldehyde, dehydrated in alcohol, defatted, and embedded in paraffin. Ganglia were serial sectioned at 5 μm, stained with hematoxylin and eosin, and examined at a total magnification of 200. A drawing tube was used to trace the outline of the section and demarcate the location of neurons containing a nucleolus. Counts were made of right and left ganglia of each mouse and averaged.

Counts of neurons in trigeminal ganglia of age-matched adult mice were made using the optical disector method (software; Bioquant, Nashville, TN; West, 1993; Harding, 1994). Counts and areas of neurons in every fifth section of the ganglia were sampled on 20-μm-thick fraction heights of a 30-μm-thick tissue section. This fraction (the z-axis) was determined using a stage encoder attached to the microscope. The top and bottom 5 μm were discarded to establish the height of the disector counting box. The length and width of the box was 50 × 50 μm². The estimated total neurons in the ganglion were calculated as products of all counts made in disectors multiplied by reciprocals of all sampling fractions. Counts were made of right and left trigeminals of each mouse and averaged.

Counts of trkC-expressing neurons were done on every tenth section of serial-sectioned ganglia processed for in situ hybridization. Transgenic and control ganglia were processed in parallel. To correct for counting errors due to variation in neuron size, recursive translation analysis was performed. Perimeters (p) of labeled neuronal profiles were drawn by camera lucida and cross-sectional areas (A) of each profile calculated from a digitized image of the drawing. The radius (r) of each profile was approximated using $r = \sqrt{(A/p)}$. The frequency histogram of the profile radii was transformed to an estimate of the true frequency histogram of neuronal radii with a computer program developed by Rose and Rohrlich (1987). Summing the frequencies in all bins of the resulting histogram gave an estimate of cell number in one section of a ganglion. The recursive translation method was repeated on equally spaced 20-μm serial sections (for example, on every n^{th} section) throughout the ganglion. Summing the number of cells across sampled sections and multiplying the total by n gave an unbiased estimate of the number of labeled cells. Differences in values were tested using a two-tailed t-test.

Results

Generation of NT-3 Overexpressing Transgenic Mice

Four K14-NT-3 (Fig. 1 A) founder mice (696-2, 694-2, 709-9,
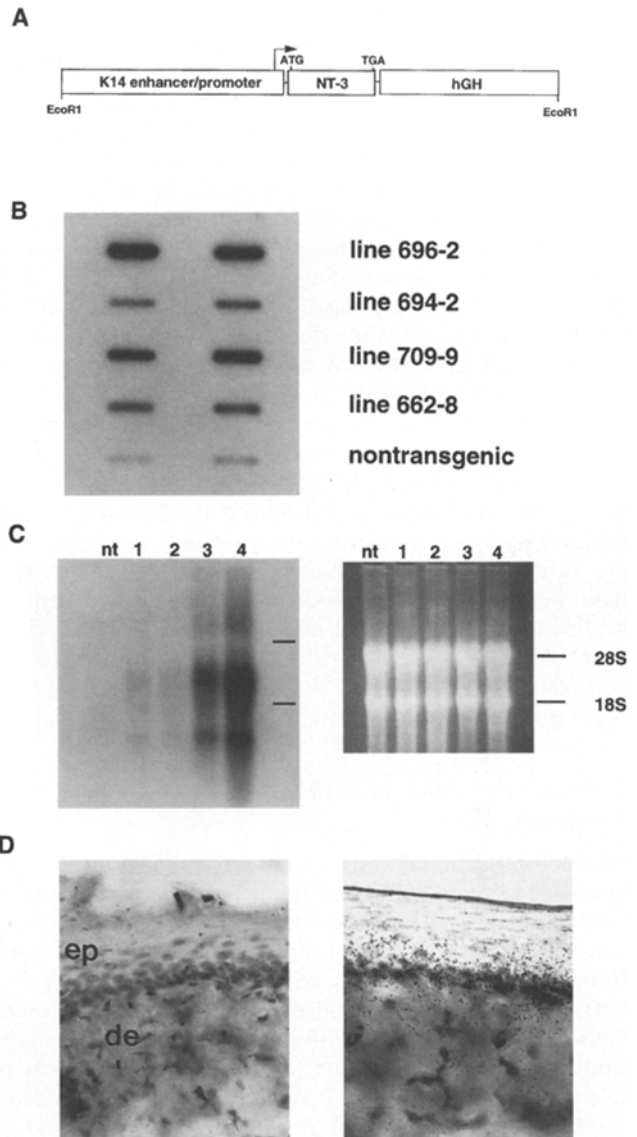

