

# Cutaneous Overexpression of Neurotrophin-3 (NT3) Selectively Restores Sensory Innervation In NT3 Gene Knockout Mice

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**ABSTRACT:** Neurotrophin-3 (NT3) is essential for development of sensory innervation to the skin. NT3 supports the postnatal survival of primary sensory neurons that mediate mechanoreception and their Merkel cell containing touch dome end organs (Airaksinen et al., 1996). In this study we determined whether NT3 overexpressed in the skin could restore innervation lost when endogenous NT3 levels were reduced. Hybrid mice that overexpress NT3 in basal keratinocytes but lack one endogenous NT3 allele (K14-NT3/NT3<sup>+/-</sup>) were compared to NT3 overexpresser (K14-NT3) mice, heterozygous knockout (NT3<sup>+/-</sup>) mice, and littermate control mice. In line with previous analyses, NT3<sup>+/-</sup> mice lost 63% of the Merkel cells associated with touch domes, 67% of touch dome units and the associated SAI inner-

vation. All of these parameters were restored to overexpresser levels in K14-NT3/NT3<sup>+/-</sup> mice. Knockout NT3<sup>+/-</sup> mice also had a 31% reduction of L4/L5 dorsal root ganglion cells and a 24% reduction of myelinated axons in the saphenous cutaneous nerve. These losses were also restored in hybrid K14-NT3/NT3<sup>+/-</sup> mice, though only to control mouse values. These results indicate that overexpression of NT3 in skin of NT3<sup>+/-</sup> knockout mice rescued most cutaneous neurons lost in NT3<sup>+/-</sup> mice, but was unable to rescue NT3-dependent neurons that project to noncutaneous sensory targets. © 2000 John Wiley & Sons, Inc. *J Neurobiol* 43: 40–49, 2000  
**Keywords:** neurotrophin-3; skin innervation; SAI neuron

The family of neurotrophin growth factors (NGF, NT3, BDNF, and NT4) are essential regulators of neuron survival and differentiation in the somatosensory system. Mice that lack neurotrophins or their respective tyrosine kinase trk receptor proteins have specific deficits in sensory neuron number and peripheral innervation patterns (Ernfors et al., 1994a,b; Fa-

rinas et al., 1994; Fundin et al., 1997; Rice et al., 1998). Neurotrophin-3 (NT3) plays a particularly important role since mice lacking NT3 (NT3<sup>-/-</sup>) lose up to 70% of their sensory neurons during embryonic development (embryonic day (E) 10.5–E13.5) and certain types of sensory complexes (Ernfors et al., 1994b; Farinas et al., 1996). One type of sensory complex lost in NT3 mutants are Merkel cell touch dome mechanoreceptors of the skin (Airaksinen et al., 1996; Fundin et al., 1997). Merkel complexes are innervated by slowly adapting type I (SAIs) neurons. Interestingly, SAI-Merkel complexes are present in skin of newborn NT3<sup>-/-</sup> knockout mice and gradually are lost postnatally. By postnatal day 14 (P14) no com-

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plexes are visible in NT3<sup>-/-</sup> skin, and only ca. 20% are left in heterozygous (NT3<sup>+/-</sup>) mice (Airaksinen et al., 1996). Thus, NT3 is not essential for embryonic SAI survival nor formation of Merkel complexes, but is required for their postnatal maintenance.

That NT3 supports embryonic neuron survival and postnatal sensory complex development indicates that the temporal and tissue-specific action of NT3 is highly regulated. To investigate how this regulation impacts sensory innervation of the skin, we used the K14 keratin promoter to generate transgenic mice that overexpress NT3 in the epidermis beginning at E11 (Albers et al., 1996). NT3 overexpresser mice had significant enhancement of the sensory system that included up to a 70% increase in sensory neurons in the trigeminal ganglia, doubling of the percentage of trkC neurons, and a major increase in the size and innervation to Merkel cell touch dome complexes. These enhancements demonstrated the role of NT3 as a target-derived growth factor in the cutaneous system and suggested that skin-supplied NT3 could entirely restore the sensory losses observed in the NT3 mutants. However, NT3 and its major receptor protein trkC are made by many other nonneuronal and neuronal tissues during embryonic and postnatal development, suggesting sources other than the skin could influence neuron survival (Schechterson and Bothwell, 1992; Tessarollo et al., 1993). For example, migrating neural crest cells and early precursor neurons grown in culture are responsive to NT3 in the culture medium, and mesenchymal tissue adjacent to developing dorsal root ganglia (DRG) express NT3 (Kalcheim et al., 1992; Buchman and Davies, 1993; Membregh and Hall, 1995; ElShamy and Ernfors, 1996). Thus, NT3 signaling could occur along the projection pathway prior to target innervation and thereby influence neuron survival and/or differentiation.

To examine how skin-derived NT3 regulates the survival of developing DRG and cutaneous neurons, we determined whether it was sufficient to restore cutaneous innervation in mice that had reduced endogenous NT3 expression. Hybrid mice that overexpressed NT3 in skin but carried an insertional mutation in one copy of the endogenous NT3 gene (K14-NT3/NT3<sup>+/-</sup>) were studied. Though NT3<sup>+/-</sup> mice exhibited substantial loss of Merkel cells and their innervation, overexpression of NT3 in their skin completely reversed these deficits to overexpresser mouse levels. In contrast, restoration of DRG neurons lost in NT3<sup>+/-</sup> mice was only to control levels. These results indicate that skin-derived NT3 could rescue most NT3-dependent cutaneous innervation, but was not sufficient to rescue all DRG sensory neurons.

