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Muscle sensory neurons require neurotrophin-3 from peripheral tissues during the period of normal cell death

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SUMMARY

To determine if muscle sensory neurons require neurotrophin-3 (NT3) during the period of normal cell death, we used an NT3-specific antiserum to deplete NT3 from peripheral tissues during this period in chick embryos. DiI staining of dorsal roots indicated that limb injections of anti-NT3 reduced the spinal projection of muscle spindle afferents. In contrast, injection of the antiserum into the spinal cord had no demonstrable effect, indicating that the reduced projection following limb injection was due to peripheral blockade of NT3 signaling. Counts of neurons retrogradely labeled from muscle and cutaneous nerves showed that peripheral blockade of NT3 selectively reduced the survival of muscle sensory neurons without affecting the survival of cutaneous sensory neurons or motoneurons. In situ hybridization with *trk*C probes indicated that, during the period of cell death, most large diameter muscle sensory neurons express *trk*C transcripts, whereas few cutaneous neurons express this receptor for NT3. We conclude that large diameter muscle afferents, including spindle afferents, require NT3 from peripheral tissues to survive the normal period of sensory neuron death in vivo.

Key words: neurotrophin-3, muscle spindle afferents, cutaneous afferents, motoneurons, trkC, cell death

INTRODUCTION

During normal embryonic development, approximately half the motoneurons and primary sensory neurons generated die after their axons reach their peripheral targets. Since expansion of the target field, treatment with target extracts or treatment with purified factors can rescue a substantial fraction of these neurons, the availability of target-derived survival factors is likely to be limited during normal development (for reviews see Levi-Montalcini, 1987; Davies, 1987; Barde, 1989; Oppenheim, 1989; Thoenen, 1991). An important class of these survival factors are the neurotrophins. Four neurotrophins that share considerable sequence identity have been characterized at the molecular level: nerve growth factor (NGF; Cohen, 1960), brain-derived neurotrophic factor (BDNF; Leibrock et al., 1989), neurotrophin-3 (NT3; Hohn et al., 1990; Jones and Reichardt, 1990; Maisonpierre et al., 1990) and neurotrophin-4/5 (NT-4/5) (Burkemeier et al., 1991; Hallbook et al., 1991).

The physiological responses of neurons to the neurotrophins are primarily mediated by a set of structurally related receptors encoded by the *trk* family of tyrosine kinase receptors. Three members of this family have recently been identified as functional high affinity receptors for neurotrophins (Klein et al., 1991; Squinto et al., 1991; Cordon-Cardo et al., 1991; Lamballe et al., 1991). Studies of ligand binding and signal transduction have shown that NGF preferentially activates the *trk*A receptor and NT-3 preferentially activates the *trk*C receptor. The interaction of neurotrophins with the *trk*B receptor are more complex, since both BDNF and NT-4/5 are potent activators of this receptor (Ip et al., 1993).

Of the neurotrophins, NGF is the most extensively characterized in terms of its biological function in vivo. NGF is a target-derived survival factor that is required by sympathetic neurons and most sensory neurons during the period of normal cell death (Levi-Montalcini and Booker 1960; Johnson et al., 1980; Davies et al., 1987; Ruit et al., 1992). It is presently unclear if the other neurotrophins act in a similar fashion. The widespread distribution of transcripts for NT3 and BDNF (Schecterson and Bothwell, 1992) and their receptors (Klein et al., 1991; Lamballe et al., 1991; Tessarollo et al., 1993) suggest that the function of these neurotrophins may be distinct from that of NGF (Lindsay et al., 1994).

Recent genetic deletion studies have demonstrated that NT-3 is essential for the normal development of various peripheral ganglia. For example, deletion of NT-3 results in a severe loss of neurons in both sensory and sympathetic ganglia (Ernfors et al., 1994; Farinas et al., 1994). In dorsal root ganglia (DRG), less than 30% of the normal complement of sensory neurons develop; losses include the muscle sensory neurons that supply muscle spindles and Golgi tendon organs, as well as other classes of sensory neurons (Ernfors et al., 1994; Farinas et al., 1994). However, since genetic deletion leads to the complete loss of NT-3 throughout embryogenesis, it is unclear when NT-3 acts to influence the development of sensory neurons.

Several lines of evidence suggest that NT-3 may act as a target-derived survival factor for some types of muscle sensory neurons. First, NT-3 transcripts have been localized to developing limb muscle during the period of sensory cell death (Henderson et al., 1993). Second, culture studies have shown that NT-3 is more effective than either NGF or BDNF at supporting the survival of muscle sensory neurons (Hohn et al., 1990; Hory-Lee et al., 1993). Third, sensory neurons that supply muscle spindles have been shown to be insensitive to NGF deprivation (Laing et al., 1988; Ruit et al., 1992), and appear to be unaffected by genetic deletion of BDNF (Jones et al., 1994), suggesting that these neurons may require a different survival factor.

In this report, we have used a specific, function-blocking antiserum to NT3 to block the action of NT3 in peripheral tissues only during the period of neuronal death in the DRG. By depleting NT3 during the period of cell death, we show that large diameter muscle afferents, including muscle spindle afferents, require NT3 to survive. Neither cutaneous sensory neurons nor motoneurons required NT3 to survive this period. Finally, large muscle sensory neurons were found to differentially express *trk*C during the period of DRG cell death, suggesting that the survival-promoting effects of NT3 on this class of sensory neurons are mediated by trkC receptors.

MATERIALS AND METHODS

In ovo injections

Fertile eggs (SPAFAS) were windowed after 3 days incubation (E3); embryos were moistened with several drops of saline containing 100 u/ml penicillin/streptomycin (Gibco) and returned to the incubator until E6. For limb injections, embryos received daily, unilateral injections of 2-4 μl of either undiluted anti-NT3 serum or non-immune rabbit serum to the right anterior thigh beginning on E6 and continuing through E9. For spinal cord injections, embryos received daily injections of 2-4 μl of serum to the central canal at the mid-thoracic level beginning on E6 and continuing through E8. Pressure injections of sera containing 0.02% fast green were administered using a broken micropipette (tip size: 10-30 μm) connected to a Picospritzer (General Valve). On E10, embryos were processed for retrograde or dorsal root labeling (see below). Approximately 50% of both control and anti-NT3-treated embryos survived. The anti-NT3 serum was a generous gift from Regeneron Pharmaceuticals. It was produced by immunizing rabbits with purified human recombinant NT3.

