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RESEARCH ARTICLE

Pacinian Corpuscle Development Involves Multiple Trk Signaling Pathways

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The development of crural Pacinian corpuscles was explored in neonatal mutant mice lacking nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) or neurotrophin-4 (NT4), or their cognate Trk receptors. Deficits of the corpuscles and their afferents were greatest in *NT3*, less in *BDNF*, and least in *NT4* null mice. Deletion of *NGF* or *p75*^{NTR} genes had little or no impact. No Pacinian corpuscles were present in *NT3;BDNF* and *NT3;NT4* double or *NT3;BDNF;NT4* triple null mice. Deficits were larger in *NT3* than *TrkC* mutants and were comparable to deficits observed in *TrkB* or *TrkA* mutants. Afferents of all corpuscles coexpressed TrkA and TrkB receptors, and some afferents coexpressed all three Trk receptors. Our results suggest that multiple neurotrophins, in particular NT3, regulate the density of crural Pacinian corpuscles, most likely by regulating the survival of sensory neurons. In addition, NT3/TrkB and/or NT3/TrkA signaling plays a greater role than NT3/TrkC signaling in afferents to developing Pacinian corpuscles. *Developmental Dynamics 231:551–563, 2004.* © 2004 Wiley-Liss, Inc.

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INTRODUCTION

Limb mechanoreceptors form at sites where somatic sensory axons contact prospective target cells (Zelená, 1994; Hippenmeyer et al., 2002). The numbers of distinct classes of skin and muscle receptors located in specific limb regions are remarkably consistent among individual rodents of the same species. Studies of genetically modified mice suggest that neurotrophins, members of a family of structurally related growth factors, play a crucial role in determining the numbers and properties of mechanoreceptors by regulating the development of the sensory neurons that innervate them. Mice carrying a deletion in the gene for nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) or neurotrophin-4 (NT4), or for their high-affinity tyrosine kinase (Trk) transmembrane receptors display deficits of functionally distinct subpopulations of sensory neurons in the dorsal root ganglia (DRGs). The neuron deficits in the neurotrophin-deficient mice are primarily due to increased neuron death during development (reviewed by Fariñas, 1999; Huang and Reichardt, 2001). Specific classes of peripheral mechanoreceptors are missing in neurotrophin null mice as a consequence of the absence of subtypes of DRG neurons. These deficits reflect the dependence of developing mechanoreceptors on their sensory innervation (reviewed by Zelená, 1994; Snider, 1994).

The Pacinian corpuscle is a rapidly adapting mechanoreceptor whose development is dependent on sensory innervation. It consists of an

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elongated axon terminal surrounded by a lamellar inner core of Schwann cell origin that is encased in a multilayered outer core of concentric lamellae of cells of perineurial origin (Pease and Quilliam, 1957; Nava, 1974). Corpuscles form not only in the dermis and hypodermis but also on the surfaces of muscle fasciae, aponeuroses, and tendons. Sensory nerve fibers initiate the development of Pacinian corpuscles (Zelená, 1978) through the inductive effect of an afferent neuron-derived neuregulin (glial growth factor 2) on the peripheral target cells (Kopp et al., 1997). Nerve section results in degeneration of Pacinian corpuscles in newborn rats, attesting to the critical dependence of the receptors on intact innervation (Zelená, 1980).

The function of the four neurotrophins in the development of Pacinian corpuscles and their afferents is not well-understood. Expression of specific Trk receptors by the cells forming the mechanoreceptor is suggestive of a role for neurotrophin/Trk signaling in the corpuscle morphogenesis. The lamellar layers of Pacinian corpuscles in rats and cats were reported to express TrkB (Stark et al., 2001), a Trk receptor with a high affinity for BDNF and NT4 that can also bind NT3 in certain cellular contexts (Barbacid, 1994). However, the functional importance of TrkB receptors is unclear because mutation in the trkB gene does not alter either the immunohistochemical or the ultrastructural characteristics of Pacinian corpuscles in mice (Gonzalez-Martinez et al., 2004). Expression of TrkA receptors, which can bind NGF as well as NT3, has been observed in the Pacinian corpuscles of humans but not corpuscles of rats or cats (Ribeiro-da-Silva et al., 1991; Vega et al., 1994; Lopez et al., 1998). In contrast, expression of TrkC, the principal Trk receptor for NT3, could not be detected by immunocytochemistry in rat or cat Pacinian corpuscles (Stark et al., 2001). The inner and outer core cells of Pacinian corpuscles in mice and several other mammalian species also express $p75^{NTR}$, a low affinity nonkinase receptor that can modify neurotrophin signal transduction by Trk receptors (Lopez et al., 1998; Albuerne et al., 2000; Stark et al., 2001). Importantly, these studies did not address whether the afferent axons that innervate Pacinian corpuscles expresses any of the three Trk receptors, or $p75^{NTR}$; thus, the potential role of Trk signaling in the development of sensory neurons innervating the corpuscles has not been fully explored.

Subsets of neurons that express TrkA, TrkB, or TrkC receptors are present in DRGs at different times during embryonic development. NT3/TrkC signaling is critical for the development of several classes of mechanoreceptive afferents capable of high frequency discharge. Large caliber afferents such as group Ia and II afferents of muscle spindles and group Ib afferents of Golgi tendon organs are two examples of afferents dependent on NT3 for their development and differentiation (Ernfors et al., 1994a; Fariñas et al., 1994; Tessarollo et al., 1994). Pacinian corpuscles are innervated by large caliber sensory neurons capable of high-frequency discharge, raising the possibility that NT3/TrkC signaling is critical for their development. However, many corpuscles develop normally in mutant mice lacking the *NT3* gene (Ernfors et al., 1994a). Thus, the role of NT3 and other neurotrophins in the assembly of the Pacinian afferent system is not well-defined.

We undertook a comprehensive study of Pacinian corpuscle development in the crus of mice with null mutations in neurotrophin or neurotrophin receptor genes. We observed that NT3 signaling through more than one Trk receptor plays a major role in supporting Pacinian corpuscle afferents and corpuscle assembly. In the absence of NT3, however, BDNF and NT4 can also support the development of a limited number of Pacinian afferents resulting in morphologically adequate Pacinian corpuscles. Preliminary reports of these data have been presented in abstract form (Šedý, 2003).

RESULTS

Uniform Size of the Crural Pacinian Corpuscle Complex

The Pacinian corpuscles are distributed in highly specific and predictable locations in the lower leg (Fig. 1). The corpuscles arise in a grape-like manner from the terminal branches of the interosseous nerve along the distal portion the fibula in the vicinity of the fibulotibial junction and the adjacent interosseous membrane (Zelená, 1978). The main, distal branch of the interosseous nerve gave rise to 27–30 (mean, 28.9 ± 0.9; $n = 12$) Pacinian corpuscles that were located along the dorsal aspect of the fibula in wild-type specimens (129/c57bl/6 background) stained for cholinesterase (ChE). A small, proximal branch of the interosseous nerve innervated an accessory group consisting of two to eight (mean, 4 ± 1.5; $n = 8$) corpuscles that were located medial to the fibula (Fig. 1A,B). We limited our study to the Pacinian corpuscles arising from the main interosseous nerve branch because of the consistency in the size of the complex. Corpuscle counts were equivalent between neonatal (28.9 ± 0.9; $n = 12$) and adult (29.2 ± 1.5; $n = 8$; $P < 0.05$) wild-type mice (129/c57bl/6 background, ChE staining).