Figure 1. Isolation and characterization of K14-NT-3 transgenic mice. Overexpression of the NT-3 cDNA in skin was driven by promoter and enhancer sequences of the human K14 keratin gene which directed transgene expression to basal keratinocytes of the epidermis (*A*). Arrow indicates transcriptional start site. Four founder mice (696-2, 694-2, 709-9, and 662-8) were identified by Southern hybridization (not shown) using a random primed [³²P]dCTP-labeled probe made to the full-length NT-3 cDNA. Transgene copy numbers were determined by film densitometry of slot blots (*B*) and were estimated to be 8 (line 696-2), 2 (line 694-2), 5 (line 709-9), and 3 (line 662-8). Relative levels of transgene expression in skin was measured by Northern analysis (*C*) of RNA isolated from back skin. The autoradiogram of the probed membrane is shown on the left, the ethidium bromide stained gel on the right. A major transcript hybridized to a ³²P-labeled NT-3 probe at the approximated size of the transgene of 2 kb. With extended exposure of the film the endogenous NT-3 transcript (1.3 kb) was also visible. Lines demarcate positions of the 28S and 18S ribosomal RNA bands. Lane *nt*, nontransgenic RNA; lane *1*, 662-8 RNA; lane *2*, 709-9 RNA; lane *3*, 694-2 RNA; and lane *4*, 696-2 RNA. (*D*) In situ hybridization using a ³⁵S-labeled antisense probe made against the NT-3 mRNA confirmed K14-NT-3 transgene expression in basal keratinocytes of the epidermis (*right*). No specific signal was detected in sections from control skin (*left*) for the 2-wk exposure period. *Ep*, epidermis; *de*, dermis.

and 662-8) and F1 offspring were identified by Southern hybridization using a probe specific to the NT-3 cDNA sequence. Homozygous K14-NT-3 offspring were viable and normal in appearance though did not reproduce well and generally had small litters. The reason for this low fecundity is unclear, though ovaries from these mice were smaller and had fewer ruptured (matured) follicles than sibling controls. Because of poor reproductive yields of homozygotes, experimental analyses were restricted to hemizygous transgenics. Screening of later generation mice was carried out using slot blot hybridization of isolated tail DNA. By film densitometry, transgene copy number of hemizygotes were estimated to be 8 (line 696-2), 2 (line 694-2), 5 (line 709-9), and 3 (line 662-8) (Fig. 1 B). Relative levels of transgene mRNA expression was determined using Northern analysis of RNA isolated from back skin (Fig. 1 C). A ^{32}P-labeled riboprobe made to the full-length NT-3 sequence hybridized at $\sim$2 kb. Hybridization was also to a smaller transcript which may be a splicing variant. That transgene expression was localized to basal keratinocytes of the skin was verified by in situ hybridization analysis of tail skin using a ^{35}S-labeled riboprobe to the NT-3 cDNA (Fig. 1 D). The two lines (696-2 and 694-2) that expressed the highest levels of the K14-NT-3 transcript were used for these studies.

Sensory Innervation of Skin Is Altered in NT-3 Transgenic Mice

Histological examination of transgenic skin showed a remarkable increase in the size of touch dome mechanoreceptors; in some cases transgenic touch domes (Fig. 2 B) were nearly double the size of control touch domes (Fig. 2 A). In dermal tissue beneath touch domes an increased cellularity was consistently observed that may represent Schwann cells associated with innervating fibers. This possibility was suggested by an increased labeling intensity in this region by an antibody (O1) that recognizes a galacto-cerebroside component of myelin protein expressed in Schwann cells (not shown). Further analysis using electron microscopy will verify the identity of these cells.

To examine the nature of innervation to the skin and touch dome units, sections of control and transgenic whisker pad and flank skin were immunolabeled using an antibody against protein PGP 9.5 (Fig. 2, C, D, G, H), which recognizes all neuronal fiber types, and an antibody against neurofilament 150 (NF 150; Fig. 2 E, control; 2 F,

transgenic), an intermediate filament protein expressed primarily in myelinated axons. An overall increase in the density of innervation to the dermis and epidermis of transgenics was apparent (Fig. 2 C, control; 2 D, transgenic), particularly in association with the larger touch dome units in the transgenic skin (Fig. 2 E, control; 2 F, transgenic) and hair follicles (Fig. 2 G, control; 2 H, transgenic). This increase in innervation density was found in all regions of the body examined which included the whisker pad, foot pad, dorsal skin, and flank skin.

The enhanced innervation to hair follicles was most apparent on the follicle where circumferential terminations are located (Fig. 2 G, control; 2 H, transgenic). These terminations are found as either unmyelinated endings associated with smaller vellus hairs or as a component of piloneural complexes (Rice et al., 1993). Piloneural complexes are baskets of neuronal endings associated with larger follicles (guard, tylotrich) at approximately the mid-follicle level. They consist of longitudinally arranged myelinated nerve endings (lanceolate endings) that are encircled by circumferentially oriented myelinated endings (Rice et al., 1993; Fundin and Rice, 1995). In NT-3 transgenic skin, the circumferential ending component of piloneural complexes was richly enhanced (Fig. 2 H) compared with the generally few number of circular endings associated with control follicles (Fig. 2 G). These endings were detectable using both PGP 9.5 and NF 150 (not shown) immunolabeling, indicating that they are primarily, though perhaps not exclusively, myelinated fibers.