Cell culture

Lumbosacral DRG isolated from E8 chick embryos were dissociated following treatment with 0.05% trypsin for 20 minutes at 37°C. The cells were preplated for 2-3 hours on tissue culture plates to reduce the number of non-neuronal cells and then plated at a density of 1000-3000 cells per well in 4-well plates (Nunc) that had been previously coated with polyornithine (1 mg/ml; Sigma) and laminin (8 μg/ml; Gibco). The growth medium was MEM containing 10% heat-inactivated horse serum and 100 u/ml penicillin/streptomycin (Gibco). The media was supplemented with either rat NT3, chicken NGF or chicken BDNF, with or without the anti-NT3 serum. Neuronal survival was determined after 20 hours in culture by comparing the number of surviving neurons to the number of cells plated. All recombinant neurotrophins were produced by baculovirus as previously described and were used at approximately 1-2 ng/ml (see Garner and Large, 1994). Anti-NT3 was used at a final dilution of 1:100.

Labeling of sensory projections

Treated embryos were washed in phosphate-buffered saline (PBS), decapitated and eviscerated. Following ventral laminectomy, all of the lumbosacral dorsal roots on both sides were pressure-injected with DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; 5 mg/ml in 90% ethanol, 10% dimethyl sulfoxide; Molecular Probes) using broken micropipettes. For reference, the ventral roots ipsilateral to the serum injection were injected with DiO (3,3′-dioctadecyloxacarbocyanine perchlorate; 3 mg/ml in 90% ethanol, 10% dimethyl sulfoxide; Molecular Probes). The spinal cords with attached DRG were then fixed in 4% paraformaldehyde (PF) in PBS for at least 48 hours at room temperature, embedded in 19% gelatin and sectioned at 50-70 μm using a vibrating microtome. The sections were mounted in Gelmount (Biomedia) and photographed immediately. In some cases, the limbs and thoracic spinal cord were also fixed, as were several additional embryos either 4 or 24 hours after a single injection on E6. These tissues were processed for immunohistochemistry (see below) to determine the distribution of the injected serum.

Retrograde labeling

E10 embryos (stage 35-36) were washed in sterile Tyrode's, decapitated and eviscerated. The lumbosacral spinal cord and DRG were exposed by dissecting away the ventral and lateral portions of each vertebra. Peripheral nerve branches of the crural plexus were exposed on both sides and either the lateral femoral cutaneous nerves or the femoral muscle nerves were pressure injected with a 25% solution of lysinated rhodamine dextran (3 kD; Molecular Probes) in 1% Triton X-100 (Sigma) using a broken micropipette (see Glover et al., 1986). The spinal cord was opened along the ventral midline and the preparations, including intact ventral roots and attached thighs, were then incubated for 24-32 hours at 32°C in sterile oxygenated Tyrode's containing 100 u/ml penicillin/streptomycin. Following dye transport, the preparations were fixed for 18 hours in 4% PF at 4°C, cryoprotected in 30% sucrose, embedded in a 2:1 mixture of 30% sucrose and OCT (Miles Scientific) and frozen on dry ice. Serial, 10 μm frozen sections were cut on a Reichert-Jung cryostat and collected on Super-Frost Plus slides (Fisher). Labeled cells were counted in every fourth section.

RNA probe synthesis

RNA probes labeled with ^{35}S-UTP (1300 Ci/mmol; New England Nuclear) were synthesized in vitro using standard protocols (Promega). For probes complementary to the extracellular domain of *trk*C, a construct encoding the entire extracellular domain of the chicken *trk*C, including 10 base pairs upstream from the start site, was used as a template. This probe should hybridize to all known splice variants of the chicken *trk*C receptor (see Garner and Large, 1994). The transcripts were then hydrolyzed to an average size of 250-350 bases (Cox et al., 1984). Sense probes synthesized from the same constructs were used as control probes.

In situ hybridization

Selected sections containing retrogradely labeled sensory neurons were fixed in 4% PF for 20 minutes, washed three times in PBS and digested for 10 minutes with proteinase K (10 μg/ml in 50 mM Tris/5 mM EDTA) at room temperature. The sections were then fixed again and washed in PBS (as above), washed in 100 mM triethanolamine and acetylated for 10 minutes in 0.25% acetic anhydride in 100 mM triethanolamine. The sections were then washed twice in 2× SSC, dehydrated through graded alcohols and air-dried.

Hybridization solution containing 6-7×10^4 cts/minute/μl was applied and covered with a Parafilm coverslip. Hybridization was performed at 50°C in a humid chamber for 8-10 hours. The hybridization solution consisted of: 50% formamide, 300 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA, 3 mM DTT, 1× Denhardt's solution, 500 μg/ml yeast tRNA, 200 μg/ml herring sperm DNA and 10% dextran sulfate. Following hybridization, the coverslips were removed by washing in 5× SSC at 50°C. The slides were washed four times in 2× SSC (37°C) and digested with RNAase A (1 μg/ml in 500 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA) for 45 minutes at 37°C. The slides

were then washed once in RNAase buffer, washed in 2× SSC at 37°C for 15 minutes, washed in 2× SSC at 56°C for 15 minutes and in 0.1× SSC at 56°C for 15 minutes. The sections were dehydrated through graded alcohols, air-dried and coated with NTB-2 nuclear track emulsion (1:1 in distilled H_2O; Kodak). The autoradiograms were exposed for 12-14 days, developed in D19 (Kodak), dehydrated through graded alcohols, equilibrated in xylene and coverslipped in DPX (Aldrich Chemical).

Immunohistochemistry

To determine the distribution of the injected antiserum, spinal cords with attached DRG and limbs of sera-injected embryos were fixed in 4% PF, processed for frozen sectioning (as above) and sectioned at 14 μm. Selected sections were rehydrated with three washes in PBS and endogenous peroxidase activity was quenched by incubating for 9 minutes in 0.6% H_2O_2 in methanol. The sections were then washed three times in PBS, incubated for 30 minutes in blocking buffer (PBS containing 1% bovine serum albumin and 1% horse serum), and incubated for 60 minutes in secondary antibody (HRP-conjugated goat anti-rabbit; Cappel; 1:500 in blocking buffer). The sections were washed three times in 0.1 M phosphate buffer and developed for 3-5 minutes using a DAB substrate kit (Vector) with nickel intensification according to the manufacturer's directions.

RESULTS

Antiserum specificity

The specificity of the anti-NT3 serum is demonstrated in Table 1. NT3, BDNF and NGF each supported the survival of a large number of sensory neurons in vitro. Addition of the anti-NT3 antiserum to the culture medium completely blocked the survival-promoting effects of NT3 but had no effect on sensory neuron survival in cultures containing chicken NGF or chicken BDNF. In addition, the NT3 antiserum specifically blocked NT3-stimulated ougrowth from DRG explants (at dilutions of 1:50 to 1:500) without affecting NGF or BDNF-stimulated outgrowth (not shown). Moreover, although the antiserum reacts with human recombinant NT-3 on western blots, it did not cross-react with either NGF or BDNF (not shown).