The stability of postnatal counts in our material is consistent with reports indicating that Pacinian corpuscles begin to develop before birth and that no new corpuscles form after birth in wild-type rodents (Zelená, 1978; Nava, 1974, 1988). In sections stained with toluidine blue, the early development of crural Pacinian corpuscles took place over a 3-day period between embryonic day (E) 16.5 and postnatal day (P) 0 (with the delivery occurring on day E19). At E16.5, a cluster of cells that were presumed to be of Schwann cells origin occupied the site adjacent to the fibula where crural Pacinian corpuscles are characteristically located; occasional axon profiles were intermingled with the cells (Fig. 2A). At E17.5, early Pacinian corpuscles were recognized as prominent round or oval axon profiles surrounded by rows of sheath cells giving rise to the lamellated inner core region and mesenchymal cells giving rise to the capsule (Fig. 2B). The axon profiles were thicker than axons comprising the parent interosseous nerve, as is characteristic of Pacinian afferent terminals. By P0, individual Pacinian corpuscles com-

prising the crural cluster could be identified as distinct entities and could be counted in sections stained with toluidine blue or for ChE.

Loss of Interosseous Nerve Afferents in Mutant Mice

Large caliber myelinated nerve fibers innervate Pacinian corpuscles. In cats, Pacinian afferents do not branch to supply more than one corpuscle (Iggo, 1985), thus Pacinian afferents and corpuscles exist in a 1:1 ratio. To determine whether the 1:1 ratio between afferents and Pacinian corpuscles exists in the mouse, we compared the number of myelinated axons in the interosseous nerve with the number of Pacinian corpuscles innervated by the nerve in plastic-embedded sections (Fig. 3A–C). In wild-type mice, the number of myelinated axons (33 $\pm$ 0.9; n = 3) exceeded by approximately 20% the number of corpuscles innervated by the interosseous nerve (27.5. $\pm$ 0.4; n = 8; Fig. 3A). The extra nerve fibers were small-caliber fibers that terminated in the vicinity of the numerous blood vessels supplying crural corpuscles. The excess fibers most likely represented vascular afferent and efferent autonomic nerve fibers. We did not observe the interosseous nerve to supply sense organs other than Pacinian corpuscles or contain motor axons to muscle fibers. We next counted the interosseous nerve fibers in *NT3* null mutants that are known to be deficient in sympathetic nerve fibers (Fig. 3B; Zhou et al., 1997). Between P11 and P14 when myelination is near completion, the number of interosseous myelinated axons (15.3 $\pm$ 0.5; n = 3) in *NT3* mutants closely approximated the number Pacinian corpuscles (15.0 $\pm$ 0.7; n = 3) innervated by the nerve. These data are consistent with a 1:1 relationship between the corpuscles and parent large caliber myelinated afferents in the absence of autonomic fibers.

Incomplete myelination precluded obtaining accurate counts of the interosseous nerve afferents in mutant and wild-type mice between P0 and P4. However, the interosseous nerves appeared thinner in all neurotrophin-deficient mice that lacked the full complements of corpuscles. Furthermore, double and triple neurotrophin null mutants that completely lacked Pacinian corpuscles also lacked an interosseous nerve (Fig. 3C). Thus, the deletion of neurotrophin genes impacts Pacinian corpuscles and their afferents equally. These observations are consistent with the hypothesis that the deficits of Pacinian corpuscles in neurotrophin-deficient mice result from deficits of afferent nerve fibers (or sensory neuron bodies) rather than from deficiencies in the peripheral target cells or abnormalities in the process of corpuscle assembly.

Impact of Single Neurotrophin Deletions

Fewer Pacinian corpuscles were present in mice lacking NT3 (Fig. 1C), BDNF, or NT4 than in wild-type mice at P0–P4 (Table 1). The corpuscle deficits ranged from 15 to 45% in the three single neurotrophin null mutants and were greatest in *NT3,* less in *BDNF,* and least in *NT4* null mice. Thus, NT3, BDNF, and NT4 support subpopulations of the Pacinian corpuscles and their afferents. In contrast, deletion of NGF had no statistically significant impact on corpuscle numbers (Table 1A). Pacinian corpuscles retained all of their principal structural features in single gene null mutants, although corpuscles of the mutants with the largest numerical deficits tended to be smaller at birth than their wild-type neonatal counterparts. However, the corpuscles became larger and acquired more lamellae in those mutants that survived past the neonatal stage, similar to wild-type corpuscles (Fig. 2C–F).

We addressed the postnatal stability of Pacinian corpuscles in neurotrophin-deficient mice by comparing numbers of corpuscles in newborn and adolescent *NT3* null mutants. Corpuscle counts were equivalent in *NT3* mutants examined between P0 and P1 (14.7 $\pm$ 0.5; n = 6) and P11 and P14 (15.0 $\pm$ 0.7; n = 3) in sections stained with toluidine blue. Thus, the absence of NT3 does not result in postnatal degeneration of the corpuscles. Similar observations were made in *BDNF* null mutants (data not shown).

Major Impact of Multiple Neurotrophin Deletions

The differential impact of deletions of single neurotrophin genes on the numbers of Pacinian corpuscles and their afferents could be due to the existence of nonoverlapping subpopulations of sensory neurons that are each dependent on a single neurotrophin for its development. Alternatively, some of the sensory neurons may be able to use more than one neurotrophin. We have addressed these possibilities by comparing deficits of Pacinian corpuscles in double and triple neurotrophin gene null mutants with deficits of corpuscles in single gene null mutants.

Observations on double and triple gene null mutants suggest a central role for NT3 signaling in supporting the development of Pacinian corpuscles and their afferents. NT3 was able to support a subpopulation of Pacinian corpuscles in the absence of BDNF and NT4. Approximately one third of the crural complement of Pacinian corpuscles survived in mice that expressed NT3 but not BDNF or NT4 (*BDNF;NT4* null mice). Moreover, the number of residual corpuscles (9–11) in *BDNF;NT4* double mutants was roughly similar to the deficit of corpuscles (11–14) in *NT3* null mice (Table 1A and B). Thus, NT3 may be critical for the development and differentiation of a subpopulation of Pacinian corpuscles and their afferents.

BDNF and NT4, when acting in concert, are capable of supporting a major subpopulation of Pacinian corpuscles on their own as reflected by the large deficit of corpuscles (65% of wild-type) in *BDNF;NT4* mutants, a deficit that occurs despite the presence of NT3. Conversely, approximately 46% of the corpuscles survived in NT3-deficient mutants that retained BDNF and NT4 function. In addition, BDNF or NT4 can support developing corpuscles in conjunction with NT3. Cooperation between BDNF and NT3 as well as between NT4 and NT3 was suggested by the complete absence of Pacinian corpuscles in mutants lack-

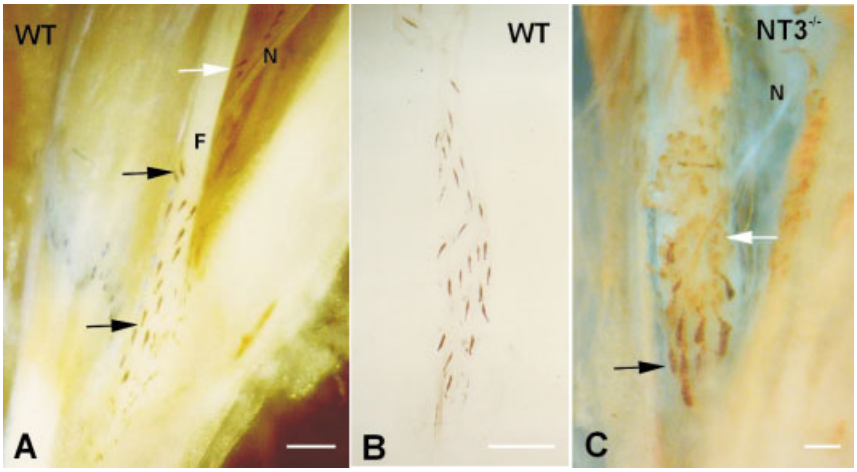

Fig. 1. Crural Pacinian corpuscles of adult wild-type (WT) and newborn *NT3* null mice in preparations stained for cholinesterase (ChE). **A:** Main corpuscle cluster (black arrows) located along the dorsal aspect of the distal end of the fibula (F) in WT mouse. A smaller, accessory group of corpuscles (white arrow) arises from a proximal branch of the interosseous nerve (N) medial to the fibula. **B:** String of WT Pacinian corpuscles is scattered along the length of the interosseous nerve isolated from the underlying fibula. **C:** The main cluster of crural Pacinian corpuscles in *NT3*$^{-/-}$ newborn. Note that corpuscles located in the center of the cluster are missing as shown by the absence of their ChE reactivity (white arrow), whereas peripherally located corpuscles are present and retain ChE activity (black arrow). Scale bars = 100 μm in A,B, 50 μm in C.

ing NT3 and either BDNF or NT4, such as in the *NT3;BDNF* or *NT3;NT4* double mutants (mice deficient in both NT3 and NGF were not available). Synergy between NT3 and BDNF, and/or NT3 and NT4 may explain the greater loss of corpuscles in *BDNF;NT4* mutants than in *NT3* null mice (Table 1A and B).