## METHODS

### Isolation of Animals

Generation of K14-NT3 transgenic overexpresser mice and NT3 knockout mice are described in Albers et al. (1996) and Ernfors et al. (1994b), respectively. Heterozygous NT3 knockout mice that overexpress NT3 in the skin (K14-NT3/NT3<sup>+/-</sup>) were established (see Fig. 1) by crossing K14-NT3 mice (line 696-2, B6 × C3 F1 hybrids) with heterozygous NT3 knockout mice (NT3<sup>+/-</sup>, strain Balb/C126; provided by Drs. Fan and Jaenisch). K14-NT3/NT3<sup>+/-</sup> F1 males and females were bred with NT3<sup>+/-</sup> mice to generate animals used in this study. Genotypes were determined by polymerase chain reaction (PCR) and Southern blot analysis. Both male and female mice between 8 weeks and 6 months of age were analyzed. Animals in this study were used in accordance with the guidelines of the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and the NIH Guide for the Care and Use of Laboratory Animals.

### Two-Site Enzyme-Linked Immunosorbant Assay of NT3 Protein

ELISAs were performed using the NT3 E<sub>max</sub> Immunoassay kit (Promega, Madison, WI) according to the manufacturer's instructions. Mice were euthanized by anesthesia overdose and flank skin was shaved, depilated, removed, weighed, and frozen on dry ice. Skin was homogenized in sample buffer [0.1 M phosphate-buffered saline (PBS), 0.4 M NaCl, 0.1% Triton X-100, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 0.1 mM PMSF, 20 K $\mu$ l/ml aprotinin, 0.5% BSA, pH 7.4] using a polytron homogenizer. Samples were spun at 13,000 rpm for 15 min at 4°C, supernatants were collected, and aliquots were assayed.

### Immunohistochemistry

Deeply anesthetized mice were transcardially perfused with 4% paraformaldehyde in phosphate buffer. Flank skin was removed and immersion fixed for up to 4 hours and then embedded in either gelatin or paraffin. Gelatin-embedded tissue was cut at 40  $\mu$ m 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), and incubated overnight at room temperature in primary antibody [anti-PGP9.5 1:5000 (Ultraclone, Isle of Wight, U.K.); anti-NF150 1:3000 (Chemicon, Temecula, CA)]; dilutions were made in

5% NGS and 0.25% Triton]. Sections were washed and incubated 1 h in a 1:500 dilution of biotinylated goat anti-rabbit secondary antibody followed by a strep-avidin complex incubation (Vector Laboratories, Burlingame, CA). Antibody binding was visualized using a nickel cobalt-enhanced diaminobenzidine reaction. Sections were washed, mounted on slides, and counterstained with methyl green. Paraffin-embedded tissue was sectioned at 10  $\mu\text{m}$ , mounted on slides, dewaxed, rehydrated, and treated with proteinase K. Sections were incubated with anti-cytokeratin 20.8 (Boehringer Mannheim, Indianapolis, IN) and anti-NF150 for 1 h at 37°C, washed, and incubated in goat anti-rabbit Cy2 and goat anti-mouse Cy3 for 1 h. Slides were coverslipped with either a phosphate-buffered 90% glycerol solution containing paraphenyldiamine or DPX mountant (BDH Laboratory Supplies, Poole, England), and viewed using a Leica confocal microscope housed in the University of Kentucky Imaging Center.

### Quinacrine Labeling

Touch domes and associated Merkel cells were identified on flank skin by depilating skin and 24 h later intraperitoneally injecting mice with quinacrine dihydrochloride (Sigma Chemical Co., St. Louis, MO) (15 mg/kg; made in PBS), a fluorescent compound that concentrates in neuroendocrine cell types (Nurse et al., 1984). Twelve to 20 h after quinacrine injection, 1  $\text{cm}^2$  of flank skin was removed, trimmed of dermal fat and connective tissue, and whole-mounted on a glass slide in antifade solution. Merkel cells associated with touch domes were counted across the entire section using a microscope equipped with fluorescent optics, and the number of Merkel cells per  $\text{cm}^2$  of flank skin was calculated.

### Saphenous Nerve Counts

Mice were deeply anesthetized and perfused with PBS followed by 4% paraformaldehyde made in phosphate buffer. Saphenous nerve segments were removed from midhigh level, postfixed for 2 h in 2% glutaraldehyde:4% paraformaldehyde, washed in phosphate buffer, dehydrated through alcohols, embedded in Spurr's resin, and cut at 0.7–0.8  $\mu\text{m}$  on an ultramicrotome. Ultrathin sections were stained and photographed on an electron microscope, and images were assembled into montages from which myelinated and unmyelinated nerve fibers were counted. Diameters of myelinated fibers were measured using the NIH Image software. Areas of 150 myelinated fibers randomly selected from each nerve sample (total = 12) were determined.

### L4/L5 DRG Cell Counts and Diameters

Neuron counts in adult L4/L5 DRG were determined using methods described by Coggeshall et al. (1990). Ganglia were serially sectioned at 5  $\mu\text{m}$  and nissel stained, and every 10th section was examined at  $\times 400$  to identify neurons with a nucleolus. To compensate for neurons with two or more nucleoli, profiles of randomly selected neurons were reconstructed, and the number of nucleoli per 100 neurons was determined. This ratio was multiplied by the total neurons counted to obtain the total number of neurons per ganglion.