Distribution of anti-NT3 serum in injected embryos

Anti-NT3 was injected unilaterally into chick limbs once a day beginning on E6 and continuing through E9. To determine the distribution of the injected anti-NT3, limbs and axial tissues of treated embryos were fixed on E10 and stained using an HRP-conjugated secondary antibody. Unilateral limb injections consistently resulted in more intense staining of the ipsilateral limb (compare Fig. 1A and B), indicating that the antiserum is more highly concentrated in the injected limb. Within the injected limb, the staining was largely uniform, with intense staining in both muscle and cutaneous targets of sensory neurons (Fig. 1A). Control injections resulted in a similar distribution of the non-immune control serum (not shown). Although clearly less intense than the injected limb, staining in the contralateral limb was well above the background found in limbs from normal embryos (not shown).

Table 1. Effects of anti-NT3 on the survival of sensory neurons cultured in the presence of different neurotrophins

	Percent survival after 20 hours in culture	
Neurotrophin added to culture medium	Neurotrophin alone	Neurotrophin + anti-NT3
NGF	59.8±5.6	62.7±6.6
BDNF	50.7±4.9	54.4±6.8
NT3	37.5±2.7	3.5±1.3
none	2.6±0.9	n.d.

Percentages are given as the mean±s.d. Data are from 3 independent experiments with duplicate cultures for each condition. Neurotrophins were added to a final concentration of 1-2 ng/ml. Anti-NT3 was used at a dilution of 1:100.

In contrast, no staining above background was detected within the spinal cord or DRG in E10 embryos following a series of four anti-NT3 injections to the limb. To verify that the spinal cord and DRG were not exposed to anti-NT3 at earlier stages, in a separate series of experiments injected embryos were fixed at 4 and 24 hours after a single injection on E6. Again, no staining above background was detected in the spinal cord or DRG 4 hours after the injection, despite the fact that surrounding tissues exhibited intense staining (Fig. 1C). A similar pattern was obtained 24 hours after a single injection; peripheral tissues were well stained (although less intensely than after 4 hours) but the spinal cord and DRG were not labeled (not shown). These results indicate that, following unilateral limb injections, anti-NT3 is concentrated within the injected limb with some spread to the contralateral limb and other peripheral tissues, but there is no detectable antiserum in the spinal cord or DRG.

To be certain that the effects of the limb injections were not due to the presence of low levels of anti-NT3 that had diffused to the DRG and spinal cord, we also analyzed embryos following direct injection into the spinal cord. 4 hours after a single injection into the central canal of an E6 embryo, intense staining was detected within the spinal cord and DRG (Fig. 1D). The axial tissues surrounding the spinal cord were also intensely stained, probably due to leakage at the injection site, but staining in limb tissues was much lighter. A similar pattern of staining was apparent 24 hours after injection (not shown).

Peripheral blockade of NT-3 reduces central projections of muscle spindle afferent fibers

Labeling of all of the lumbosacral dorsal roots in control embryos revealed the characteristic pattern of spinal cord projections typical of sensory afferents in normal embryos at this stage (Davis et al., 1989); muscle spindle afferent collaterals project ventrally toward the motor columns, while other sensory fibers are confined to the dorsal laminae (Fig. 2A). These ventrally projecting fibers include the most rapidly conducting sensory axons, group Ia fibers, and they will be abbreviated as Ia fibers. There was no detectable effect of control serum injections on the Ia fiber projection in any of the embryos examined (n=8).

In embryos that received limb injections of anti-NT3, there was an obvious reduction in the number of ventrally projecting Ia collaterals in the spinal cord ipsilateral to the injection (Fig. 2C,E). A reduction in the central projections of Ia fibers was consistently observed on the ipsilateral side in at least three segments of each anti-NT3-treated embryo (total of 36 asymmetric segments in 8 embryos). The effect of the anti-NT3 was selective in that there was no noticeable change in the density of sensory fibers within the dorsal horn. Although the anti-NT3 treatment reduced the number of Ia collaterals in the spinal cord, the growth of the remaining Ia collaterals appeared normal. In all