To further explore whether populations of Pacinian corpuscles have overlapping or nonoverlapping neurotrophin requirements for survival, we compared the number of corpuscles in *BDNF;NT4* double gene mutants with the number of corpuscles present in *BDNF* and *NT4* single gene mutants. If a subpopulation of corpuscles used both BDNF and NT4, then the deficit of corpuscles in *BDNF;NT4* mutants would be expected to be greater than the sum of deficits in *BDNF* and *NT4* mutants, because the absence of one of the neurotrophins would be compensated by the other neurotrophin in single gene mutants. Indeed, the loss of corpuscles in *BDNF;NT4* mutants (65%) was greater than com-

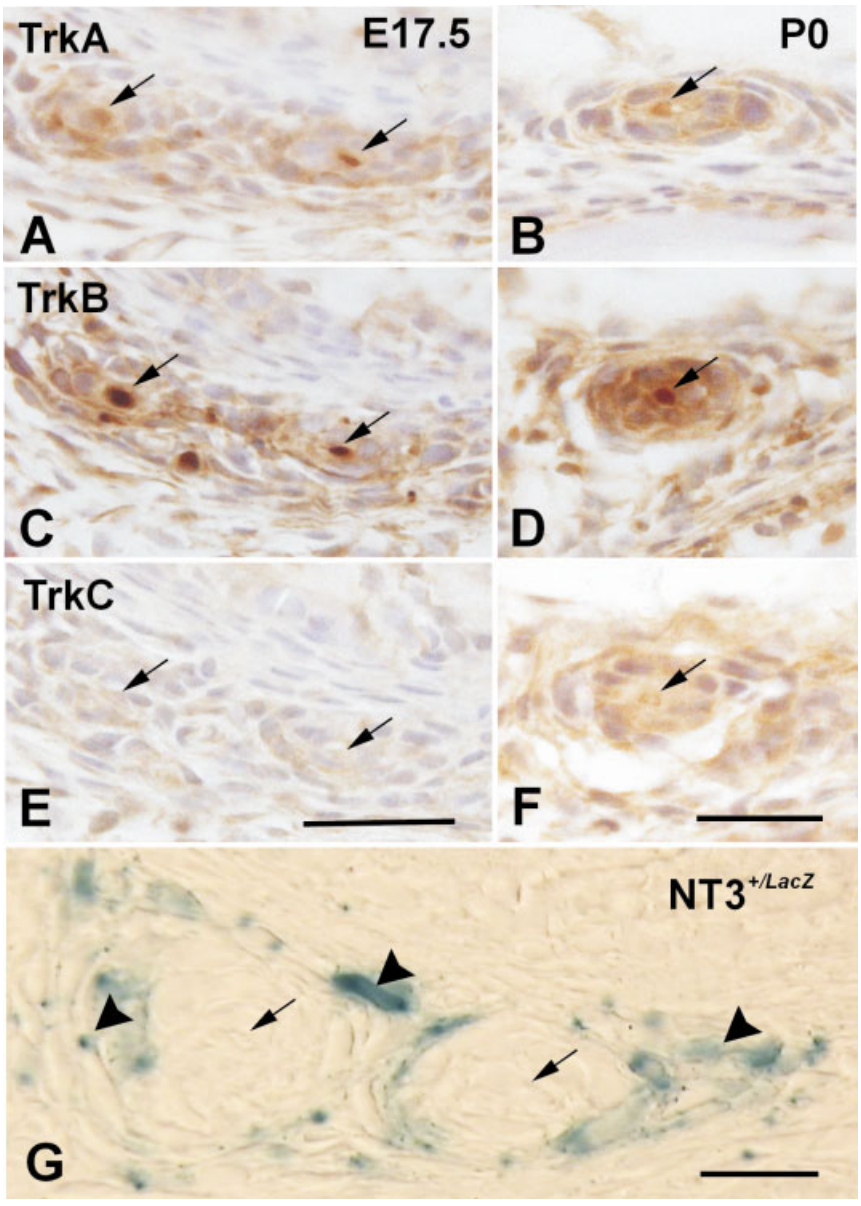

Fig. 4. Expression by Pacinian corpuscles of TrkA, TrkC, and TrkB receptors by protein immunocytochemistry in wild-type mice and NT3 by the *LacZ* reporter in *NT3*$^{+/lacZ}$ mouse. **A–F:** Afferent axons (arrows) of embryonic day (E) 17.5 embryonic corpuscles colocalize TrkA (A) and TrkB (C) but not TrkC (E) receptors, whereas postnatal day (P) 0 newborn corpuscles colocalize all three Trk receptors (B,D,F). **G:** Expression of the *lacZ* marker (arrowheads) is limited to the outer capsular cells in corpuscles of *NT3*$^{+/lacZ}$ newborn; the inner core lamellae and afferent axon show no lacZ reactivity. Scale bars = 20 μm in E (applies to A,C,E), in F (applies to B,D,F), 25 μm in G.