Merkel Cells Associated with Touch Dome Complexes Are Increased in Number

SA1 afferents that innervate touch domes terminate on specialized neuroendocrine cells known as Merkel cells. Merkel cells are thought to act as transducers of tactile stimulation, presumably via release of neurotransmitters stored in dense-core granules in the cell (Chen et al., 1973; Gottschaldt and Vahle-Hinz, 1981; Pasche et al., 1990). To determine whether Merkel cell numbers increase in conjunction with the increase in touch dome area and innervation, control and transgenic mice were injected with quinacrine, a fluorescent compound that is selectively concentrated in neuroendocrine cell types (Nurse and Diamond, 1984). Transgenic skin had a statistically significant ($P < 0.001$) increase in the number of Merkel cells per touch dome (Fig. 3 A). Since overexpression of NT-3 increased the

Figure 2. Histological and immunocytochemical analysis of transgenic skin. Flank skin from control (*A*) and transgenic (*B*) mice was stained with hematoxylin and eosin. A conspicuous feature of the transgenic skin was the enlarged touch dome mechanoreceptor units (*area in brackets*). Also noted was an increase in cellularity beneath transgenic touch domes (*arrowheads*), which may represent Schwann cells associated with innervating fibers. Innervation to skin was detected by immunolabeling tissue sections using anti-PGP9.5 (*C, D, G,* and *H*) and anti-NF150 (*E* and *F*) antibodies. PGP9.5 immunoreactivity was typically more extensive in transgenic skin (*D*) compared to control skin (*C*), particularly in areas surrounding hair follicles and touch domes. The increase staining may have both sensory and sympathetic fiber components. Touch domes of NT-3 mice (*F*) labeled with NF 150 antibody were larger and appeared to have greater innervation density than control skin touch domes (*E*). Putative Merkel cells are indicated by arrows in *E*. The number of circular endings comprising the piloneural complexes of NT-3 transgenic skin (*H, arrowheads*) were greatly enhanced compared with circular endings of control mouse piloneural complexes (*G, arrowheads*). *G* and *H* show endings on guard hairs of whisker pad skin. Similar enhancement was found associated with hair follicles of flank skin. Open arrows in *G* and *H* point to a cluster of free nerve endings in the epidermis ("bush endings"; Rice et al., 1993). The bush ending in *H* is out of the plane of focus and so appears less dense than example in *G*. Staining of muscle tissue is nonspecific background. *Ep,* epidermis; *de,* dermis; *hf,* hair follicle; *h,* hair; *n,* nerve fibers; *sg,* sebaceous gland; *m,* muscle. Bars, *A–D,* 50 μm; *E–F,* 25 μm; *G–H,* 25 μm.
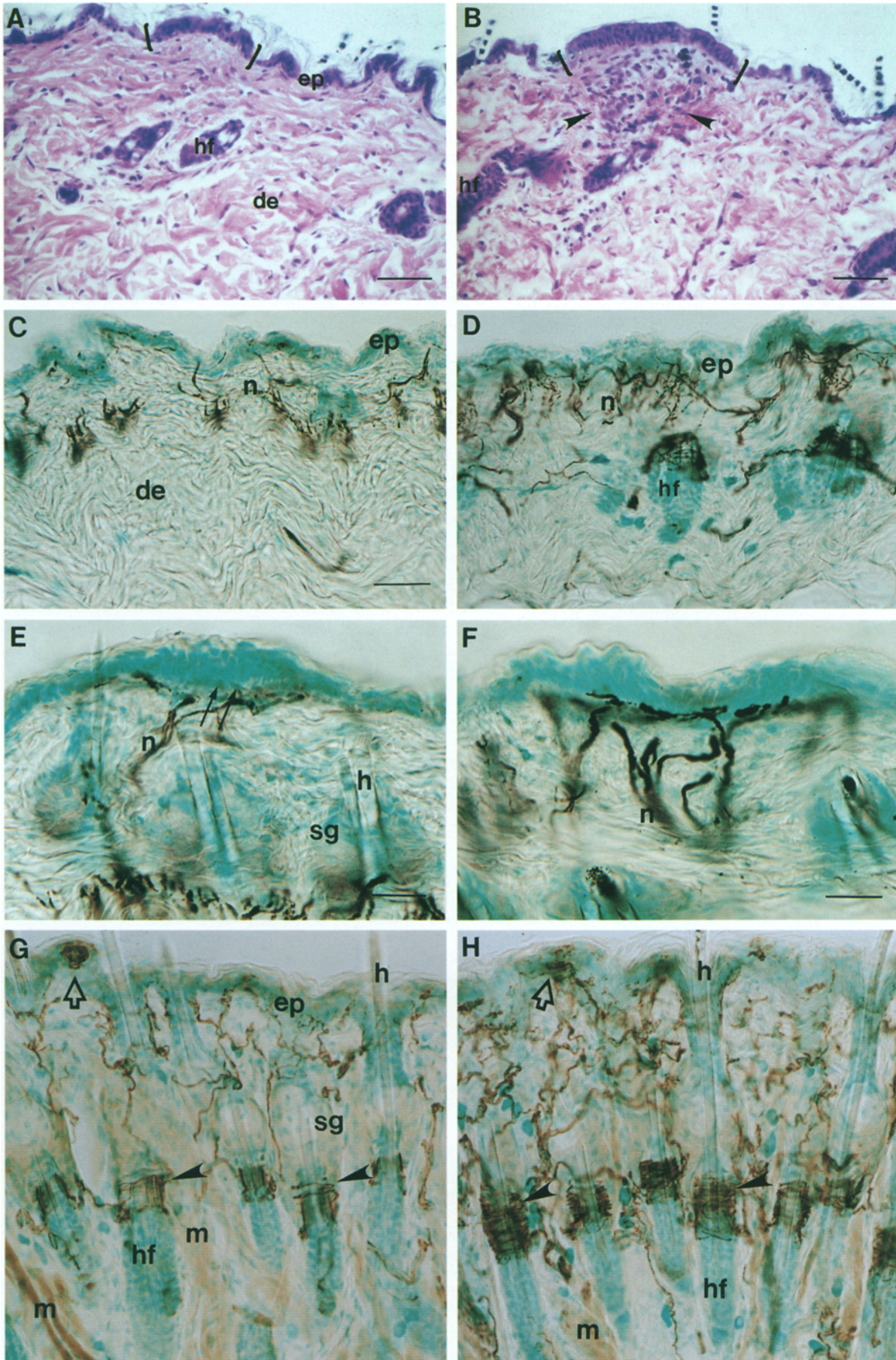
number of termination sites of the SA1s (i.e., Merkel cells), we examined whether the overall number of touch domes was affected by their overabundance. Counts of touch domes in a 1 cm² area of control and transgenic flank skin (taken from identical regions) showed no difference between control and transgenics (Table I). Thus, excess NT-3 in the skin, though able to increase the number of Merkel cells, did not increase the number of touch dome end organs.