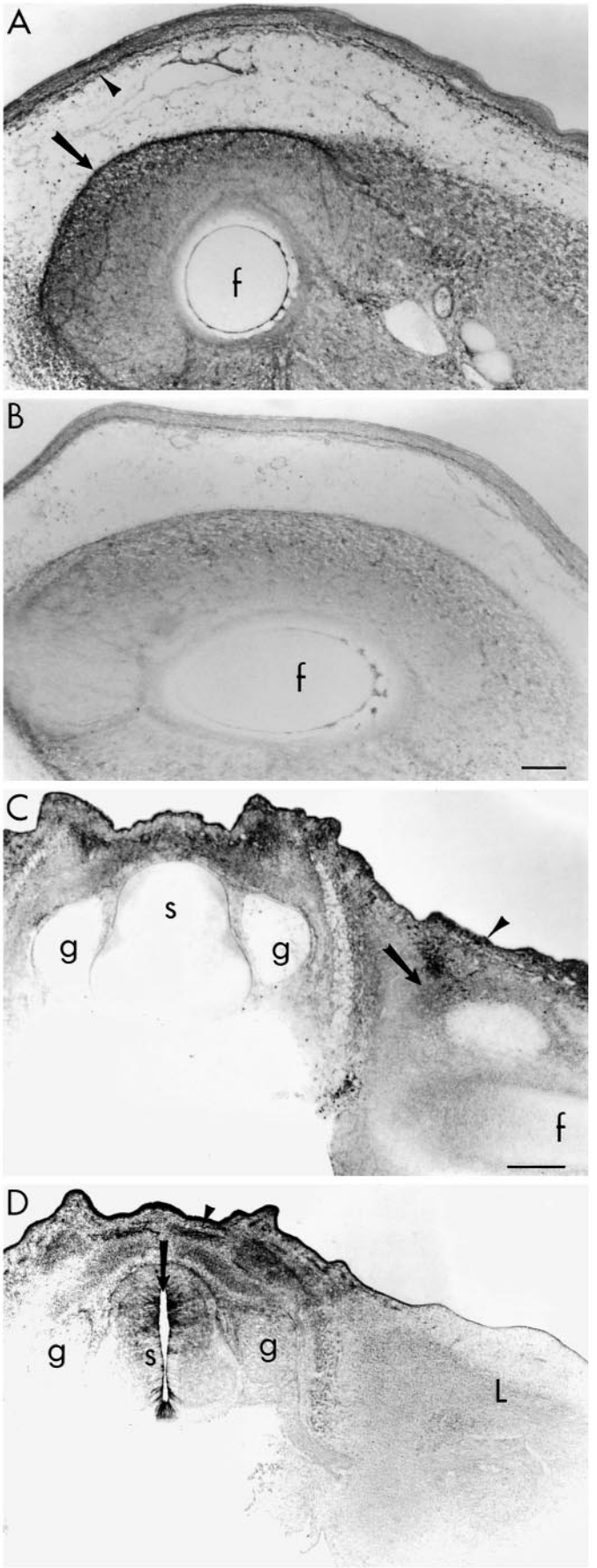


Fig. 1. Distribution of injected anti-NT3 serum. (A,B) Distribution of anti-NT3 in ipsilateral (A) and contralateral (B) limbs at E10 following 4 unilateral injections of anti-NT3 to the limb. Anti-NT3 was injected once a day beginning on E6 and continuing through E9. In the ipsilateral limb (A), staining is intense in both muscle (arrow) and skin (arrowhead). A similar distribution is evident in the contralateral limb (B), although the staining is less intense. (C) Distribution of anti-NT3 4 hours after a single injection to the limb on E6. Despite intense staining in both the muscle (arrow) and skin (arrowhead) in the ipsilateral limb and in axial tissues, the serum was not detected in dorsal root ganglia (g) or spinal cord (s). (D) Distribution of anti-NT3 4 hours after a single injection to the central canal (arrow) on E6. The serum was detected in the spinal cord (s), dorsal root ganglia (g) and axial tissues (arrowhead). Weak staining was also seen in both limbs (L). A and B are cross sections of E10 limbs. C and D are transverse sections of E6 embryos; f, femur. Scale bars, 200 μm.

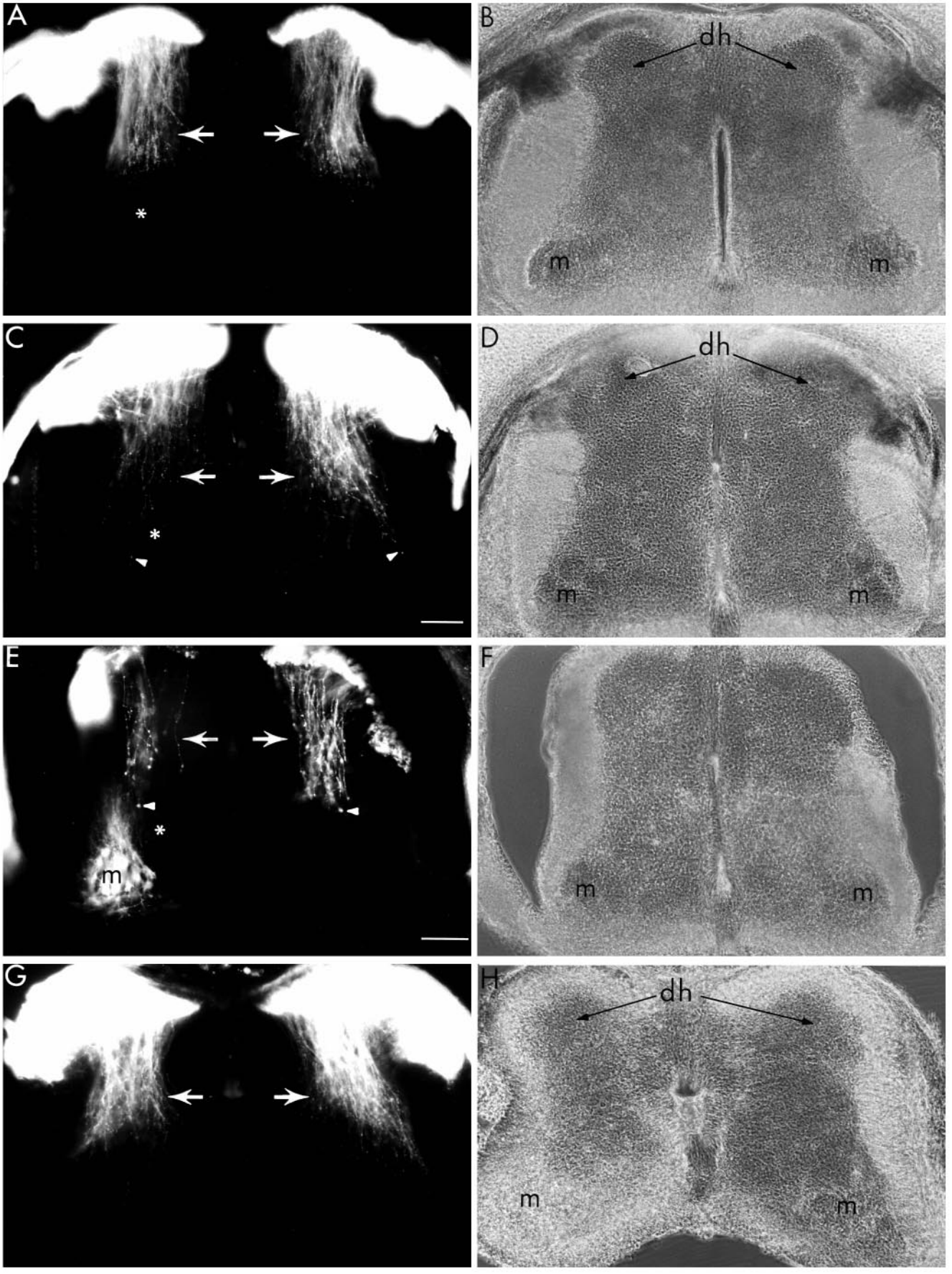
embryos examined, the remaining Ia collaterals on the ipsilateral side were directed toward the motor column. Moreover, the remaining ipsilateral fibers extended as far ventrally as those on the contralateral side (Fig. 2C,E).

In contrast, central administration of anti-NT3 had no apparent effect on the development of Ia collaterals. Following spinal cord injection of anti-NT3, the projection of Ia collaterals was indistinguishable from that seen in control embryos (Fig. 2G; n=4). Thus, the loss of Ia fibers following limb injections must be due to peripheral blockade of NT3 signaling rather than to diffusion of the antiserum to DRG or spinal cord.