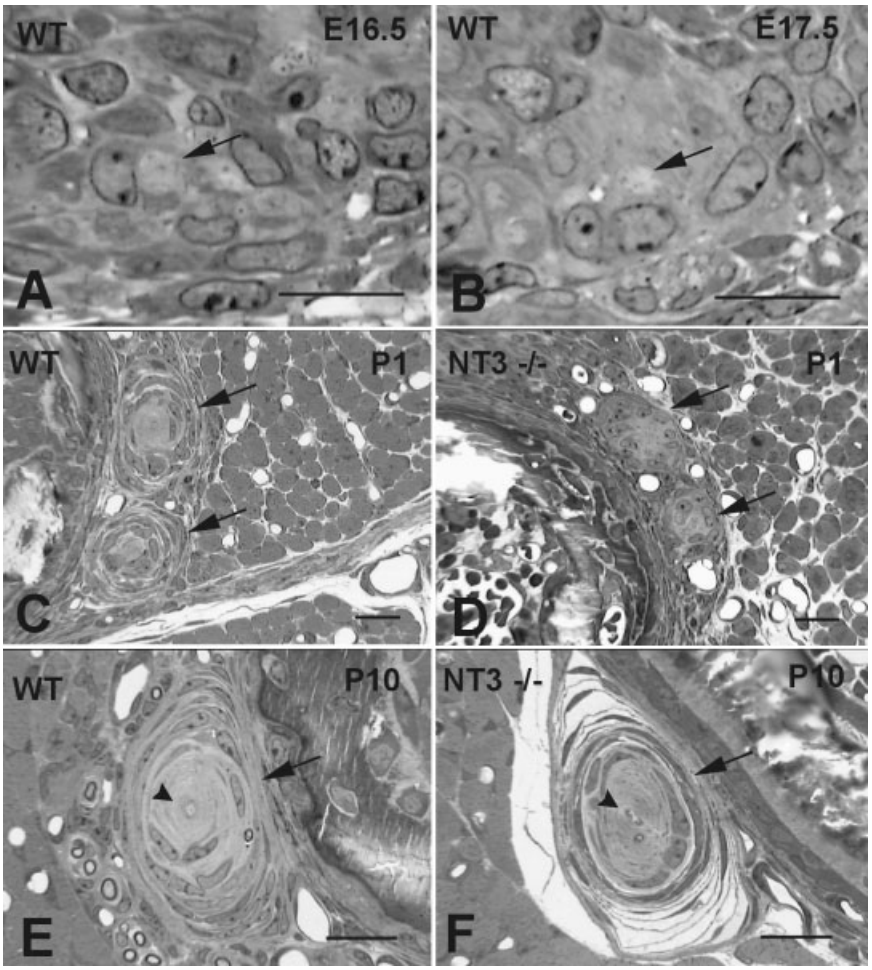

Fig. 2. Development of Pacinian corpuscles in wild-type (WT) and *NT3* null mice in toluidine blue staining. **A,B:** Nascent corpuscles (arrows) at embryonic day (E) 16.5 (A) and E17.5 (B). **C,D:** Distinct corpuscles of WT (C) and *NT3* mutant (D) mice appear morphologically similar at P1 except that the mutant corpuscles are smaller than WT corpuscles (arrows). **E,F:** By postnatal day (P) 10, the size and layering of the inner and outer core cells of WT (E) and mutant (F) corpuscles (arrows) are comparable; arrowheads denote afferent axon terminals. Scale bars = 20 μm in A–F.

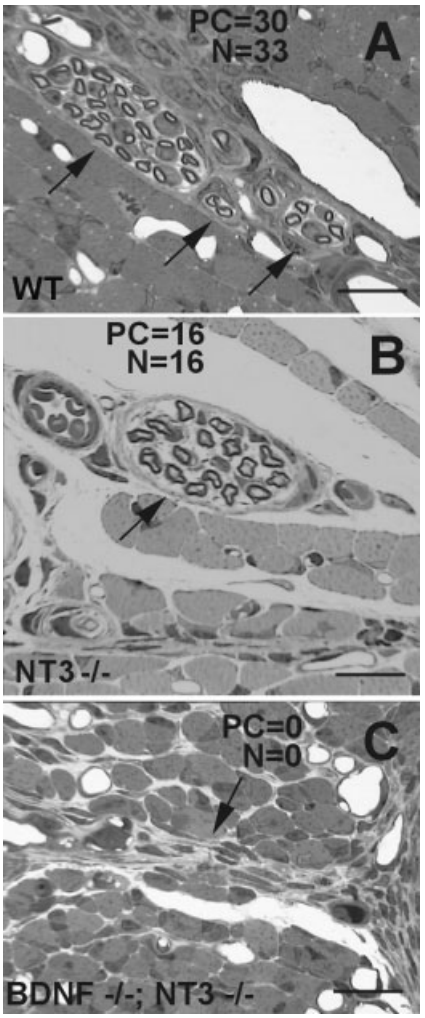

Fig. 3. Loss of the interosseous nerve afferents in neurotrophin mutant relative to wild-type (WT) mice. **A:** The interosseous nerve (arrows) contains 33 myelinated nerve fibers (N) and innervates 30 Pacinian corpuscles (PC) in WT mouse. **B:** NT3 deficiency in *NT3*$^{-/-}$ mouse results in an approximately 40% deficiency of interosseous nerve afferents (arrows) and Pacinian corpuscles. **C:** The interosseous nerve and Pacinian corpuscles are absent entirely in *BDNF;NT3* double null mutant. Note that the numbers of nerve fibers and Pacinian corpuscles in A–C refer to the particular counts in the individual animal tissues depicted. The arrow points to the tissue cleft occupied by the interosseous nerve in WT mice. Scale bars = 10 μm in A–C.

bined corpuscle deficits in *BDNF* and *NT4* mutants (43%), suggesting that approximately 20% of Pacinian corpuscles can use both BDNF or NT4 for survival (Table 1A and B). Thus, at least some of the corpuscles that are missing in *BDNF;NT4* mutants may be nonselective in terms of their neurotrophin requirements and could be supported by either BDNF or NT4.

Furthermore, the absence of corpuscles in *NT3;BDNF* and *NT3;NT4* double null mutants indicates that neither BDNF alone nor NT4 alone can support the formation and/or survival of any subpopulation of Pacinian corpuscles and their afferents in the absence of NT3. Thus, the surviving corpuscles in *BDNF* or *NT4* single gene mutants are likely supported jointly by the two residual neurotrophins, one of which is NT3. Furthermore, corpuscles were absent in *NT3;BDNF;NT4* triple mutants, indicating that no subpopulation of Pacinian corpuscles can be supported by NGF alone (Table 1B). The inability of BDNF, NT4, or NGF to support corpuscle development on their own, in the absence of NT3, analyzed in conjunction with corpuscle deficits in *BDNF;NT4* double mutants, reflects synergistic interaction between NT3 and one of the neurotrophins that signal through TrkB. Thus, a subset of Pacinian corpuscles and their afferents may be capable of deriving developmental support from more than one neurotrophin.

Corpuscle Deficits in NT3 Absence Are Not Rescued by BDNF

We used a mouse model in which the *NT3* gene has been replaced by the *BDNF* gene (*B/N* allele) to investigate whether deficits in Pacinian corpuscles and afferents caused by

TABLE 1. Numbers of Crural Pacinian Corpuscles in Null Mutant Mice Lacking One or More Neurotrophins[a]

Genotype	No. of corpuscles	% of WT	% Deficit	Interosseous nerve
A. Single gene null mutants				
WT	27.5 ± 0.4; N = 8	100%	—	+
NGF−/−	26.0 ± 0.7; N = 4	96%	5%	+
NT4−/−	23.5 ± 0.5; N = 4*	85%	15%	+
BDNF−/−	19.7 ± 0.6; N = 9*	72%	28%	+
NT3−/− (P0–P1)	14.7 ± 0.5; N = 6*	54%	46%	+
NT3−/− (P12–P16)	15.0 ± 0.7; N = 3*	55%	45%	+
B. Multiple gene null mutants				
BDNF−/−; NT4−/−	9.5 ± 0.6; N = 4*	35%	65%	+
NT3−/−; BDNF−/−	0 ± 0; N = 3	0%	100%	−
NT3−/−; NT4−/−	0 ± 0; N = 3	0%	100%	−
NT3−/−; BDNF−/−; NT4−/−	0 ± 0; N = 3	0%	100%	−

[a]Values are means ± SEM of counts of corpuscles in semiserial plastic sections in neonatal (P0–P4) or adolescent (P12–P16) neurotrophin mutants and their wild type (WT) littermates. WT values are pooled counts from several WT mice of each of the mutant lines (129/Balb/C background). Presence (+) or absence (−) of the interosseous nerve in samples is indicated. Values marked with an asterisk are significantly different from WT values at $P < 0.05$.