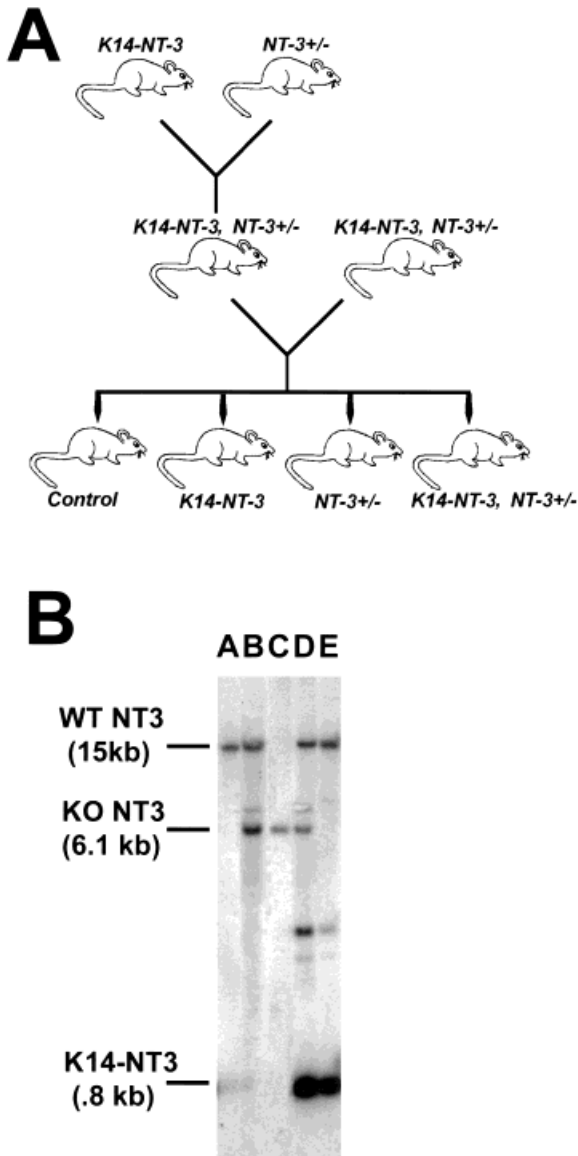
### Statistical Analysis of Data

Analysis of variance was used to compare NT3 protein level, Merkel cell number, touch dome number, saphenous nerve counts, and DRG cell counts and sizes across genotypes. Individual means were compared using the Fisher's least significant difference (LSD) procedure. The  $\alpha$  level was set at  $p < .05$ .

## RESULTS

### Expression of the K14-NT3 Transgene Could not Rescue Homozygous Knockout Mice from Postnatal Death

The effect of NT3 overexpression in skin on the phenotype of NT3 knockout mice was determined by first isolating male and female hybrid mice that carried the K14-NT3 transgene and were heterozygous for the NT3 knockout allele [K14-NT3/NT3<sup>+/-</sup>; Fig. 1(A)]. Hybrid mice were bred with heterozygous NT3 knockout mice (NT3<sup>+/-</sup>) since homozygous mice died postnatally. Of 123 adult mice that were genotyped using PCR and Southern blotting [Fig. 1(B)], none were found to carry both the K14-NT3 transgene and the double (homozygous) knockout of the NT3 gene (K14-NT3/NT3<sup>-/-</sup>). Genotypes of 50 P0 mice (day of birth) were also determined and, though 4 NT3<sup>-/-</sup> mice were born, no K14-NT3/NT3<sup>-/-</sup> mice were generated. These results suggest either the dual transgenic phenotype enhanced embryonic death of homozygous knockout mice (which typically die postnatally) or that the K14-NT3 transgene sequence was integrated at a chromosomal site that prohibited independent segregation from the endogenous NT3 allele. We therefore focused our analysis on K14-NT3/NT3<sup>+/-</sup> mice. Using these mice,



**Figure 1** Generation of hybrid transgenic mice. (A) Heterozygous NT3 knockout mice that overexpress NT3 in the skin (K14-NT3/NT3<sup>+/-</sup>) were isolated by crossing K14-NT3 mice with NT3<sup>+/-</sup> knockout mice. K14-NT3/NT3<sup>+/-</sup>F1 males and females were bred with NT3<sup>+/-</sup> mice to generate animals used in this study. (B) Southern blot analysis of genomic DNA isolated from tails of each mouse line and restriction cut with *Bam*HI enzyme. A full-length NT3 <sup>32</sup>P-labeled probe hybridized to a 15-kb fragment from wild-type alleles, 6.1-kb fragment from mutant alleles, and a 0.8-kb fragment from the NT3 transgene. Shown are control (lane A), NT3<sup>+/-</sup> (lane B), NT3<sup>-/-</sup> (lane C), NT3/NT3<sup>+/-</sup> hybrid (lane D), and K14-NT3 transgenic (lane E) samples.

we determined whether enhanced levels of NT3 in skin could rescue the severe loss of Merkel cells and SAI type neurons that occurred in the heterozygous knockout genotype.