Anti-NT3 selectively enhances the death of large muscle sensory neurons

To determine if the observed reduction in the central projection of Ia afferents resulted from decreased survival of muscle sensory neurons, as opposed to a change in their central projections, we counted the number of sensory neurons retrogradely labeled from individual muscle and cutaneous nerves. Femoral muscle nerve injections exclusively label muscle sensory neurons in the DRG, mainly in lumbosacral (LS) segments 2 and 3. These injections also label the femorotibialis motor pool, within the same segments of the spinal cord (Honig, 1982). Injection of the lateral femoral cutaneous nerve exclusively labels cuta-

Fig. 2. Peripheral but not central injection of anti-NT3 reduces Ia afferent projections in the spinal cord. Fluorescence (A,C,E,G) and phase contrast (B,D,F,H) images of spinal cords in transverse section after DiI labeling of the dorsal roots. In control embryos in which one limb was injected with non-immune serum (A,B; E10), Ia afferent fibers (arrows) extend toward the motor columns (m). An approximately equal population of Ia fibers is revealed both ipsilateral (*) and contralateral to the injected limb. The fibers of other sensory neurons have begun to arborize within the dorsal horn (dh). After four injections of anti-NT3 to one limb, (C-F), the Ia afferent projection is reduced on the ipsilateral side (*). There were fewer Ia fibers (arrows) on the ipsilateral side at both comparable (C,D; E10) and earlier (E,F; stage 35) stages of development. Those Ia fibers that are present on the ipsilateral side extend as far ventrally (arrowhead) toward the motor columns (m) as do Ia fibers on the contralateral side (arrowhead). Dorsal horn (dh) projections did not appear to be affected by limb injection of anti-NT3. After anti-NT3 injections to the spinal cord (G,H; E10), Ia collaterals (arrows) appear normal and extend toward the motor columns (m). Dorsal horn (dh) projections also appear normal after spinal cord injections. Scale bars = 100 μm.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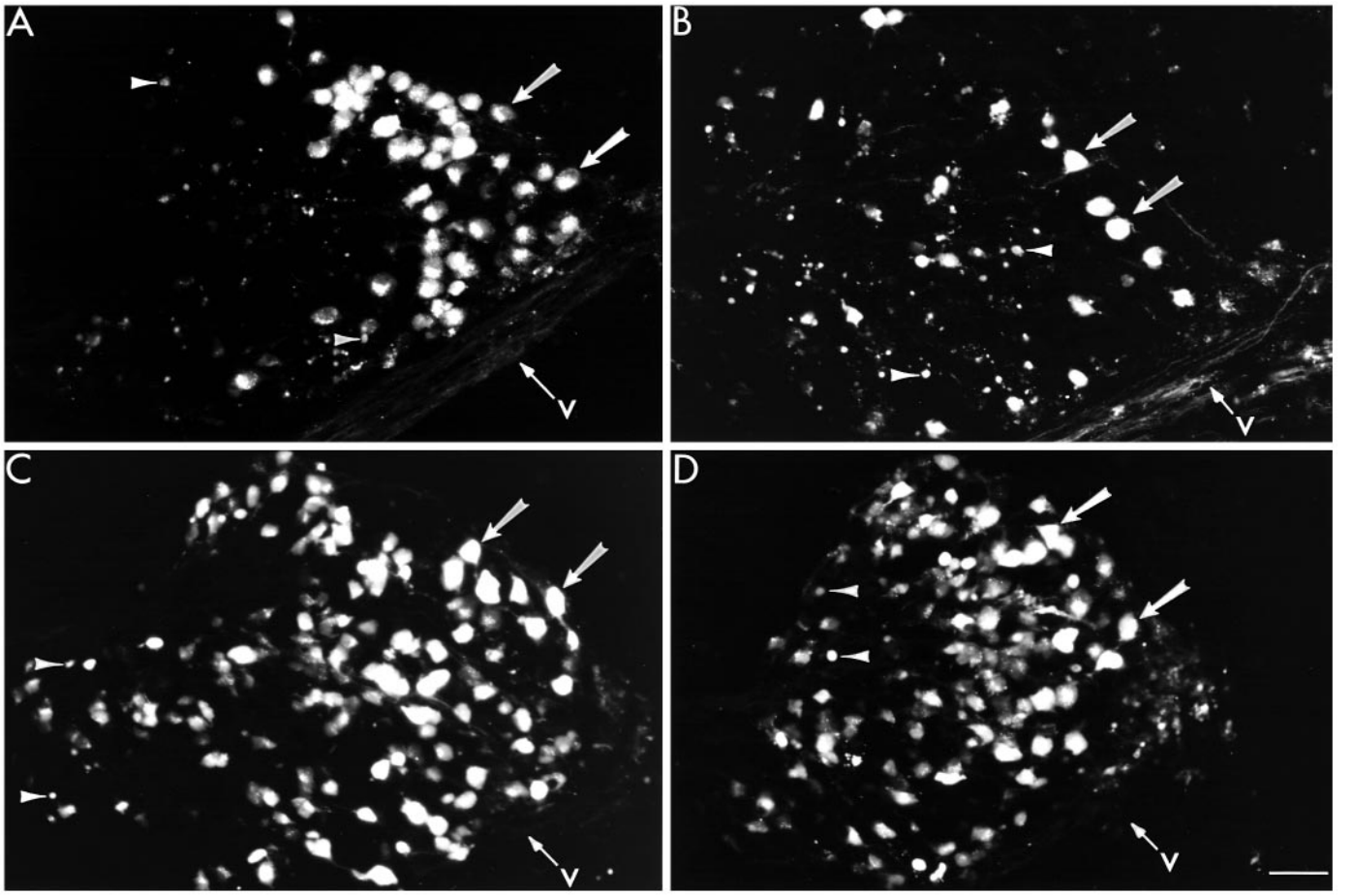

Fig. 3. Anti-NT3 injection to the limb selectively eliminates large diameter muscle sensory neurons. Different DRG populations were identified by retrograde labeling from muscle nerves (A,B) and cutaneous nerves (C,D). (A) Retrograde labeling of the femoral nerve in control embryos identifies many large diameter muscle sensory neurons (arrows), which are often clustered along the ventral and lateral aspects of the DRG. Small diameter neurons (arrowheads) that project to muscle are also labeled, as is the ventral root (v). (B) Anti-NT3 injection to the limb drastically reduces the number of large diameter muscle afferents (arrows, compare with A) that are labeled from the femoral nerve. This treatment does not appear to affect the number of small diameter muscle afferents (arrowheads). (C) Retrograde labeling of the lateral femoral cutaneous nerve in control embryos labels both large (arrows) and small (arrowheads) diameter cutaneous sensory neurons but does not label the ventral root (v) or motoneurons (not shown). (D) Anti-NT3 injection to the limb does not obviously affect either large (arrows) or small (arrowheads) diameter cutaneous neurons labeled from the lateral femoral cutaneous nerve. Dorsal is left and lateral is up in each panel. Scale bar = 50 μm.

neous neurons, mainly in LS 1 and 2 (Honig, 1982). The selectivity of cutaneous labeling was verified by the absence of dye in motoneurons. One embryo was rejected on the basis of non-selective cutaneous nerve labeling.