TABLE 2. Deficits of Crural Pacinian Corpuscles in Null Mutant Mice Lacking Neurotrophin Receptors or the p75^{NTR} Receptor and a Neurotrophin[a]

Genotype	Number of Pacinian corpuscles	% of WT	% Deficit
A. Counts from ChE-stained whole-mounts			
trkC$_E$+/+	28.9 ± 0.9; N = 12	100%	—
trkC$_E$−/−	25.5 ± 0.9; N = 8*	88%	12%
trkC$_K$+/+	25.6 ± 0.8; N = 10	100%	—
trkC$_k$−/−	23.0 ± 0.7; N = 8*	90%	10%
B. Counts from semiserial plastic sectioning			
WT	27.5 ± 0.4; N = 8	100%	—
trkC$_E$−/−	24.6 ± 0.6; N = 5*	89%	11%
trkA−/−	17.0 ± 0.6; N = 4*	62%	38%
trkB−/−	14.3 ± 0.3; N = 7*	52%	48%
p75^{NTR}−/−	24.3 ± 0.4; N = 7*	88%	12%
p75^{NTR}−/−; BDNF−/−	16.5 ± 0.5; N = 2*	60%	40%
p75^{NTR}−/−;NT4−/−	19.7 ± 0.3; N = 3*	71%	29%

[a]Values represent means ± SEM of counts of the corpuscles in neonatal (P0–P4) neurotrophin mutant and wild type (WT) littermates. Trk mutants have a 129/c57b1/6 background, whereas neurotrophin and *p75^{NTR}* mutants have a 129/Balb/C background. Counts differed between WT mice of *TrkC$_E$* and *TrkC$_K$* mutant strains examined in (ChE) cholinesterase stained whole-mounts, and the counts are listed separately. Counts were statistically equivalent among WT mice of the *TrkA*, *TrkB*, and *TrkC$_E$* lines examined in plastic embedded sections, and the listed value represents the mean of pooled WT counts. Values marked with an asterisk are significantly different from corresponding WT values at $P < 0.05$.

the absence of NT3 can be rescued by BDNF. This model eliminates activation of TrkA and TrkC by the product of the *NT3* locus while preserving only activation of TrkB receptors by ectopic BDNF in a spatiotemporal pattern that is characteristic of endogenous NT3 (Coppola et al., 2001). Comparison of counts of Pacinian corpuscles between *B/N* (16.5 ± 0.3; n = 8) and *NT3* (14.8 ± 0.6; n = 9) mutants at P0–P14 showed a minor (6%) rescue effect of the *B/N* allele relative to wild-type (27.5 ± 0.4; n = 8) values. Thus, expression of BDNF instead of NT3 at sites where NT3 is normally expressed does not negate the greater part of the adverse impact of NT3 absence on numbers of Pacinian corpuscles. The inability of the ectopic BDNF (and consequent TrkB activation) to rescue most of the corpuscle deficits caused by NT3 absence suggests a critical requirement of a subpopulation of corpuscle afferents for NT3 (and NT3/TrkC and/or NT3/TrkA signaling) at an early stage of development. A substantial number of NT3-dependent DRG neurons are not rescued by the *B/N* allele and die in *B/N* mutants (Coppola et al., 2001). These missing DRG neurons may include afferents subserving Pacinian corpuscles, thus accounting for the corpuscle deficit in *B/N* mutants.

Impacts of *TrkB* or *TrkA* Deletions Exceeds That of *TrkC* Deletion

To further elucidate the signaling pathways involved, we compared the impact of deleting the full-length TrkA, TrkB, or TrkC isoforms, and a truncated TrkC isoform on the development of the Pacinian afferent system (Table 2). Deletion of *TrkA* or *TrkB* resulted in a 38% and 48% deficit of Pacinian corpuscles, respectively, whereas only 11% of the corpuscles were missing in *TrkC* mutants. Thus, the developing Pacinian corpuscles depend more on TrkB and TrkA than on TrkC signaling.

We next compared the impact of *TrkC$_E$* deletion with that of *TrkC$_K$* deletion. Deleting *TrkC$_E$* eliminates all known TrkC isoforms, whereas deleting *TrkC$_K$* removes only the tyrosine kinase domain of TrkC (Tessarollo et al., 1997). The Pacinian corpuscle deficits in *TrkC$_E$* and *TrkC$_K$* mutants were comparable, suggesting that the absence of the kinase domains of TrkC is sufficient to account for the impaired development of the corpuscles and their afferents (Table 2A).

All neurotrophins bind to the low affinity neurotrophin receptor p75^{NTR}

receptor, which lacks a tyrosine kinase-signaling domain. Neurotrophin binding to this receptor may facilitate or impede Trk signaling, depending on the cellular context (Barker and Shooter, 1994; Chao and Hempstead, 1995). Deletion of *p75^{NTR}* alone had only a minor impact on Pacinian corpuscle numbers (Table 2B). The small deficit of corpuscles in *p75^{NTR}* null mutants suggests a limited facilitatory effect of p75^{NTR} on neurotrophin signaling in the Pacinian system. Deletion of *p75^{NTR}* in addition to deletion of either *BDNF* or *NT4* (*p75^{NTR};NT3* double mutants were not available) enhanced the corpuscle deficits relative to those observed in *BDNF* or *NT4* single gene mutants, suggesting that any facilitatory effect of p75^{NTR} is not limited to a single neurotrophin (Table 2B). Thus, deletion of *p75^{NTR}* had the greatest effect when only NT3 were present and BDNF or NT4 were deficient.

TABLE 3. Pacinian Corpuscle Counts in Heterozygous (+/−) or Null (−/−) *NT3* and *TrkC* Mutants[a]

	No. of corpuscles	% deficit (vs. WT)
A. Counts from semiserial plastic sectioning		
WT	27.5 ± 0.4; N = 8	—
NT3+/−	22.0 ± 0.3; N = 5	20%
NT3−/−	14.8 ± 0.5; N = 6	46%
B. Counts from ChE stained whole-mounts		
trkC$_E$+/+	28.9 ± 0.9; N = 12	—
trkC$_E$+/−	29.3 ± 0.8; N = 6	0%
trkC$_E$−/−	25.5 ± 0.9; N = 8	12%

[a]Note the gene dose effect of eliminating one copy of the *NT3* gene. This effect is absent in *TrkC* mutants. Also note that the effects of eliminating *NT3* and *TrkC* genes are not equivalent. These data suggest that NT3 signaling involves receptors other than TrkC.

Impacts of *NT3* and *TrkC* Gene Dosage Are Discordant

Numbers of muscle spindles correlate with neurotrophin gene dose and, hence, presumably with concentration of neurotrophins in tissues. Deletion of one copy of the *NT3* gene, which presumably decreases the level of NT3 in tissues by 50%, results in a 50% loss of muscle spindles, whereas deletion of two *NT3* gene copies results in a 100% loss of spindles (Ernfors et al., 1994a; Fan et al., 2000). Similarly, the deficit of Pacinian corpuscles in *NT3$^{+/-}$* mice was approximately half of the deficits observed in *NT3* null mice (Table 3). Thus, NT-3 influences the development of the NT3-dependent subpopulation of Pacinian corpuscles in a dose-dependent manner.

If the effects of NT3 on development of the Pacinian corpuscles were mediated solely by NT3/TrkC signaling, then the magnitude of impacts of *NT3* and *TrkC* gene deletions would be expected to be similar. A gene dose-dependent effect was not observed in the *TrkC* null mutants that lacked the principal Trk receptor-binding site of NT3. Deletion of both copies of *NT3* had a much greater impact (46% deficit) than deletion of both *TrkC* genes (11% deficit). The differential impact of *NT3* and *TrkC* deletions was also evident in heterozygous mutants. *TrkC$^{+/-}$* mutants did not show any deficit in numbers of corpuscles, whereas there was a deficit in *NT3$^{+/-}$* mice (Table 3). Thus, in the absence of one or both copies of the *TrkC* gene, NT3 appears to be capable of a compensatory signaling through TrkA or TrkB receptors. Whether NT3 signals exclusively through TrkC or uses all three Trk receptors in wild-type mice could not be addressed directly in our gene deletion experiments. However, the possibility that NT3 signaling might occur through Trk receptors other than TrkC, even in the presence of TrkC, was suggested by the differential distribution of the corpuscle loss in *NT3* and *TrkC* null mutants. In *NT3* null mice, the deficits of Pacinian corpuscles were often more noticeable in the central part of the crural cluster, whereas the corpuscles were missing diffusely in both the central and peripheral regions of the crus in *TrkC$_E$* and *TrkC$_K$* null mice (Fig. 1C).