Overexpression of NT-3 Increases the Number of Sensory and Sympathetic Neurons and Axonal Diameter

Cell culture studies have shown that addition of NT-3 to culture medium supports the survival of subpopulations of sensory neurons. To determine whether elevated levels of NT-3 may similarly enhance survival of sensory neurons in vivo, the number of neurons in trigeminal and dorsal root sensory ganglia of adult NT-3 transgenic and control mice were counted (Table II). We found that NT-3 transgenic mice had a 70% increase in the number of trigeminal neurons and a 42% increase in DRG neurons when compared with control ganglia. Somal areas of trigeminal neurons in transgenic ganglia also were, on average, 23% larger (DRG neurons were not measured). This response may reflect an increase in axonal branching of NT-3–responsive neurons.

Because NT-3 is known to affect sympathetic neuron survival, the number of neurons in adult SCG was measured. This analysis showed a 32% increase in SCG neurons. The average number of SCG neurons in NT-3 transgenics ($n = 3$) was 24,372 ± 255 compared with the average control value ($n = 3$) of 18,495 ± 963 ($P < 0.005$).

NT-3 is known to support the survival of myelinated sensory neurons of large axonal caliber that innervate
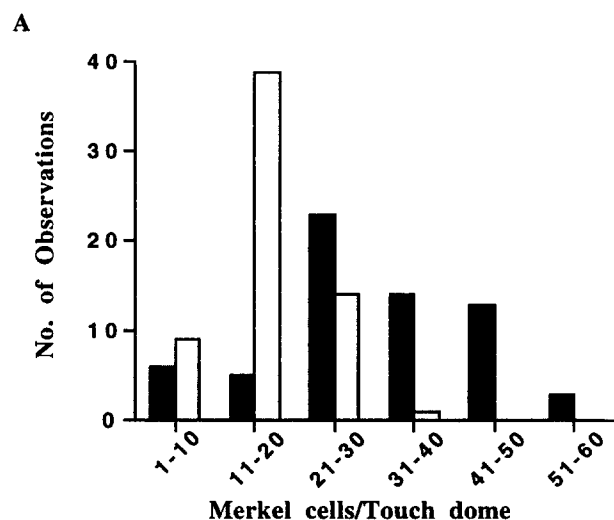
A

Figure 3. Enhanced expression of NT-3 increases the number of Merkel cells. The number of Merkel cells per touch dome unit was increased in NT-3 transgenics (*black bars*) when compared with control measures (*white bars*). Quinacrine-labeled Merkel cells associated with touch domes were counted on flank skin from three transgenic and three control mice using a Zeiss microscope equipped with FITC optics.

Table I. Measure of Touch Dome Density

Sample	Touch domes/cm²	Average
Transgenic line 696-2	23	
	27	
	25	25
Controls	21	
	29	
	25	25

Touch dome density on adult transgenic and control skin was determined on 1 cm² whole mount skin sections isolated from corresponding regions of the flank. Analysis was performed on three transgenic and three control mice. Although transgenic touch domes were larger in size and had more Merkel cells, the density of touch dome units per cm² of flank skin (25 U/cm²) was unchanged between transgenic and control samples.

proprioceptive end organs of muscle (Ernfors et al., 1994; Farinas et al., 1994). To determine if overexpression of NT-3 in epidermis affected the character of nerve fibers innervating the skin, cross-sectional profiles of the cutaneous saphenous nerve were analyzed. Comparison of nerve profiles on a gross level showed the average area of transgenic nerves (Fig. 4, *right*: 34,016 mm²; $n = 3$) to be nearly double the area of control nerves (Fig. 4, *left*: 17,196 mm²; $n = 3$). This increase was reflected by an increase in the number of axons counted on photographic montages made from electron microscopic images of nerves. Myelinated axon counts from control (521 ± 6.8 SEM) and transgenic (839 ± 11.8 SEM) mice showed transgenic nerves to have ~60% more myelinated fibers ($P < 0.0001$) than controls (Table III). A 65% increase in the number of unmyelinated fibers was also measured in transgenic samples (3,726 ± 221) compared with control (2,248 ± 331) samples. There was no difference in the percent of each fiber type between control and transgenic samples: both had ~19% myelinated fibers and 81% unmyelinated fibers.