## Overexpression of NT3 in Skin Rescues Cutaneous Axons in NT3<sup>+/-</sup> Mice

The saphenous nerve is a purely cutaneous nerve that innervates skin of the medial calf and foot, which is useful for evaluation of sensory axon parameters. Previous assessment of axon number and physiology in NT3<sup>+/-</sup> knockouts showed a 24% loss of myelinated axon profiles and 80% loss of SAI fibers (Airaksinen et al., 1996). In the present study, analysis of axon parameters (Table 1) also showed NT3<sup>+/-</sup> mice had 24% fewer myelinated axons than control mice (452 vs. 593;  $p < .05$ ). In comparison, nerves from K14-NT3 mice had 27% more myelinated axons (754 vs. 593;  $p < .05$ ). Hybrid K14-NT3/NT3<sup>+/-</sup> myelinated axon counts were intermediate in number, i.e., there were 31% more myelinated axons than counted in NT3<sup>+/-</sup> nerves (656 vs. 452;  $p < .05$ ) and 13% fewer than K14-NT3 nerves (656 vs. 754;  $p < .05$ ). Compared to controls, the number of myelinated axons in K14-NT3/NT3<sup>+/-</sup> mice did not differ significantly (656 vs. 593;  $p > .05$ ). Thus, the number of myelinated axons in hybrids was restored to control levels, but was slightly (though significantly) lower than overexpresser levels. Counts of unmyelinated axons showed significant reduction in NT3<sup>+/-</sup> nerves ( $p < .05$ ) with recovery in K14-NT3/NT3<sup>+/-</sup> hybrids to levels statistically equivalent to control and K14-NT3 values (Table 1).

The diameter of myelinated axons rescued in K14-NT3/NT3<sup>+/-</sup> mice were determined and plotted as a frequency distribution histogram (Fig. 2). This analysis showed NT3<sup>+/-</sup> mice had preferential loss of large myelinated axons ( $> 3.5 \mu\text{m}$ ) and a 12% reduction in average axon diameter [Fig. 2(C)]. In contrast, large axon profiles were prominent in nerves of K14-NT3 transgenic mice [Fig. 2(B)], as reflected by a 22% increase in the average axon diameter relative to controls [Fig. 2(A)]. In K14-NT3/NT3<sup>+/-</sup> hybrid nerves [Fig. 2(D)], large axon profiles ( $> 3.5 \mu\text{m}$ ) were partially restored, suggesting predominant rescue of large cutaneous neurons. In particular, very large axons ( $5.6 \mu\text{m}$  diameter) that appear in K14-NT3 nerves also were present in the K14-NT3/NT3<sup>+/-</sup> hybrids, suggesting specific enhancement of this population by K14-NT3 transgene expression.

## Overexpression of NT3 Allows Partial Rescue of L4/L5 DRG Neurons in Hybrids

Though axons projecting to the skin of K14-NT3/NT3<sup>+/-</sup> mice were enhanced, counts of DRG neurons indicated that a partial rescue of this cell population occurred (Table 2). Counts of L4/L5 neurons from

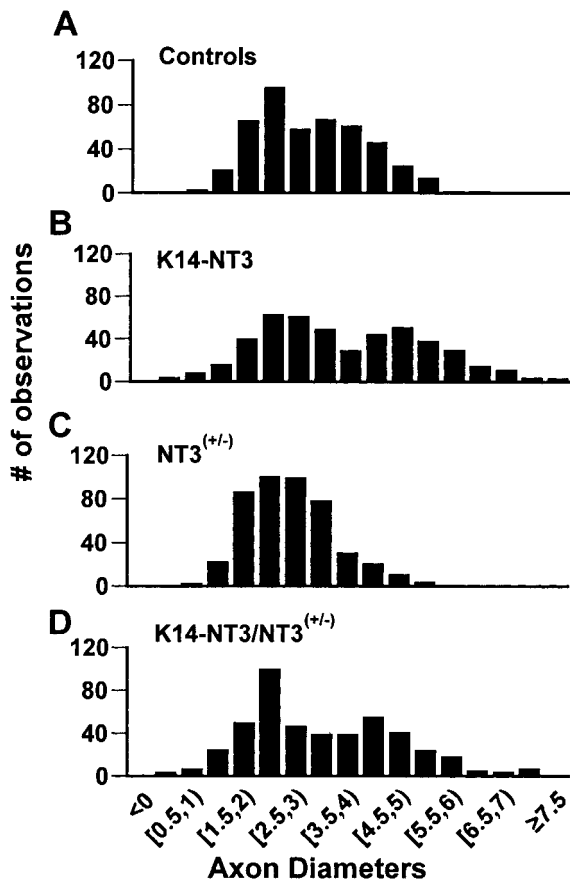
**Table 1** Number of Myelinated and Unmyelinated Nerve Fibers in the Saphenous Nerve. Axons comprising saphenous nerves from control (n = 3), K14-NT3 (n = 3), NT3<sup>+/-</sup> (n = 3), and K14-NT3/NT3<sup>+/-</sup> (n = 3) mice were counted using images of ultrathin sections collected using an electron microscope. K14-NT3 nerves had 27% more myelinated axons compared to control nerves, whereas NT3<sup>+/-</sup> nerves had 24% less. The number of axons in K14-NT3/NT3<sup>+/-</sup> hybrids was not significantly different from controls. Hybrid axon number was however, 13% less than overexpresser values ( $p < .05$ ), suggesting that not all myelinated axons projecting to the skin were rescued. In comparison, all unmyelinated axons lost in NT3<sup>+/-</sup> mice were restored to overexpresser levels. Values are means  $\pm$  SEM.