NT3 Expression

Many sensory neurons derive their neurotrophin support from target tissues. To address whether NT3 is expressed in Pacinian corpuscles, we used P0–P1 neonates that had either a homozygous (*NT3$^{lacZ/lacZ}$*) or heterozygous (*NT3$^{+/lacZ}$*) *lacZ* reporter gene substitution for *NT3*. Both *NT3$^{+/LacZ}$* and *NT3$^{lacZ/lacZ}$* newborns showed no lacZ expression in the central axon or among the layers of the inner core of the corpuscle; however, moderate lacZ expression was detected in cells forming the external capsule (Fig. 4G). NT3 protein immunocytochemistry yielded similar results. Moderate NT-3 immunoreactivity was detected in the external capsular lamellae and, to a lesser degree, in the connective tissues surrounding the corpuscles in wild-type E18.5 embryos and P0–P1 neonates.

Trk Expression

We used Trk protein immunocytochemistry to examine whether afferent axons of Pacinian corpuscles express TrkA, TrkB, or TrkC receptors in late embryonic and neonatal wild-type mice (Fig. 4A–F). At E17.5, the central axon terminal, but not the corpuscle inner core, displayed strong TrkB and moderate TrkA immunoreactivity in all Pacinian corpuscles. TrkC immunoreactivity was not detected at E17.5. At P0, afferents in all corpuscles continued to colocalize TrkA and TrkB by immunocytochemistry. In addition, afferent axons in most but not all Pacinian corpuscles were also moderately immunopositive for TrkC at P0. The outer capsule showed weak TrkC immunoreactivity and the inner core was weakly immunopositive for TrkA in newborn Pacinian corpuscles. Control preabsorption with preimmune peptide or incubation with normal goat serum instead of pri-

mary antibody did not yield any detectable staining.

DISCUSSION

Our study shows that the development of a full complement of crural Pacinian corpuscles in mice requires the concurrent presence of NT3, BDNF, and NT4. Deletion of any of the three neurotrophins resulted in measurable deficits in numbers of corpuscles and their afferent nerve fibers. The impact was greatest for NT3, intermediate for BDNF and least for NT4. Deletion of the *NGF* gene had no statistically significant impact. A major function of neurotrophins is developmental regulation and maintenance of DRG neurons (reviewed by Snider, 1994). The lack of specific markers for afferent neurons to Pacinian corpuscles makes it difficult to determine the impact of neurotrophin deletions on the development of the neurons. However, several lines of evidence suggest that the corpuscle deficits result from deficits of the corresponding sensory neurons in DRGs of neurotrophin-deficient mice: (1) development of Pacinian corpuscles is exquisitely neuron-dependent, and no corpuscles survive in limbs of nerve-sectioned neonates (Zelená, 1978); (2) deficits of crural corpuscles correlated with deficits of parent afferents in the interosseous nerve (and presumably with deficits of the corresponding neuron bodies) in neurotrophin mutant mice; (3) subpopulations of neurons are missing in DRGs of mice lacking *NT3*, *BDNF*, or *NT4* genes (Klein et al., 1993; Ernfors et al., 1994a; Liu et al., 1995); and (4) DRG neurons of *NT3* mutants fail to express parvalbumin (Ernfors et al., 1994a; Airaksinen and Meyer, 1996), a calcium binding protein that is expressed by afferents of several classes of mechanoreceptors including Pacinian corpuscles (Del Valle et al., 1994; Vega et al., 1996).

The inability of the *B/N* allele (*BDNF* in place of *NT3*) to fully rescue Pacinian corpuscles provides further evidence that the corpuscle deficits in mutants result from deficits of the DRG sensory neurons rather than from defective corpuscle assembly associated with the absence of neurotrophins in peripheral target tissues. In *B/N* mutants, ectopic BDNF is expressed in a spatiotemporal pattern that is characteristic of endogenous NT3 (Coppola et al., 2001). If Pacinian corpuscles required peripheral BDNF for their assembly, and corpuscle deficits in *BDNF* mutants resulted from the absence of peripheral BDNF, then one might expect no corpuscle deficits in *B/N* mutants that express excess BDNF in peripheral tissues. However, numbers of Pacinian corpuscles were decreased, as are the numbers of neurons in DRGs and nerve fibers in peripheral nerves, in *B/N* mutants (Coppola et al., 2001). Thus, the number of afferents projecting to the periphery, rather than the neurotrophin concentrations in target tissues, is the likely determinant of numbers of Pacinian corpuscles in the mutants.

Nevertheless, NT3 and to a lesser degree BDNF, may play specific local roles in the development of peripheral target cells in addition to supporting the survival of the innervating neurons. For example, Merkel receptor cells are dependent on NT3 during the perinatal period (Fundin et al., 1997; Szeder et al., 2003). In our preparations, the surviving Pacinian corpuscles in *NT3* and *NT3;BDNF* null mutant newborns were smaller than corpuscles in wild-type newborns. Thus, neurotrophin signaling pathways that regulate specific aspects of the corpuscle morphology, such as the development of lamellation or rate of corpuscle maturation, may exist. However, changes in numbers of crural corpuscles undoubtedly reflected the impact of neurotrophin deletions on the numbers of neurons innervating the Pacinian corpuscles.

NT3 and Pacinian Corpuscle Development

Our study indicates that NT3 is the principal neurotrophin responsible for the development of afferents to Pacinian corpuscles and, hence, corpuscle assembly. Deficits in numbers of corpuscles and their afferents were greater in *NT3* mutants (46%) than in *BDNF* (28%) or *NT4* (15%) null mutants. Moreover, NT3 was the only neurotrophin that could support corpuscles in the absence of the other two neurotrophins. Neither BDNF nor NT4 could support corpuscles when NT3 was absent.

NT3 is required for the survival of sensory neurons that innervate muscle spindles and Golgi tendon organs. Proprioceptive DRG neurons and their larger caliber axons, as well as muscle spindles and tendon organs, are missing in *NT3* null mutants (Ernfors et al., 1994a; Fariñas et al., 1994; Tessarollo et al., 1994). Both proprioceptive and Pacinian neurons are large, express parvalbumin and are capable of high-frequency discharge. Neurons innervating Pacinian corpuscles may be supported by NT3 in dose-dependent manner similar to proprioceptive neurons. Corpuscle deficits were twice as large in mutants lacking two copies of the *NT3* gene than in mutants lacking one copy of the *NT3* gene. Neuron loss in *NT3* mutants (60%) exceeds the estimated number of proprioceptive neurons (20%) residing in the lumbar DRGs (Ernfors et al., 1994a; Klein et al., 1994; Fariñas et al., 1996). Thus, neurons of Pacinian corpuscles may represent one of the subpopulations of nonproprioceptive neurons that are missing in DRGs of *NT3* null mice. Loss of specific cutaneous afferents in *NT3* mutants, such as D-hair afferents of hair follicles and slowly adapting afferents innervating touch dome, suggests that neurons other than proprioceptive afferents depend on NT3 signaling (Airaksinen and Meyer, 1996; Koltzenburg et al., 1996). Vibrissa-related Merkel cell afferents are also eliminated during development in *NT3* null mutants (Airaksinen et al., 1996; Fundin et al., 1997; Szeder et al., 2003).

Presently, Pacinian corpuscles are the only large, encapsulated mechanoreceptors of the limb known to be dependent on more than one neurotrophin. Other limb mechanoreceptors such as muscle spindles and Golgi tendon organs depend solely on NT3, and their numbers in hindlimbs are not affected by the deletion of *BDNF, NT4,* or *NGF* (Ernfors et al., 1994a,b; Liu et al., 1995). A

minority of cutaneous receptors, such as the Ruffini afferents of the mystacial pad, depends on a single neurotrophin, BDNF, for survival and differentiation (Fundin et al., 1997; Rice et al., 1998). In contrast to DRG neurons that supply limb proprioceptors, cranial proprioceptive neurons, which are located in the trigeminal nucleus and innervate masticatory muscles, can be segregated into several subpopulations that are each developmentally dependent on a different neurotrophin (Fan et al., 2000). Thus, afferents to crural Pacinian corpuscles resemble cranial proprioceptors in terms of their developmental dependence on more than one member of the neurotrophin family.