To determine whether NT-3 expression affected the size of myelinated axons, diameters were measured of 150 axons chosen in a uniform random manner (Fig. 5). Total diameters (axon plus myelin sheath; Fig. 5, *A* and *D*), axon alone (Fig. 5, *B* and *E*), and myelin sheath alone (Fig. 5, *C*

Table II. Number and Somal Area of Neurons of Sensory Ganglia of NT-3 Transgenic and Control Mice

Sample	L4/L5 DRG neuron number	Trigeminal neuron number	Trigeminal neuron area (± SEM)
Transgenic line 696-2			
	18,153	48,469	234.3 ± 8.6
	19,448	41,719	235.1 ± 8.4
	20,667	40,969	270.6 ± 9.1
Average	19,423 ± 726	43,719 ± 2900	246.7 ± 8.7
Controls			
	14,860	25,312	189.2 ± 8.0
	14,403	25,312	201.2 ± 8.2
	11,766	26,719	211.5 ± 8.1
Average	13,658 ± 964	25,781 ± 574	200.6 ± 8.1

Cell counts of L4/L5 DRG and counts and somal areas of trigeminal ganglion neurons from three transgenic and three control mice were measured. Transgenic DRGs had a 42% increase in neuron number whereas trigeminal ganglia had a 70% increase in neuron number and a 23% increase in area. Areas of DRG neurons were not measured.
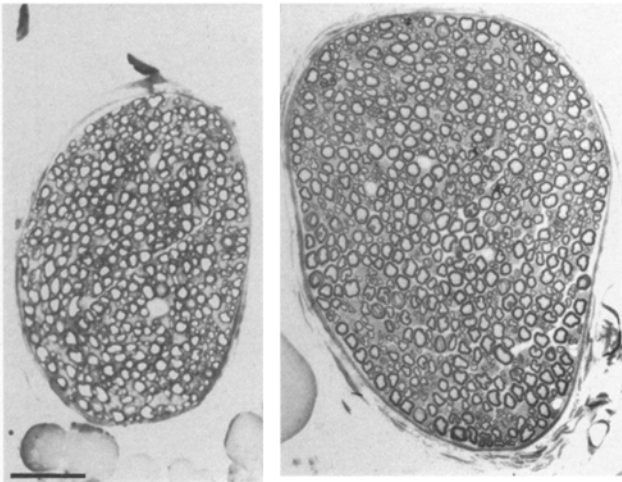
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Figure 4. Enhanced expression of NT-3 increases the number of myelinated and unmyelinated sensory neurons. Cross-sectional areas of cutaneous saphenous nerves ($n = 3$) of transgenic mice (*right*; 34,016 μm^2) were nearly double in size compared with areas of nerves ($n = 3$) from age-matched control mice (*left*; 17,196 μm^2, $P < 0.001$). Bar, 50 μm.

and *F*) were measured and plotted as frequency distribution histograms. Total diameter measures indicated a shift from a unimodal distribution in control mice (Fig. 5 *A*), to a bimodal distribution in transgenic mice (Fig. 5 *D*; $P < 0.0001$). Though this shift suggested that the population of small- to mid-sized axons was depleted and the population of large-sized axons enhanced, replotting the distribution to represent the total number of axons in each category showed an increased number of axons in both the small and large subclasses (not shown). Little to no change was found in the number of medium-sized axons.

To determine whether the increase in fiber size resulted from an increase in axon thickness, myelin thickness, or both, separate measures of each were performed. Axon thickness was larger in transgenic nerve samples (Fig. 5 *E*) when compared with control nerve measures (Fig. 5 *B*; $P < 0.005$). Measures of myelin thickness in control (Fig. 5 *C*) and transgenic (Fig. 5 *F*) also showed transgenics to have,

Table III. Number of Myelinated and Unmyelinated Nerve Fibers in NT-3 Transgenic (NT-3 TG) and Control Saphenous Cutaneous Nerves

	Unmyelinated nerve fibers		Myelinated nerve fibers	
	NT-3 TG	Control	NT-3 TG	Control
Nerve count	3726 ± 221	2248 ± 331	839 ± 11.8	521 ± 6.8
Percent increase	65	NA	60	NA
Percentage of total nerve count	82	81	18	19

Table III shows counts of fibers obtained from ultrathin sections of nerves from three transgenic and three control mice. Counts from photographic montages of electron microscopic images showed a 65% increase in unmyelinated fibers and a 60% increase in myelinated fibers in transgenic nerves. Though the percent of unmyelinated and myelinated fibers increased, the overall percent of the two fiber types was unchanged. Counts are $\pm$ SEM.

on average, a thicker myelin sheath, with a significant shift to the large end of the distribution plot compared with control values ($P < 0.0001$).

Overexpression of NT-3 Leads to Increased Numbers of TrkC Receptor Expressing Neurons

The biological effects of NT-3 are primarily mediated through the trkC receptor tyrosine kinase (Lambelle et al., 1991). To determine if increased availability of NT-3 affected the number of sensory neurons that express trkC, in situ hybridization using a ^{35}S-labeled antisense probe to the trkC mRNA was carried out on trigeminal ganglia (Fig. 6). Ganglia from adult mice of the 696-2 and 694-2 transgenic lines showed on average a 123% increase in the number of trkC-expressing neurons compared with controls (Table IV). Thus, elevation in target field synthesis of NT-3 can increase the number of neurons responsive to this ligand.