	Control	K14-NT3	NT3 <sup>+/-</sup>	K14-NT3/NT3 <sup>+/-</sup>
Myelinated	593 $\pm$ 21	754 $\pm$ 37*	452 $\pm$ 15*	656 $\pm$ 36
Unmyelinated	2744 $\pm$ 249	3169 $\pm$ 174	1957 $\pm$ 150*	3081 $\pm$ 281

\* Indicates significantly different from controls.

NT3<sup>+/-</sup> mice (9906) were 31% less compared to control values (9906 vs. 14,451;  $p < .05$ ), whereas K14-NT3 DRG neurons were increased by 46% (14,451 vs. 21,084;  $p < .05$ ). Counts of DRG neurons

in hybrid K14-NT3/NT3<sup>+/-</sup> mice (12,370) were between control (14,451) and knockout (9906) values. This suggests that only a partial rescue of NT3-dependent neurons occurred. In addition, the number of neurons rescued in hybrid DRG was 41% lower than the number rescued in K14-NT3 DRG ( $p < .05$ ). Thus, overexpression of NT3 in skin could restore the neuron number to control values, but not to overexpresser levels, indicating nonepidermal sources of NT3 were required for complete DRG development.



**Figure 2** A population of large diameter myelinated axons is restored in NT3<sup>+/-</sup> saphenous nerves by peripheral NT3 expression. Diameters of a randomly selected population of myelinated saphenous neurons are plotted to show distribution of fiber sizes. The average size of myelinated nerve fibers is larger in K14-NT3 transgenic mice and smaller in NT3<sup>+/-</sup> mice relative to control nerves. Also note reappearance of the largest myelinated axon diameters in the K14-NT3/NT3<sup>+/-</sup> hybrid profiles.

### Overexpression of NT3 Restores Sensory Innervation to the Skin of NT3<sup>+/-</sup> Knockouts

To examine how the level of NT3 in skin relates to innervation density, we first measured the relative levels of NT3 peptide in flank skin of each mouse line. The NT3 protein level in skin of K14-NT3 mice was *ca.* three-fold higher than control littermate values ( $17.53 \pm 2.37$  vs.  $6.19 \pm 1.46$ ,  $p < .05$ ) as measured by enzyme-linked immunosorbant assay (ELISA). This increased NT3 was not released into the general circulation, since ELISA measure of NT3 peptide in serum from overexpresser mice showed no change from control values (J. Suicek, Regeneron Pharmaceuticals, personal communication). Similar to overexpresser mice, skin of K14-NT3/NT3<sup>+/-</sup> mice had a threefold increase in peptide compared to NT3<sup>+/-</sup> skin ( $16.63 \pm 1.76$  vs.  $4.34 \pm 0.62$ ,  $p < .05$ ). Thus, transgene expression completely restored the level of NT3 in NT3<sup>+/-</sup> flank skin to a value indistinguishable from K14-NT3 mouse skin ( $p > .05$ ).

To evaluate skin innervation on the histologic level, sections of flank skin and whisker pad skin were immunolabeled with antibodies to PGP 9.5, a general neuronal marker, neurofilament 150 (NF 150) a marker of myelinated axons, and keratin 20.8 (K20) an intermediate filament protein specific for Merkel cells (Moll et al., 1984). Labeling of NT3<sup>+/-</sup> mouse skin with NF150 and K20 [Fig. 3(C)]. showed that touch dome mechanoreceptor units were reduced

**Table 2** Number of Neurons in L4/L5 Dorsal Root Ganglia. Cell counts of L4/L5 DRG neurons from control (n = 4), K14-NT3 (n = 4), NT3<sup>+/-</sup> (n = 4), and K14-NT3/NT3<sup>+/-</sup> (n = 4) mice were measured. Enhanced NT3 expression in NT3<sup>+/-</sup> skin resulted in restoration of DRG number to control values, but not to overexpresser levels (*p* < .05).

	Control	K14-NT3	NT3 <sup>+/-</sup>	K14-NT3/NT3 <sup>+/-</sup>
Cell counts				
Mean (± SEM)	14,451 ± 1367	21,084 ± 1801*	9,906 ± 1322*	12,370 ± 1214

\* Indicates significantly different from controls.

in size and had fewer myelinated SAI fibers compared to control mouse touch domes [Fig. 3(A)]. Touch domes were extremely difficult to locate in NT3<sup>+/-</sup> mice and, though twice the number of NT3<sup>+/-</sup> samples were viewed, few were found. In contrast, K14-NT3/NT3<sup>+/-</sup> mice [Fig. 3(D)] had enlarged touch domes with associated myelinated fibers present at a density comparable to K14-NT3 overexpressers [Fig. 3(C)]. In addition, the skin of NT3<sup>+/-</sup> mice had an overall decrease in nerve density, which appeared to be restored to overexpresser levels in K14-NT3/NT3<sup>+/-</sup> mice (not shown).