Limb injection of anti-NT3 resulted in a selective reduction in the survival of large muscle sensory neurons. In all anti-NT3 embryos examined (n=5), there was an obvious loss of large diameter muscle afferents ipsilateral to the antiserum injection. The anti-NT3 treatment appeared to eliminate selectively the largest muscle afferents, which occupy the ventral and lateral margins of the DRG with no noticable effect on small diameter muscle sensory neurons (compare Fig. 3A and B). However, the anti-NT3 treatment had no obvious effect on the overall size of the ganglion. The treatment also had no obvious effect on the survival of either large or small diameter cutaneous sensory neurons (compare Fig. 3C and D).

Cell counts of retrogradely labeled sensory neurons confirmed that peripheral NT3 deprivation selectively eliminated large diameter muscle afferents (Fig. 4A; n=5). Only large diameter neurons (≥10 μm) were counted because these are more reliably labeled with retrograde tracers (Oakley, unpublished observations) and because only large diameter sensory neurons express receptors for NT3 (Mu et al., 1993; Oakley et al., 1993). For quantitative comparisons, the number of labeled muscle afferents in LS2 and LS3 were summed and compared to those labeled in control embryos. The effects of limb injection of anti-NT3 were especially pronounced ipsilateral to the antiserum injection, where fewer than 50% of the large muscle afferents survived NT3 deprivation as compared to controls (P<0.001). A significant but less dramatic effect was detected contralateral to the antiserum injection with about 70% of the large muscle afferents surviving the treatment (P<0.05). These results suggest that the observed loss of Ia collaterals ipsilateral to the anti-NT3 injection was due to the more severe loss of muscle sensory neurons on the ipsilateral side.

In contrast, cell counts of large diameter cutaneous neurons showed that limb injection of anti-NT3 had no detectable effect on the survival of these neurons (Fig. 4B; n=5). For quantita-

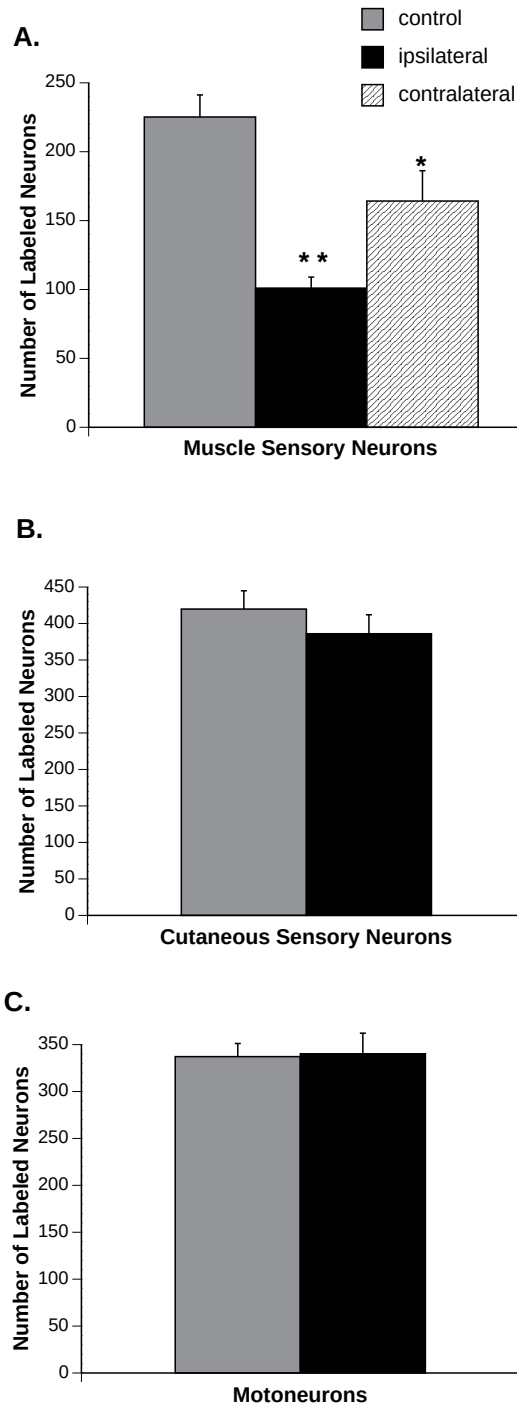

Fig. 4. Peripheral NT3 deprivation selectively enhances the death of large muscle sensory neurons. Quantitative analysis of the survival of identified muscle sensory neurons (A), cutaneous sensory neurons (B) and motoneurons (C). (A) Limb injection of anti-NT3 reduced the survival of large diameter muscle sensory neurons ipsilateral to the serum injection (**, $P<0.001$). A significant (*, $P<0.05$) but less dramatic reduction was also detected in the contralateral side. In contrast, limb injection of anti-NT3 did not significantly alter the survival of either large diameter cutaneous sensory neurons (B) or motoneuron (C) populations. Muscle sensory neurons and motoneurons were counted in segments LS2 and LS3 of the same embryos. Cutaneous sensory neurons were counted in segments LS1 and LS2. Statistical comparisons were made using the one-tailed Student's *t*-test. Error bars show s. e. m.

tive comparisons, the number of labeled cutaneous afferents in LS1 and LS2 were summed and compared to those labeled in control embryos. We found no significant difference between control and anti-NT3-treated embryos. These results indicate that, unlike large muscle afferents, large cutaneous afferents are not vulnerable to peripheral NT3 deprivation during the period of normal cell death in the DRG.

We also examined the femorotibialis motor pool to determine if motoneurons are vulnerable to peripheral NT3 blockade during this period. There were no overt differences in the overall appearance or distribution of motoneurons in anti-NT3-treated embryos as compared with control embryos (compare Fig. 5A and B). Moreover, counts of labeled motoneurons in LS2 and 3 showed that anti-NT3 treatment had no significant effect on motoneuron survival (Fig. 4C). These results further demonstrate the highly specific effects of peripheral NT3 deprivation; limb injections of anti-NT3 enhanced the death of the large diameter muscle sensory neurons that supply the femorotibialis muscle without altering the survival of the motoneurons that supply this same muscle.