Discussion

Our studies of transgenic mice that overexpress NT-3 show that increased levels of NT-3 produced by the epidermis caused an increase in the number of neurons in adult sensory and sympathetic ganglia and alteration of neuronal innervation to the skin. Expression of NT-3 caused a 42–70% increase in sensory neuron number indicating that some, though not all, neurons were affected. This result differs from the 100% rescue found in ganglia of NGF-overexpressing mice (Albers et al., 1994) and illustrates the functional specificity of neurotrophins on sensory neurons. In addition, these in vivo results are in agreement with cell culture studies of trigeminal neurons isolated from E12 embryos in which application of NT-3 led to a 65% increase in survival compared with a 100% survival of cultures supplemented with NGF (Buchman and Davies, 1993). As well, NT-3 gene knockout mice had up to a 61% loss of trigeminal neuron populations (Farinas et al., 1994), suggesting that NT-3 transgene expression increased the survival of the population of sensory neurons lost in NT-3 knockouts.

In addition to increasing sensory neuron number, NT-3 overexpression increased the number of SCG-sympathetic neurons 32% over control values. This increase is less than that measured in cell culture studies in which NT-3 supplementation supported survival of dividing neuroblasts isolated from either chick (Dechant et al., 1993) or rat (Birren et al., 1993; DiCicco-Bloom et al., 1993) sympathetic ganglia. Addition of NT-3 (3 ng/ml) to cultures of sympathetic neuroblasts isolated from E14.5-old rat embryos increased the number of surviving cells by 75–90% (Verdi and Anderson, 1994). In mice lacking NT-3, an approximate 50% loss of SCG neurons was measured in newborn animals. Both the culture and knockout data show a higher percentage of NT-3-responsive sympathetics than the 32% measured in the NT-3 overexpressers. This difference may occur because only a portion of the SCG neuronal population innervate the skin and thus have direct access to the higher NT-3 level.

Analysis of NT-3 and trkC gene knockout mice have shown that proprioceptive IA and IB sensory neurons and
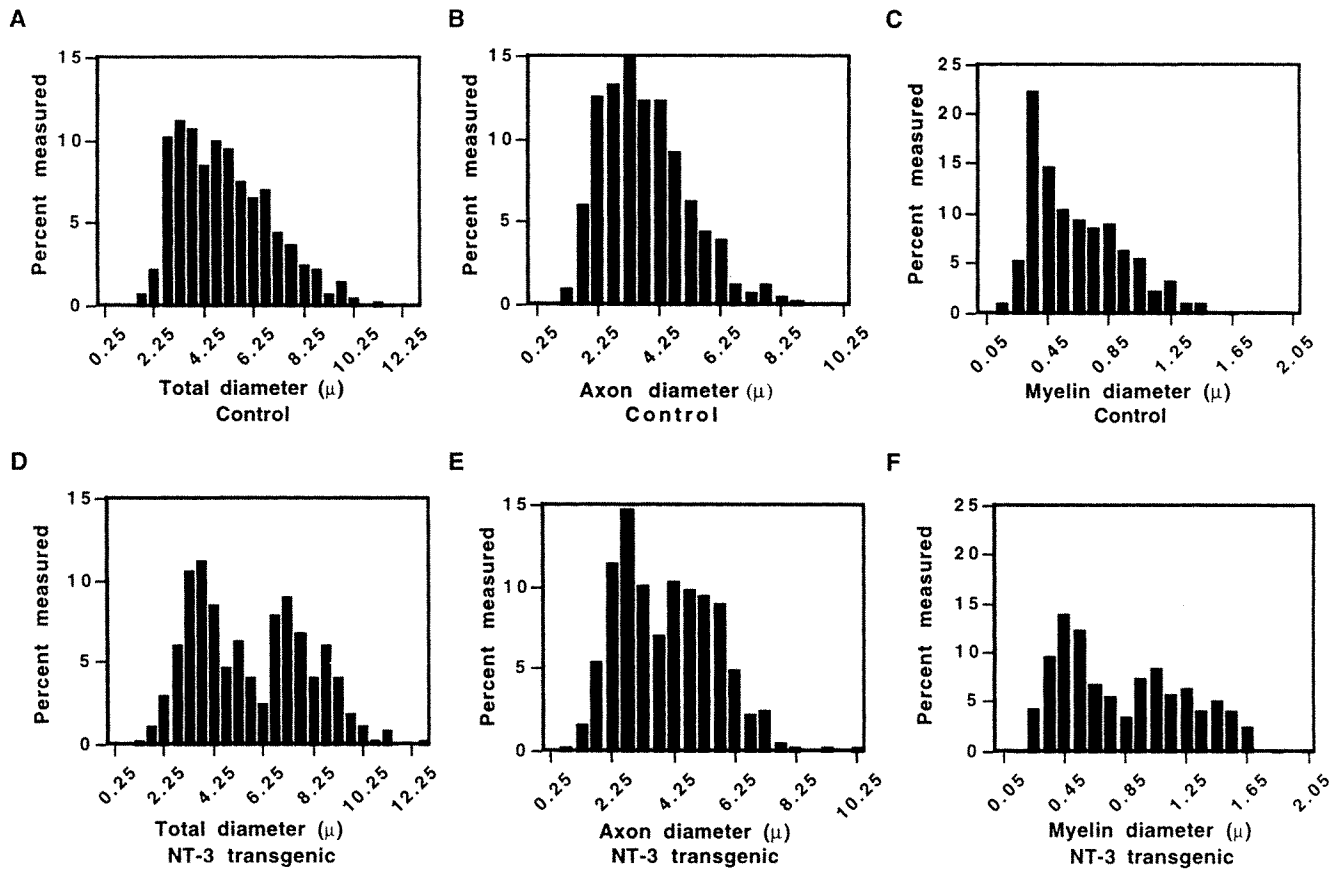
A