Previous analysis of NT3 overexpresser skin showed a major enhancement of hair follicle innervation with an increased density of fibers comprising piloneural complexes [Figs. 3(E–H)] (Albers et al., 1996). Piloneural complexes have longitudinally arranged myelinated nerve fibers (lanceolate endings) encircled by circumferentially oriented myelinated endings [Fig. 3(E)] (Rice et al., 1993; Fundin et al., 1995). The density of this innervation was drastically reduced in NT3<sup>+/-</sup> mice, particularly the circular ending component [Fig. 3(G)]. In contrast, hair follicles labeled in K14-NT3 [Fig. 3(F)] and K14-NT3/NT3<sup>+/-</sup> [Fig. 3(H)] skin had enriched circumferential endings (arrows in Fig. 3), which in some cases totally obscured the lanceolate endings. Thus, the NT3-dependent innervation to touch domes and hair follicles that was reduced in NT3<sup>+/-</sup> skin could be restored and enhanced by overexpression of NT3 in the epidermis.

### Overexpression of NT3 in Skin of NT3<sup>+/-</sup> Mice Completely Restores Merkel Cell Number in Touch Dome End Organs

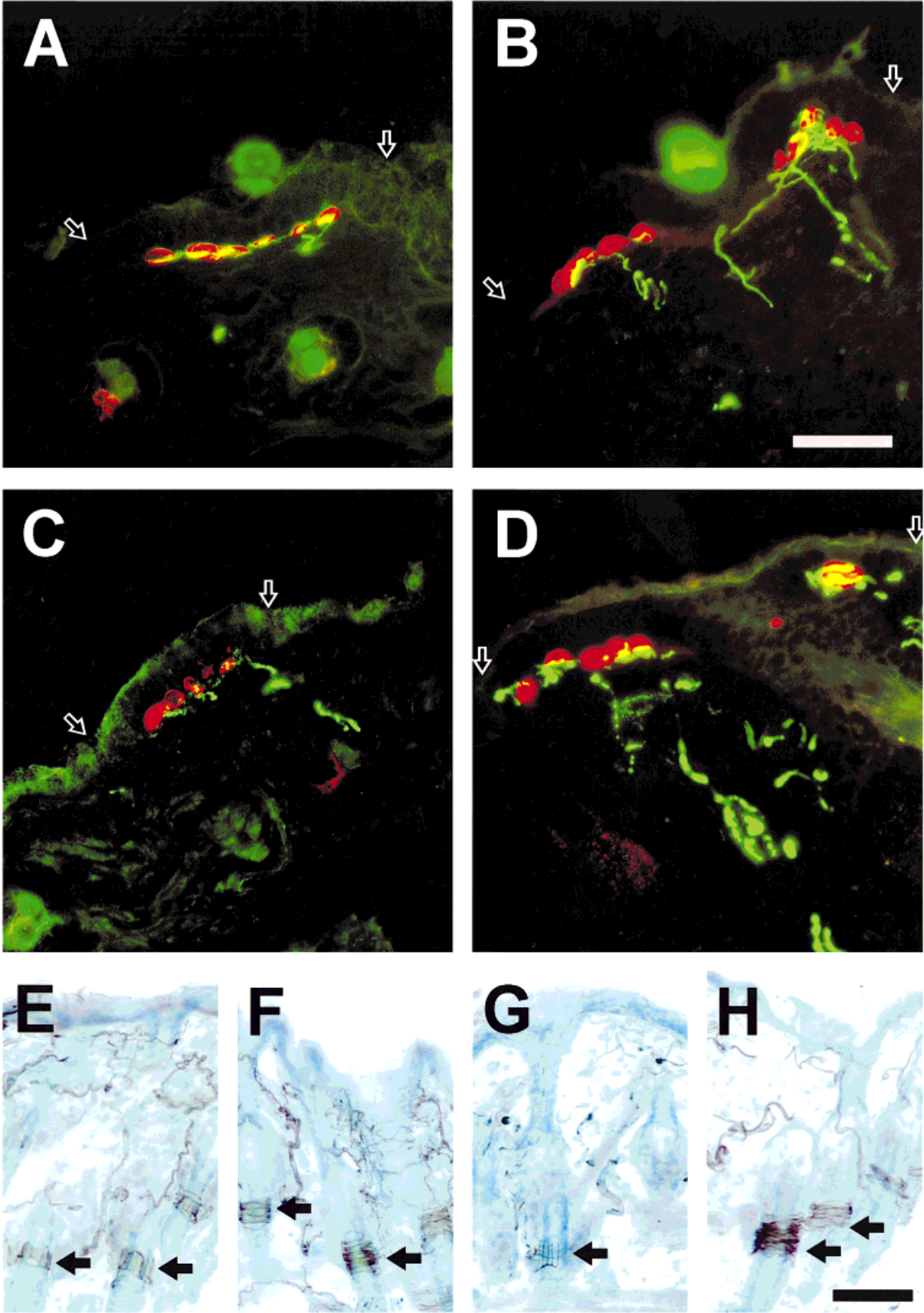
The effect of cutaneous NT3 overexpression on Merkel cells was determined by injecting mice with quinacrine dihydrochloride, a fluorescent compound that concentrates in neuroendocrine cell types (Nurse et al., 1984). Analysis of flank skin of NT3<sup>+/-</sup> mice showed a substantial reduction in the number of Merkel cells per touch dome [Fig. 4(A)] compared to control skin (*p* < .05), whereas K14-NT3 transgenic

mice had a substantial increase in Merkel cells per touch dome [Fig. 4(A), *p* < .05]. A large increase in Merkel cell number was also found in touch domes of K14-NT3/NT3<sup>+/-</sup> hybrids, demonstrating that K14-NT3 transgene expression rescued all Merkel cells even though endogenous NT3 was reduced.