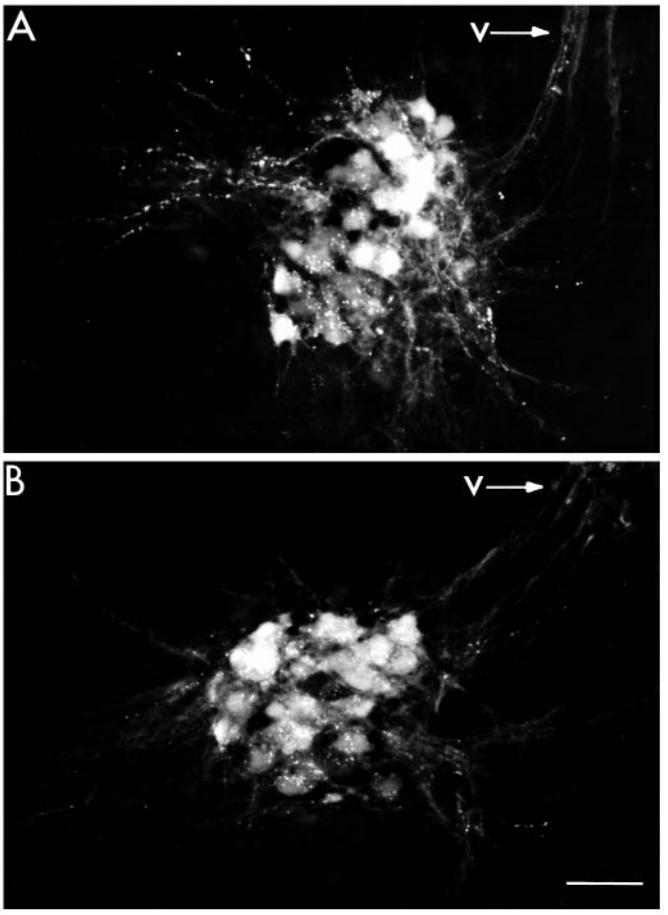

Fig. 5. Peripheral NT3 deprivation does not alter motoneuron survival. The ipsilateral femorotibialis motor pool is shown in transverse sections of spinal cord in limb injected control (A) and anti-NT3 (B) treated embryos by retrograde labeling from the femoral nerve. The femorotibialis pools in anti-NT3 treated embryos were not obviously different from controls in either size or distribution. Dorsal is left and lateral is up. v, ventral root axons. Scale bar = 50 μm.

Differential expression of trkC in muscle sensory neurons

To determine if the selective dependence of muscle afferents on NT3 is correlated with selective expression of NT3 receptors by these neurons, we examined the expression of *trk*C transcripts in identified sensory neurons. For these experiments, sensory neurons were retrogradely labeled from muscle or cutaneous nerves in normal E10 embryos near the end of the cell death period. Following hybridization with a probe specific for *trk*C transcripts, we found that the majority of large diameter muscle afferents express NT3 receptors. In contrast, cutaneous neurons that expressed *trk*C were rare (compare Fig. 6A and B). Counts of identified afferents confirmed that at least 60% of the large muscle afferents (≥10 μm in diameter) expressed *trk*C whereas only about 2% of the large cutaneous afferents were positive for *trk*C transcripts (Table 2).

DISCUSSION

We have used a highly specific antiserum to block NT3 signaling in peripheral tissues during the period of naturally occurring cell death in the DRG. Our results demonstrate that, following limb injection of a function-blocking antiserum to NT3, large diameter muscle sensory neurons, including muscle spindle afferents, selectively died. In contrast, the survival of large cutaneous neurons and motoneurons was not affected by peripheral NT3 deprivation. Moreover, we show that most large muscle sensory neurons express *trk*C during the period of DRG cell death, whereas few cutaneous neurons express this receptor for NT3. These results indicate that many large muscle sensory neurons require peripherally derived NT3 to survive the period of neuronal death in vivo and that the effects of NT3 are likely to be mediated by activation of trkC.

The distribution of the injected anti-NT3 strongly suggests that NT3 signaling was blocked only in peripheral tissues. Previous studies have shown that NT3 is expressed in several tissues of early embryos including skin, muscle, DRG and spinal cord, where it is localized to motoneurons (Schecterson and Bothwell, 1992; Henderson et al., 1993; Elkabes et al., 1994). Because the injected anti-NT3 was detected in the skin and muscle but not in the spinal cord or DRG, we can attribute the effect of the antiserum to neutralization of NT3 derived from peripheral limb tissues. Moreover, since direct injection of the spinal cord with anti-NT3 had no effect on Ia afferent projections, our results cannot be due to the diffusion of antiserum from peripheral to central sites. The restricted distribution of the antiserum following limb injections is likely the result of early development of neural barriers to the exchange of circulating proteins (Delorme et al., 1970; Risau et al., 1986). While these results strongly suggest that muscle-derived NT3 is required for the survival of large muscle sensory neurons, we cannot rule out the possibility that NT3 from motoneuron axons in the limb also supports these neurons. Our results further suggest that NT3 derived locally from DRG cells or from the spinal cord is insufficient to support the survival of a full complement of muscle sensory neurons during this period of development.

Peripheral NT3 blockade selectively enhanced the death of large muscle sensory neurons. Anti-NT3 treatment led to the loss of more than 50% of large muscle afferents. Since the NT3 deprivation was limited to the period of cell death in the DRG (Carr and Simpson, 1978; Hamburger et al., 1981; Bhattacharyya et al., 1992), the selective loss of muscle afferents can be attributed to enhanced cell death rather than to effects on proliferation and differentiation of these neurons. The neurons vulnerable to peripheral NT3 deprivation include muscle spindle afferents as evidenced by the reduced projection to motoneurons in the spinal cords of anti-NT3-treated embryos. These neurons are a small subpopulation of the DRG and are,

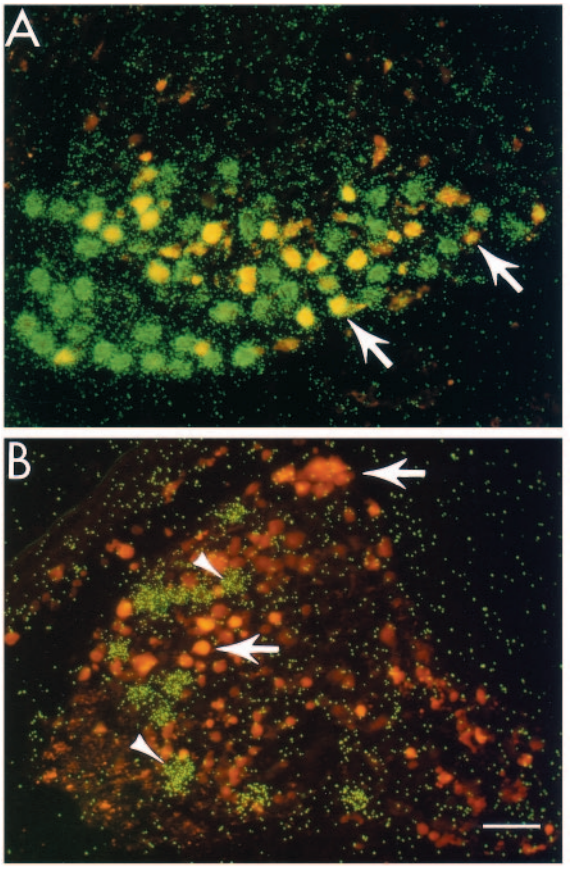

Fig. 6. Differential expression of *trk*C in muscle vs. cutaneous sensory neurons. Expression of *trk*C in retrogradely labeled muscle (A) and cutaneous (B) sensory neurons in E10 DRG. (A) Many muscle sensory neurons (red label) express *trk*C transcripts (green grains); double labeled neurons (arrows) appear yellow in this image. (B) In contrast, cutaneous sensory neurons (red label) that express *trk*C (green grains) were quite rare. The image in (B) is typical of most sections in that none of the identified cutaneous neurons (arrows) were also labeled with the *trk*C probe, despite the fact that other unidentified neurons in the section express *trk*C transcripts (arrowheads). Scale bar = 50 μm.