Percent measured

Total diameter (μ)
Control

B

Percent measured

Axon diameter (μ)
Control

C

Percent measured

Myelin diameter (μ)
Control

D

Percent measured

Total diameter (μ)
NT-3 transgenic

E

Percent measured

Axon diameter (μ)
NT-3 transgenic

F

Percent measured

Myelin diameter (μ)
NT-3 transgenic

Figure 5. NT-3 increases the diameter of myelinated axons. To determine whether the size of myelinated nerves of transgenics were altered from control nerves, diameters of a randomly selected population of myelinated axons from three control (*A, B,* and *C*) and three transgenic (*D, E,* and *F*) mice were measured. Total diameters (axon plus myelin sheath; *A* and *D*), axon alone (*B* and *E*), and myelin sheath alone (*C* and *F*) were measured. Total diameter measures of axons were clearly increased at the large end of the distribution in transgenic nerves (*D*) compared with control (*A*) samples ($P < 0.0001$). Measures of axon diameters alone (*B* and *E*), showed a similar increase ($P < 0.005$) at the large end of the distribution. Measures of myelin thickness of control (*C*) and transgenic (F) nerve fibers showed transgenics to have increased myelin ($P < 0.0001$) in axons composing the large end of the distribution.

the muscle spindle organs they innervate are dependent on NT-3 for development (Ernfors et al., 1994; Farinas et al., 1994; Klein et al., 1994; Kucera et al., 1995*a,b*). The loss of proprioceptive neurons could not, however, account for the overall 50–80% loss of sensory neurons in DRGs of NT-3 (−/−) animals, suggesting that other sensory neurons were dependent on NT-3 for development and maintenance. Our studies identify two sensory endings in skin as responsive to NT-3: circular endings of the piloneural complexes associated with hair follicles and touch dome complexes and the SA1 afferents that innervate them. These observations are supported by the increase in the number of PGP 9.5- and NF 150-positive processes innervating the piloneural complexes and touch domes and complementary studies by Airaksinen et al. (1996) on the neuronal physiology of skin from NT-3 knockout mice. In contrast to the enhancement of touch domes by NT-3 overexpression, mice lacking NT-3 had fewer touch dome units, Merkel cells, and SA1 fibers. Furthermore, physiological analysis of NT-3 (+/−) mice also showed depletion of D hair endings, neuronal fiber types whose termination site is thought to be on hair follicles (Perl, 1992). Given the major enhancement of circular endings associated with the

piloneural complex observed in NT-3 transgenics, it may be that these endings are the functional termination site of D hair endings. Future physiological analysis of the NT-3 overexpresser skin will determine if this is the case.

It is not clear how overexpression of NT-3 led to the increase in touch dome size and number of associated Merkel cells. Additional NT-3 appears to have modified both the survival and projection pattern of sensory neurons as well as the development of their target, the Merkel cell. As Merkel cells develop they become associated with nerve endings (Pasche et al., 1990) and, from denervation studies, many are dependent on this innervation for survival (Nurse et al., 1984). Thus, as muscle spindles are dependent on IA proprioceptive afferent innervation, Merkel cells associated with touch domes appear dependent on SA1 innervation.

Enhanced innervation of touch dome units in NT-3 skin was accompanied by an increase in number of myelinated axons in the saphenous nerve. Myelinated fibers and their myelin sheaths were also, on average, thicker in diameter. The increase in myelin suggests an increase in the number of associated Schwann cells, as indicated by the increased cellularity and galactocerebroside immunoreactivity in the
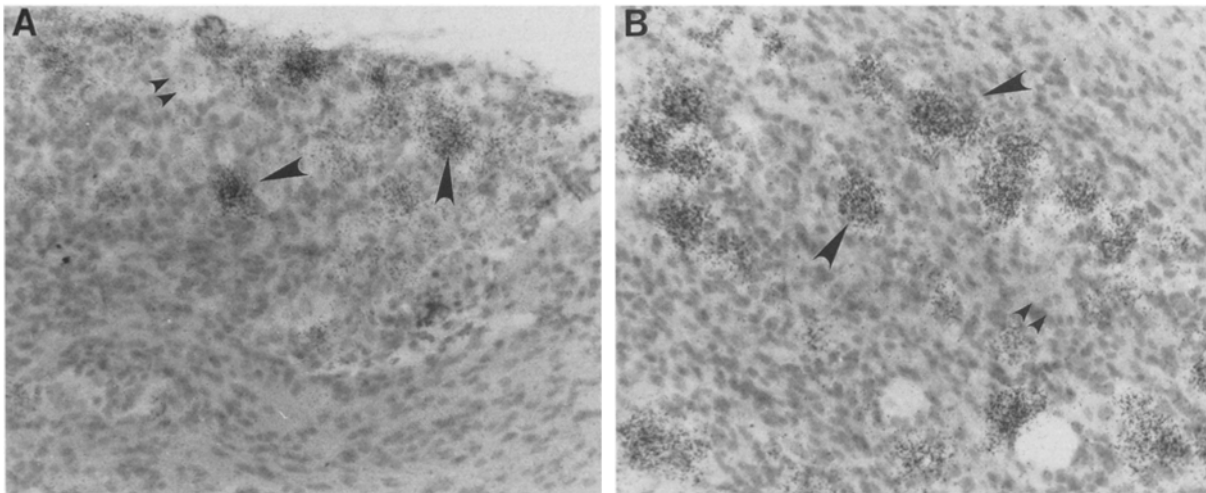
Figure 6. In situ hybridization of sensory neurons expressing trkC mRNA. Ganglia sectioned on a cryostat at 20-μm thickness were hybridized with an ^{35}S-labeled probe made to the catalytic form of the trkC receptor mRNA. Shown are sections of control (*A*) and transgenic (*B*) ganglia that were processed in parallel for in situ hybridization and counterstained with hematoxylin and eosin. Large arrowheads indicate hybridization-positive profiles; small double arrowheads indicate unlabeled neurons.