Since the level of NT3 in skin clearly affected both SAI innervation and the number of Merkel cells per touch dome, we examined whether the overall number of touch dome end organs was changed in each transgenic line. Consistent with previous findings, touch domes with associated Merkel cells were dramatically reduced in NT3<sup>+/-</sup> mice [Fig. 4(B), *p* < .05] and unchanged in K14-NT3 transgenics (*p* > .05) (Airaksinen et al., 1996; Albers et al., 1996). Also of interest is that NT3 overexpression completely rescued touch dome number in K14-NT3/NT3<sup>+/-</sup> hybrids (*p* < .05), indicating that peripheral sensory organs could be established and maintained even when endogenous NT3 was reduced.

## DISCUSSION

Mouse genetic hybrids were generated by mating NT3 knockout mice with mice that overexpress NT3 in the epidermis. In agreement with previous studies (Airaksinen et al., 1996), analysis of NT3<sup>+/-</sup> mice showed loss of more than half the normal complement of Merkel cells. In contrast, hybrid K14-NT3/NT3<sup>+/-</sup> mice, which had half the normal level of endogenous NT3 but threefold higher NT3 in the skin, showed complete rescue of Merkel cells and associated axons, such that no difference between hybrid and overexpresser sensory receptors was apparent. Since Merkel cells of touch domes are highly dependent on neuronal innervation and are lost following deafferentation (English, 1974; Nurse et al., 1984), Merkel cell reduction in NT3<sup>+/-</sup> mice was probably caused by the loss of SAI innervation. Thus, complete rescue of Merkel cells in K14-NT3/NT3<sup>+/-</sup> mice likely reflects total restoration of their SAI innervation. This interpretation is consistent with the equal innervation density observed upon neurofilament immunolabeling of



touch domes in hybrid and K14-NT3 mice. These results indicate that skin-derived NT3 is responsible for the postnatal development of the SAI-Merkel cell sensory complex and that endogenous NT3 expression either during embryonic development or postnatally by central or projection pathway tissues is non-essential.

Though the complete rescue of Merkel cell innervation in K14-NT3/NT3<sup>+/-</sup> hybrids suggests embryonic survival of SAI neurons is completely dependent on target-derived NT3, studies of NT3 knockout mice indicate SAIs have a shared trophic dependence. For example, nearly all neurons in DRG of homozygote NT3<sup>-/-</sup> knockouts that express trkC die by E11-E12, though NT3<sup>-/-</sup> mice are born with SAI innervation and only lose it postnatally (Airaksinen et al., 1996; Farinas et al., 1996, 1998; Liebl et al., 1997). In addition, innervation to Merkel cells in dorsal mouse skin begins to appear at E15 (Pasche et al., 1990), a time when cell loss in NT3<sup>-/-</sup> DRG is complete (Liebl et al., 1997). Merkel cell innervation coincides temporally with an increase of trkC expression in a subpopulation of DRG neurons in NT3<sup>-/-</sup> mice (Liebl et al., 1997; Farinas et al., 1998). The possibility exists therefore, that this new trkC population represents SAI neurons that upon contacting their Merkel cell targets in the skin, switch to a NT3 trophic dependence. Conversely, SAI neurons may from the onset of embryonic differentiation, possess multiple trophic responsiveness that could provide support in the absence of NT3. If SAI neurons have shared trophic dependence and are responsive to NT3 during embryonic development, it would be expected that the increase in DRG neurons in overexpresser mice is attributable in part to the SAI population. However, if a complete switch in trophic dependency to NT3 occurs in SAIs at E15 (as described above), the increase in innervation to Merkel complexes in NT3 overexpressers and hybrid mice should not be due to increased survival of precursor SAI neurons,

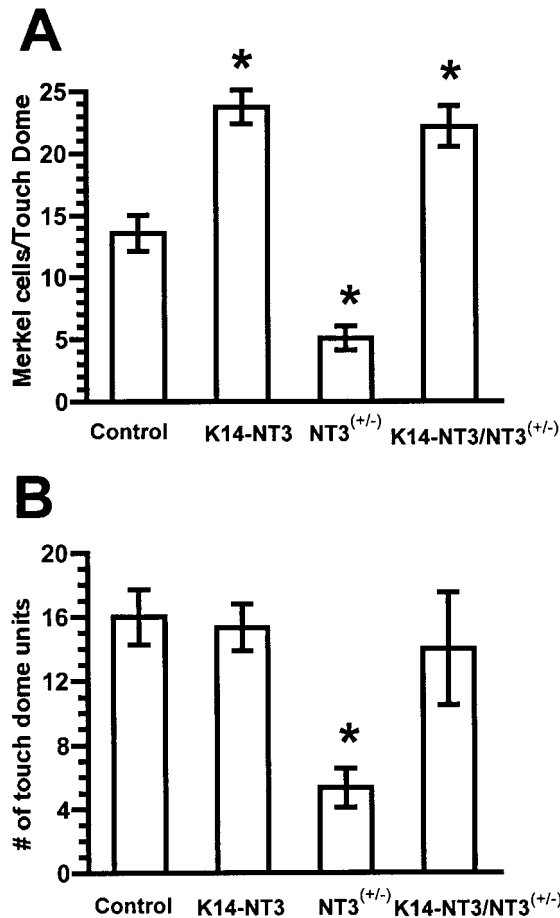
which at early times would be nonresponsive to NT3, but rather to sprouting of SAI peripheral afferents. Because cytochemical markers for SAI neurons are not currently available, the absolute number of SAI neurons in the DRG cannot be morphologically defined, but instead will require physiological characterization.