Table 2. Differential expression of *trk*C in large muscle vs. cutaneous sensory neurons

	Counts of retrogradely labeled cells		
Cell type	*trk*C +	Total	% *trk*C +
Muscle sensory neurons	723	1175	61.5
Cutaneous sensory neurons	31	1532	2.0

Counts are of large diameter (≥10 μm) DRG neurons from 4-5 embryos for each cell type. The retrograde label and *trk*C hybrids were imaged simultaneously, as in Fig. 6.

in fact, a subpopulation of the sensory neurons that project to muscle. Based on *trk*C expression (see below), we estimate that muscle spindle afferents constitute approximately 10% of the lumbar DRG in the chick at E10 (Oakley et al., 1993).

The effects of peripheral NT3 depletion were highly selective. NT3 deprivation did not significantly alter the survival of large cutaneous afferents. In addition, depletion of peripheral NT3 did not alter motoneuron survival, despite the fact that the antiserum was administered during the period of motoneuron death (Hamburger, 1975). This strongly suggests that limb motoneurons do not require NT3 from peripheral sources for survival. Similarly, normal numbers of facial and trigeminal motoneurons survive in mice lacking the NT3 gene (Ernfors et al., 1994; Farinas et al., 1994). These results indicate that NT3 selectively supports the survival of some muscle afferents in vivo, as suggested by previous in vitro studies (Hohn et al., 1990; Hory-Lee et al., 1993).

Previous gene deletion studies in mice have shown that NT3 is necessary for the development of muscle spindle afferents as well as other sensory neurons (Ernfors et al., 1994; Farinas et al., 1994). Because the gene coding for NT3 was inactivated in all tissues and throughout development, however, these studies have defined neither the time of action of NT3 nor its source. Our results extend these findings by demonstrating that muscle spindle afferents require NT3 from peripheral tissues to survive the period of cell death in the DRG. Moreover, we have shown that NT3 deprivation during this restricted period selectively decreases the survival of large muscle sensory neurons without affecting the survival of large cutaneous sensory neurons. In transgenic mice, the complete absence of NT3 at all developmental stages leads to a much more severe loss of up to 78% of all DRG neurons and 50% of sympathetic neurons (Ernfors et al., 1994; Farinas et al., 1994). Since cutaneous neurons make up the majority of neurons in the DRG, NT3 clearly influences the development of these neurons, probably at earlier stages of development. This conclusion is also supported by a previous study, which showed that early NT3 deprivation (E2-E5) reduces the number of neurons present in the quail DRG at the onset of cell death by more than 35% (Gaese et al., 1994). In contrast, we have shown that only a small subpopulation of muscle sensory neurons are vulnerable to NT-3 deprivation during the period of cell death, in that there was no obvious change in the number of small diameter neurons projecting to muscle.

Together, these findings suggest that NT3 influences the proliferation, differentiation or survival of a variety of peripheral neuron precursors, a conclusion supported by recent culture studies (Kalcheim et al., 1992; Buchman and Davies, 1993; DiCicco-Bloom et al., 1993; Wright et al., 1992). Our results indicate that, in addition to the early and rather widespread effects of NT3 on many types of peripheral neurons, NT3 acts quite specifically as a survival factor for large muscle sensory neurons during the interaction of these neurons with their targets. This later function of NT3 is therefore more analogous to the action of NGF on small diameter cutaneous neurons (Davies, 1987; Ruit et al., 1992).

The selective vulnerability of large muscle afferents to NT3 deprivation is correlated with the differential expression of *trk*C, the preferred receptor for NT3, in these neurons. Our results show that the majority of large muscle afferents, but few cutaneous afferents, express *trk*C during the period of normal cell death in the DRG. This is in substantial agreement with the findings of McMahon and colleagues, who have demonstrated a similar differential distribution of *trk*C transcripts in large muscle afferents in the adult rat (McMahon et al., 1994). However, they reported *trk*C transcripts in approximately 10% of cutaneous afferents, whereas we found only 2% of large cutaneous neurons expressing these transcripts. This discrepancy could result from species or developmental differences. Although NT3 deprivation produced by antiserum injection eliminated more than half of all large muscle afferents, many of these neurons survived the treatment. Incomplete blockade of NT3 signaling by the antiserum probably explains at least part of this incomplete effect. In addition, some muscle afferents might be less vulnerable to NT3 deprivation. For example, BDNF may be capable of supporting some muscle afferents, because at least some of these neurons probably coexpress both *trk*B and *trk*C (McMahon et al., 1994).

The expression of *trk*C by large muscle afferents during the period of normal cell death in the DRG suggests that this receptor mediates NT3 signaling during this period. Since both NT3 deprivation during DRG cell death (this study) and genetic deletion of the catalytic form of *trk*C (Klein et al., 1994) result in the loss of muscle spindle afferents, *trk*C probably mediates the survival-promoting effects of NT3 on this subpopulation of muscle sensory neurons. Furthermore, since *trk*C deletion results in the complete absence of muscle spindle afferent fibers in the spinal cord (Klein et al., 1994), all spindle afferents probably express *trk*C and require NT3 for survival. Mice with *trk*C deletions exhibit only a modest reduction in DRG neurons (~ 20%; Klein et al., 1994), however, as compared to the more drastic reductions reported for genetic deletion of NT3 (Ernfors et al., 1994; Farinas et al., 1994). These differences suggest that early effects of NT3 on DRG precursors may be mediated by a different receptor (Farinas et al., 1994).

In conclusion, our results demonstrate that muscle spindle afferents require NT3 from peripheral tissues in order to survive the period of normal cell death in the DRG. Since these neurons express *trk*C during this period and since trkC-mediated signaling is required for their development (Klein et al.,1994), we suggest that NT3 activates trkC and prevents the death of many of these neurons during normal development.

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