dermis subjacent to transgenic touch domes. In rat, developing Schwann cells associated with the sciatic nerve have been shown to synthesize the full-length and truncated isoforms of the trkC receptor (Offenhauser et al., 1995), and therefore should be sensitive to NT-3. Increased levels of NT-3 may stimulate Schwann cell mitogenesis, similar to the mitogenic effect it has on cultures of NIH3T3 fibroblasts transfected with trkC receptor DNA (Lambelle et al., 1991). In addition, NT-3 has been shown to support oligodendrocyte development in the CNS (Barres et al., 1994).

Table IV. Tabulation of Results of In Situ Hybridization Analysis of Trigeminal Sensory Neurons for trkC Receptor Expression

Sample	Age	Number		Average number ± SEM
		trkC	neurons	
	weeks			
Transgenic line 696-2	7	259		288 ± 22 (*n* = 4)
	9 (*n* = 2)	325	240	
	26	327		
Transgenic line 694-2	23 (*n* = 2)	479	205	362 ± 53 (*n* = 5)
	44	294		
	63 (*n* = 2)	474	359	
Controls	7 (*n* = 2)	73	108	146 ± 17 (*n* = 8)
	9	116		
	23	189		
	26	158		
	44 (*n* = 2)	127	182	
	63	214		

In situ hybridization was carried out on control and transgenic ganglia using ^{35}S-labeled riboprobes to trkC mRNA. TrkC-expressing sensory neurons were increased in NT-3 transgenics as indicated by the recursive translated corrected values. Total number of hybridization positive neurons were counted in every tenth section of ganglion isolated from various aged adult mice. *t*-test analysis showed no statistical difference between the number of trkC neurons in the two transgenic lines (*P* > 0.150). A statistically significant difference was measured between the 696-2 line and controls (*P* > 0.008) and the 694-2 line and controls (*P* > 0.0002. *n*, the number of ganglia analyzed.

In combination with platelet-derived growth factor, NT-3 promoted DNA synthesis and supported survival of cultured oligodendrocytes from rat optic nerve.

The distribution of nerve fiber diameter size was also shifted in transgenics. The nature of this shift, in which the population of small- and large-diameter myelinated fibers were both increased and midsize fibers were less affected, suggests that NT-3 supported specific populations of myelinated cutaneous nerves. Small, unmyelinated axon populations were also increased by 65%, reflecting in part the 32% increase of sympathetic postganglionic neurons. Another possible contributor to the unmyelinated pool, however, is small unmyelinated sensory neurons. Studies of adult rat DRG correlating trk expression with neuronal diameter have shown NT-3–dependent, trkC expressing neurons to be primarily of large diameter, trkA neurons to be small diameter, and trkB neurons to be of mixed sizes (Ruit et al., 1992; Mu et al., 1993; McMahon et al., 1994). Studies of trk expression and neuronal size in trigeminal sensory ganglia in our laboratory are in general agreement with these findings, though our analysis also identified a population of trkC expressing neurons of small diameter (Goodness, T.P., K.M. Albers, and B.M. Davis, unpublished data). In addition, studies by DiStefano and colleagues (DiStefano, P.S., personal communication) have found retrograde transport of NT-3 by small-diameter neurons, a result that also supports the existence of a population of small, NT-3–dependent neurons.

In summary, we have isolated transgenic mice that overexpress NT-3 neurotrophin in skin. Additional NT-3 synthesis in the cutaneous target field appeared to have rescued neurons from developmental apoptotic death and thereby increased the number of sensory and sympathetic neurons. Another possibility however, is that excess NT-3 acted in a mitogenic manner to increase the number of developing neurons. Further studies to examine the mitotic and apoptotic populations in sensory and sympathetic ganglia at various developmental timepoints will address this issue. NT-3 transgenic mice exhibited hypertrophied touch

dome end organs, an increased density of circular endings associated with piloneural complexes, an increase of trkC-expressing sensory neurons, and an increase in number of myelinated and unmyelinated fibers. These results are consistent with a role for NT-3 as a target-derived neurotrophin that acts to modulate the development of neuronal innervation to the skin.

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