Subpopulations of Pacinian Corpuscles and Their Afferents

The numbers of crural Pacinian corpuscles provide an index of numbers of DRG sensory neurons subserving the corpuscles because the ratio of afferents comprising the murine interosseous nerve and Pacinian corpuscles can be approximated as 1:1. A 1:1 ratio of receptors and Pacinian afferents has also been reported in cats (Iggo, 1985) and in quail for Herbst corpuscles (Zelená et al., 1997). Thus, the size of deficits of crural Pacinian corpuscles in mice deficient in a particular neurotrophin reflects the sizes of the DRG subpopulations of Pacinian neurons that are dependent on that same neurotrophins for their development.

Our results suggest the presence of two major subpopulations of DRG neurons projecting to crural Pacinian corpuscles that differ in their dependence on neurotrophins. One subpopulation, composing a little over 40% of all crural Pacinian neurons, appears to be dependent on NT3 signaling. Corpuscle deficits in *NT3* and *B/N* null mutants support the existence of this population. This neuron subpopulation may be the only one that is dependent on a single neurotrophin for its development and/or survival. BDNF cannot substitute for NT3 as shown by the failure of the *B/N* allele to completely rescue corpuscle deficits in the absence of NT3. The *B/N* allele produces biologically active BDNF in a spatial/temporal distribution similar to that of NT3 expression (Coppola et al., 2001). The inability of the *B/N* allele to rescue Pacinian corpuscle deficits in the absence of NT3 is similar to the failure of ectopic BDNF in *B/N* mutants to rescue proprioceptive deficits caused by *NT3* deletion (Coppola et al., 2001). Thus, a subpopulation of neurons to Pacinian corpuscles may be strictly dependent on NT3 signaling, similar to the limb proprioceptive system (Ernfors et al., 1994a; Coppola et al., 2001).

A second subpopulation of neurons to Pacinian corpuscles appears to be supported jointly by BDNF and NT4. This subpopulation may comprise approximately 60% of the crural Pacinian neurons, as indicated by corresponding corpuscle loss in *BDNF;NT4* mutants. Some of the neurons may be capable of using either BDNF or NT4 because the loss of corpuscles in *BDNF;NT4* double mutants exceeded the sum of corpuscle deficits in *BDNF* and *NT4* single mutants; thus, an overlap in the trophic requirements may exist between the populations of BDNF- and NT4-dependent afferents. Moreover, some corpuscles and their afferents may require the concomitant and collaborative presence of NT3 and BDNF or NT3 and NT4 for their proper development. Double *NT3;BDNF* or *NT3;NT4* mutants had no corpuscles and no interosseous afferents, although single *NT3*, *BDNF* or *NT4* mutants contained Pacinian corpuscles. Thus, some Pacinian neurons require NT3 only for survival and/or differentiation, whereas others can use BDNF and/or NT4, or a combination of the three neurotrophins.

The presence of a few cranial proprioceptors and masseter muscle spindles in the combined absence of NT3, BDNF, NT4, and NGF suggests that trophic factors other than neurotrophins can support developing proprioceptors (Fan et al., 2000). Likewise, the lanceolate afferents of the mystacial pad are supported by trophic factors other than neurotrophins, such as glial-derived neurotrophic factor (Buj-Bello et al., 1994; Fundin et al., 1997). In contrast, the interosseous nerve and Pacinian corpuscles were entirely absent in triple *NT3;BDNF;NT4* mutants. These data suggest that factors other than members of the neurotrophin family are incapable of providing compensatory trophic support for the developing crural Pacinian system in mice.

Role of Trk and p75^{NTR} Receptors

Wild-type murine Pacinian corpuscles expressed TrkA, TrkB, and TrkC not only in layers of their capsule (Ribeiro-da-Silva et al., 1991; Vega et al., 1996; Lopez et al., 1998; Stark et al., 2001) but also, and more importantly, in the central afferent axon located in their core. All afferents subserving Pacinian corpuscles coexpressed TrkA and TrkB, and some afferents coexpressed all three Trk receptors. The significantly greater corpuscle deficits in *NT3* than in *TrkC* mutants indicate that most NT3 signaling occurs through TrkA and TrkB receptors rather than TrkC receptors. The presence of substantial corpuscle deficits in *TrkA* or *TrkB* mutants, exceeding deficits observed in *NGF* or *BDNF* mutants, provides additional support for this assertion. The greater loss of DRG neurons in *NT3* than in *TrkC* mutants is also consistent with interaction of NT3 with TrkA and TrkB receptors (Ernfors et al., 1994a; Klein et al., 1994). NT3 signaling through TrkA and TrkB receptors has been demonstrated in a variety of neurons including DRG innervating sense organs such as Merkel cells or neurons innervating cochlear organs (Fundin et al., 1997; Rice et al., 1998; Coppola et al., 2001).

Each of the three *Trk* null mutants, *TrkA*, *TrkB*, and *TrkC*, showed deficits of Pacinian corpuscles. These deficits are consistent with the observation that the Trk receptors are expressed or coexpressed on the surface of afferents that innervate Pacinian corpuscles. Coppola et al. (2001) reported that expression of TrkC, but not TrkB or TrkA, is downregulated in embryonic DRGs of *B/N* mutants. Thus, the persistence of corpuscle deficits in *B/N* mutants to a degree similar to that observed in *NT3* mutants indicates that a sub-

population of Pacinian neurons normally uses TrkC signaling or concomitant TrkC and TrkA signaling to mediate the NT3 function. However, the presence of significantly larger corpuscle deficits in *B/N* than in *TrkC* mutants suggests that concomitant TrkC and TrkA signaling rather than exclusive TrkC signaling is required by the NT3-dependent neurons to Pacinian corpuscles to survive and/or differentiate. TrkC signaling may be mediated through activation of the kinase, because the magnitude of impact of the $TrkC_E$ mutation, in which all TrkC protein is missing, and $TrkC_K$ mutation in which tyrosine kinase activity is missing, was equivalent.

Pacinian corpuscle deficits in $p75^{NTR}$ mutants were small and of similar magnitude as the deficits observed in *TrkC* mutants. Deficits in $p75^{NTR}$*;BDNF* and $p75^{NTR}$*;NT4* double gene mutants were greater than in the corresponding single neurotrophin gene mutants. These observations are consistent with a role for $p75^{NTR}$ in facilitating neurotrophin signaling through their cognate Trk receptors. The p75 receptor has been shown to act in synergy with NT3 to enhance the survival of limb proprioceptive neurons when tissue levels of NT3 are a limiting factor (Fan et al., 1999).

Expression of Trk receptors in the DRG neurons is dynamically regulated during embryogenesis (Piñón et al., 1996; Fariñas et al., 1998; Coppola et al., 2001), and DRG neurons may change their neurotrophin signaling patterns during development (Davies, 1994). Thus, deficits of Pacinian corpuscles in *TrkA*, *TrkB* or *TrkC* mutants may result from a neuronal loss at different stages of DRG development and may reflect patterns of neurotrophin dependences at different stages of DRG development, including an early stage before the formation of the corpuscles. An analysis of the full range of neurotrophin functions in Pacinian corpuscle development and maintenance would require studies of individually identifiable Pacinian neurons at multiple stages of prenatal and postnatal development.