Analysis of skin innervation in K14-NT3/NT3<sup>+/-</sup> mice indicate that SAI neuron and hair follicle afferents were completely restored to overexpresser levels, though DRG neuron number was only partially restored. One DRG neuron population probably not rescued in hybrid mice are Ia proprioceptive neurons that innervate muscle spindles. These neurons die in NT3<sup>-/-</sup> mice before target innervation (Kucera et al., 1995). They have known dependence on NT3 derived from muscle and/or the surrounding mesenchymal tissues along their projection pathway (Ernfors et al., 1994b; Farinas et al., 1994; Kucera et al., 1995; Wright et al., 1997). Since K14-NT3 transgene expression is epithelial specific, does not elevate NT3 level in blood, and does not alter the number of spindles in muscles (D. Wright, personal communication), proprioceptive neurons were probably not rescued in NT3<sup>+/-</sup> mice by K14-NT3 expression.

Interestingly, our analysis of the cutaneous saphenous nerve indicates that some neurons lost in the DRG may be ones that innervate the skin. Myelinated saphenous axons that project to the skin were rescued in K14-NT3/NT3<sup>+/-</sup> mice, but only to control nerve levels. The 27% enhancement of myelinated fibers in overexpresser nerves was not achieved in K14-NT3/NT3<sup>+/-</sup> hybrids. This partial recovery suggests skin-derived NT3 could not rescue all NT3-dependent projections to the skin in NT3<sup>+/-</sup> mice. It may be that a population of cutaneous neurons exists that is either dependent on NT3 at early times in development before the transgene-derived NT3 was available (prior to E11/E12), or dependent on NT3 from a noncutaneous source(s). Precedence for early dependence is

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**Figure 3** Immunocytochemical analysis of transgenic and control skin. Flank skin from control (A), K14-NT3 (B), NT3<sup>+/-</sup> (C), and K14-NT3/NT3<sup>+/-</sup> (D) mice was immunolabeled using anti-NF150 to detect myelinated neurons (green) and anti-K20 to detect Merkel cells (red). Touch domes of K14-NT3 mice (B) were larger and had greater innervation compared to control touch domes (A). Touch domes of NT3<sup>+/-</sup> mice were much smaller, harder to find, and had relatively low innervation density (C) compared to control touch domes (A). Touch domes of K14-NT3/NT3<sup>+/-</sup> skin (D) were similar in size and innervation density to K14-NT3 overexpressers (B). Whisker pad skin from control (E), K14-NT3 transgenic (F), NT3<sup>+/-</sup> (G), and K14-NT3/NT3<sup>+/-</sup> hybrids (H) was immunolabeled using anti-NF150. Circular endings comprising piloneural complexes of K14-NT3 transgenic skin (F, arrows) were greatly enhanced compared to circular endings in control complexes (E, arrows). The number of circular endings in NT3<sup>+/-</sup> piloneural complexes (G, arrows) was greatly reduced relative to controls, though completely recovered in K14-NT3/NT3<sup>+/-</sup> mice (H, arrows).



**Figure 4** The number of quinacrine-labeled Merkel cells and touch dome number are restored in NT3<sup>+/-</sup> skin by transgene expression. The number of Merkel cells per touch dome (A) was substantially decreased in NT3<sup>+/-</sup> mice, though increased in K14-NT3 transgenics relative to controls. The loss of Merkel cells in NT3<sup>+/-</sup> mice was completely recovered to overexpresser levels in K14-NT3/NT3<sup>+/-</sup> mice. The number of touch domes per area of skin (B) was also significantly reduced in NT3<sup>+/-</sup> mice compared to controls. This loss was completely recovered in K14-NT3/NT3<sup>+/-</sup> mice. \* Significantly different from control values.

found in trigeminal sensory neurons, where a large fraction of neurons are first dependent on NT3 and/or BDNF during early development (E10–E11). Many neurons switch this dependency to NGF at about E12/E13 as the whisker pad skin becomes innervated (Buchman and Davies, 1993). Hence, in a manner similar to proprioceptor neurons innervating muscle, a subpopulation of neurons in K14-NT3/NT3<sup>+/-</sup> mice that innervate skin may die prior to their outgrowth to the periphery due to reduced levels of endogenous NT3 and unavailability of transgene-derived NT3. These neurons would, hypothetically, be dependent on endogenous sources of NT3 prior to target out-

growth and would switch neurotrophic dependence (e.g., to NGF or BDNF) during target encounter.

In summary, we generated hybrid transgenic mice to evaluate the role of skin-derived NT3 in DRG neuron survival and development of cutaneous sensory innervation. Our findings indicate that noncutaneous sources of NT3 are not required for SAI–Merkel or piloneural complex development, though they may be required for development of other myelinated cutaneous afferents. Future studies to define the electrophysiological properties of NT3 responsive neurons will test this possibility.

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