Target Expression of NT3

The distinct TrkA, TrkB, and TrkC immunoreactivity of the central afferent axon is consistent with the possibility that developing neurons to Pacinian corpuscles derive neurotrophin support from perineurial or other peripheral tissues as they make their way into the limb. NT3 expression was limited to cells of the external lamellae, and excluded the afferent axon. Pacinian corpuscles have also been shown to express NGF (Vega et al., 1994), but they have been not examined for BDNF or NT4 expression. NT3 synthesized by target tissues may not only support the survival of sensory neurons, but also facilitates axon branching, axon guidance, and target cell differentiation (Patel and Snider, 2002). For example, NT3 produced by muscle spindles is required for not only the survival, but also the synaptic function, of their group I and II afferents (Mendell et al., 2001). Mice that overexpress NT3 in epidermal keratocytes have enlarged touch domes with an enhanced number of Merkel cells, greater density of innervating afferents, and increased numbers of sensory neurons in DRGs (Albers et al., 1996). Developing limbs strongly express NT3 in the premuscle mass at the time afferents exit from DRGs and grow toward their prospective peripheral targets (Henderson et al., 1993). Thus, multiple extraganglionic sources of NT3 may be available to Pacinian neurons during a critical time in development when they are dependent on NT3, which is not expressed in an autocrine manner within the DRGs (Fariñas et al., 1996). Similar consideration may apply to BDNF and NT4 that are also expressed by developing limbs (Henderson et al., 1993). Thus, neurotrophins derived from peripheral tissues could retrogradely support neuron development and survival and, thus, Pacinian corpuscle formation.

CONCLUSIONS

Dependency of Pacinian corpuscles on more than one neurotrophin or Trk receptor is consistent with expression by many DRG neurons of different Trk receptors either simultaneously or sequentially during development (Fariñas et al., 1996; Piñón et al., 1996). From an evolutionary perspective, development of the phylogenetically oldest receptors such as cutaneous innervation appears to be regulated by a competitive balance between the promoting and suppressing effects of several neurotrophins (Rice at al., 1998), whereas development of the phylogenetically youngest receptors, such as limb proprioceptors, is governed by a single neurotrophin (Ernfors et al., 1994a). Pacinian corpuscles and their afferents may then represent a class of receptors in a transitional phase between the nonselective and the more selective patterns of neurotrophin dependence.

EXPERIMENTAL PROCEDURES

Animals and Genotyping

Newborn and young adult *NGF* (129/Balb/c; Crowley et al., 1994), *NT3* (129/balb/c; Ernfors et al., 1994a), *BDNF* (129/balb/c; Ernfors et al., 1994b), *NT4* (129/balb/c; Liu et al., 1995), $NT3^{lacZ/lacZ}$ (129/c57bl/6; Fariñas et al., 1996), *TrkA* (129/c57bl/6; Smeyne et al., 1994), *TrkB* (129/c57bl/6; Klein et al., 1993), $TrkC_E$ (129/c57bl/6; Tessarollo et al., 1997), $TrkC_K$ (129/c57bl/6; Klein et al., 1994), and $p75^{NTR}$ (129/balb/c; Lee et al., 1992) null mutant mice as well as *B/N* mice in which the *NT3* gene was replaced by the *BDNF* gene (129/c57bl/6; Coppola et al., 2001) were obtained from overnight matings of adult males and females that were heterozygous for the mutated genes. $TrkC_E$ null mutant mice do not express any known isoforms of TrkC, whereas $TrkC_K$ null mutants have a mutation in the TrkC tyrosine kinase domain that renders it inactive but does not affect truncated isoforms of TrkC lacking the kinase domain (Klein et al., 1994). Standard two-step polymerase chain reactions and Southern blot analysis of DNA extracted from tail tissue were used to genotype mice as null mutant (−/−), heterozygous mutant (+/−), or wild-type (+/+) for the *NGF*, *NT3*, *BDNF*, *NT4*, *TrkA*, *TrkB*, *TrkC*, or $p75^{NTR}$ genes. Double (*NT3;BDNF*, *NT3;NT4*, or *NT4;BDNF*) or triple (*NT3;BDNF;NT4*) null mutants were generated by

mating mice heterozygous for single gene deletions. Wild-type (WT) littermates served as controls. In studies of WT embryos, the morning after an overnight mating of males to females was considered E0.5. Fetuses were removed in the morning of days E16.5 or E17.5.

Counts of Pacinian Corpuscles in the Crus

Neonatal, adolescent, and young adult mutants and their wild-type littermates were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.) and were perfused with paraformaldehyde–glutaraldehyde fixative between postnatal days (P) 0 and 4, 12 and 16, or 28 and 30. Fetus were removed from dams anesthetized with sodium pentobarbital by Cesarean section. Hindlimbs were removed and embedded in resin. Counts of crural Pacinian corpuscles were obtained from the resin-embedded specimens that were serially sectioned in the transverse plane at 1 μm thickness from the ankle to the knee and stained with toluidine blue. Every 10th section was stained with toluidine blue and examined with a light microscope. Pacinian corpuscles in newborn mice are considerably longer than 10 μm, so that corpuscles could be accurately counted. Pacinian corpuscles were identified as multilamellar, elongated structures innervated by large caliber axons. In a separate procedure, the fixed hindlimbs of neonates and adults were fixed, skinned, and processed for ChE activity. The triceps muscle and deep flexors were then removed to expose the interosseous nerve that runs along the interosseous membrane in between the fibula and tibia. The exposed crural cluster of Pacinian corpuscles was visualized in ChE staining according to Karnovsky and Roots (1964) with the addition of Triton X-100 into the incubation medium. Stained corpuscles were counted with the aid of a dissecting microscope either in situ (newborns) or after shank excision (adults).

Counts of the corpuscles from plastic-embedded sections were statistically equivalent between wild-type 129/C57Bl/6 and 129/Balb/C mice and, thus, were pooled for analyses. However, counts differed slightly between wild-type mice of $TrkC_E$ and $TrkC_K$, mutant strains when examined in ChE staining. These data were not pooled and are listed separately in Table 2A. The TrkC data were analyzed by one-factor analysis of variance and post hoc comparisons. All other data were transformed by square root before analysis, but were reported as untransformed values (mean ± SEM). Means of variables (wild-type vs. mutant) were compared by a Student's *t*-test using $P < 0.05$ as the limit for statistical significance.

NT3 and Trk Immunocytochemistry

Expression patterns of TrkA, TrkB, and TrkC in Pacinian corpuscles were studied in embryonic, newborn, and adult wild-type mice by immunostaining for the receptors. Animals were perfused with 4% paraformaldehyde fixative (PFA) under sodium pentobarbital anesthesia (50 mg/kg body weight, i.p.). In adults, lower legs were removed and crural corpuscles together with their interosseous nerve were isolated. The isolated corpuscle cluster was then postfixed with PFA for 2 hr, rinsed in phosphate-buffered saline (PBS), and cryprotected in 15% and 30% sucrose. Tissues were embedded in the OTC compound (Sakura Finetek USA, Inc.), frozen in liquid nitrogen, and cut in serial 10-μm-thick sections in a cryostat. Pacinian corpuscles of embryos or newborns were processed in transverse sections of the whole crus. The sections were pretreated in normal goat serum in PBS 1:10, incubated overnight at 4°C with anti-TrkA, anti-TrkB, or anti-TrkC antibodies (numbers sc-118, sc-12, and sc-117, respectively, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:50–1:300. Peroxidase-labeled goat anti-rabbit served as a secondary antibody (Sigma, St. Louis, MO). The reaction product was visualized by treatment with 3,3′-diaminobenzidine tetrahydrochloride and H_2O_2. Some sections were counterstained with hematoxylin. Negative controls included preabsorption with an appropriate blocking peptide or substitution of normal goat serum for the primary antibody.

LacZ Histochemistry

Sites of *lacZ* reporter gene expression in $NT3^{+/lacZ}$ and $NT3^{lacZ/lacZ}$ mutants were visualized by using β-galactosidase histochemistry. Legs of neonatal (P0–P1) were fixed with 4% PFA, cryoprotected in 30% sucrose, frozen, cut at 10 μm thickness in a cryostat, and stained overnight at 37°C in X-gal solution at pH 7.3 according to Fariñas et al. (1996). Deposits of a blue reaction product indicated sites of the *lacZ* reporter expression. Pacinian corpuscles of wild-type mice were devoid of X-gal reactivity.

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