A Local Action of Neurotrophin-3 Prevents the Death of Proliferating Sensory Neuron Precursor Cells

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Summary

The role of neurotrophin-3 (NT-3) in early development of the dorsal root ganglion was investigated. Excessive cell death in the dorsal root ganglion of mice that carry a deleted NT-3 gene (NT-3$^{-/-}$ mice) preceded the period of programmed cell death, detected by the TUNEL method, and caused a reduction in the number of proliferating precursors without altering the proportion of proliferating cells to total number of neurons. Furthermore, the majority of proliferating cells detected by bromodeoxyuridine incorporation also stained with the TUNEL method. NT-3 mRNA was expressed locally in the embryonic, but not the postnatal dorsal root ganglion. Most cultured early embryonic NT-3$^{-/-}$ neurons died in the absence of exogenous NT-3 as did the wild-type neurons when cultured with NT-3 neutralizing antibodies, suggesting that NT-3 acts locally to prevent the death of proliferating sensory precursor cells during neurogenesis. Thus, NT-3 may inflict constraints on the number of proliferating precursor cells and thereby affect the number of neurons generated during development of the peripheral nervous system.

Introduction

Neurotrophic factors play a crucial role in the survival of sensory neurons in the developing vertebrate nervous system. Exogenous administration of the prototypic neurotrophic factor nerve growth factor (NGF) to the chick embryo promotes survival of the dorsal root ganglion sensory neurons that would otherwise have died during the period of programmed cell death (Hamburger et al., 1981), and neutralizing NGF in vivo with an anti-NGF antibody leads to excessive cell death of dorsal root ganglion neurons (Johnson et al., 1980). NGF is a member of a gene family of neurotrophic factors also including brain-derived neurotrophic factor (BDNF) (Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3 (NT-3) (Ernfors et al., 1990a; Hohn et al., 1990; Jones and Reichardt, 1990; Kaisho et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990), and neurotrophin-4/5 (NT-4) (Berkemeier et al., 1991; Hallböök et al., 1991; Ip et al., 1992). All neurotrophin members have more recently been shown to promote the survival of spinal and cranial sensory neurons to varying degrees in culture (Lindsay et al., 1985; Davies et al., 1986; Leibrock et al., 1989; Ernfors et al., 1990a; Hohn et al., 1990; Maisonpierre et al., 1990; Hallböök et al., 1991; Ibáñez et al., 1993), while BDNF and NT-3 also have been shown to be crucial in vivo for development of the chicken (Hofer and Barde, 1988; Gaese et al., 1994). Mice carrying a deletion in the genes encoding the neurotrophins have been generated. These mice develop with severe sensory deficits including a reduction of 70% of the lumbar dorsal root ganglion neurons in the NGF$^{-/-}$ mice (Crowley et al., 1994), 30% in the BDNF$^{-/-}$ mice (Ernfors et al., 1994b; Jones et al., 1994), and 60% in the NT-3$^{-/-}$ mice (Ernfors et al., 1994a; Fariñas et al., 1994).

The survival of sensory neurons during the period of programmed cell death may depend on neurotrophins produced by their peripheral target fields (see Barde, 1989). Distinct subpopulations of dorsal root ganglion neurons subserve different sensory modality responsiveness and contain different cytochemical properties and central terminations within the spinal cord (see Carr and Nagy, 1993), and the different classes of sensory neurons appear to some extent to depend on a particular neurotrophin. Whereas the survival and function of neurons mediating pain sensation depend on NGF (see Lewin and Mendell, 1993), NT-3 supports neurons mediating limb proprioception in culture (Hohn et al., 1990; Hory-Lee et al., 1993) and in vivo (Ernfors et al., 1994a; Fariñas et al., 1994; Oakley et al., 1995) as well as mechanoreceptive neurons supplying Merkel sensory organ innervation (Arvidsson et al., 1995, Soc. Neurosci., abstract).

Neurotrophins exert their physiological effects by binding to tyrosine kinase receptors. NGF binds and activates the trkA receptor (Hempstead et al., 1991; Klein et al., 1991), BDNF and NT-4 share the signal-transducing receptor trkB (Berkemeier et al., 1991; Glass et al., 1991; Klein et al., 1991; Soppet et al., 1991; Squinto et al., 1991; Ip et al., 1992), and NT-3 preferentially binds and activates trkC (Lamballe et al., 1991). Most, but not all, dorsal root ganglion neurons express mRNA for a trk receptor (McMahon et al., 1994). The mRNA expression of trkA and trkC receptors is mostly confined to different subpopulations of dorsal root ganglion neurons during late embryogenesis and in the adult (Mu et al., 1993; McMahon et al., 1994). Whereas close to 50% of the sciatic nerve afferents express trkA and belong to the small size cutaneous, muscle, and visceral afferents, trkC mRNA is expressed by 17% of the neurons that belong to the large size population which project to the muscle (McMahon et al., 1994). Importantly, during neurogenesis, trkC mRNA is expressed by many neurons in the dorsal root ganglion, but is rapidly downregulated below the detection limit in most neurons, and a few days later, only the subpopulation of large size neurons continue to express trkC mRNA (Ernfors et al., 1992).

Although most sensory neurons express trkA, trkB, and/or trkC at early embryonic stages (Ernfors et al., 1992; Klein et al., 1990; Martin-Zanca et al., 1990), these neurons survive independently of neurotrophins in culture (Vogel and Davies, 1991). In contrast with tissue culture studies, in vivo results have shown that NT-3 is required for survival in the dorsal root ganglion already

at the time of ganglion formation in the chick, since administration of anti-NT-3 antibodies at this stage leads to an elimination of neurons (Gaese et al., 1994). Although cultured early neurons survive independently of NT-3, the morphological maturation of isolated chick dorsal root ganglion neurons is accelerated by the addition of NT-3 to the medium (Wright et al., 1992), and the proliferation of chick neural crest cells and early rat dorsal root ganglion neurons can be stimulated by NT-3 in culture (Kalcheim et al., 1992; Memberg and Hall, 1995), suggesting that NT-3 may also play a role in the differentiation of peripheral precursor cells and as a mitogen in the early sensory ganglion.

The expression of NT-3 mRNA in the embryonic dorsal root ganglion (Ernfors et al., 1992) opens up the possibility that these neurons survive in culture because NT-3 is provided locally, as has been shown for BDNF in cultured adult dorsal root ganglion neurons (Acheson et al., 1995). To examine the role of NT-3 in early development, we have scored the survival of cultured sensory neurons in the presence of anti-NT-3 antibodies and of neurons lacking the NT-3 gene. Furthermore, we have monitored the neuronal numbers, the number of proliferating cells, and the number of apoptotic cells in vivo in mice lacking one, or both, copies of the NT-3 gene at several embryonic stages. Our results show that NT-3 may act in a local fashion to support the survival of proliferating sensory precursor cells.

Results

Early Embryonic Loss of Sensory Neurons in NT-3$^{-/-}$ Mice

NT-3$^{-/-}$ mice display an absence of 60% of the lumbar dorsal root ganglion neurons after birth (Ernfors et al., 1994a; Fariñas et al., 1994). To determine the developmental period of loss of these neurons, we prepared sections of the L5 dorsal root ganglion from embryonic day 11 (E11), E12, E14, and E17 NT-3$^{-/-}$ and age-matched control mice and counted the number of neurons. Already at E11, the dorsal root ganglion of NT-3$^{-/-}$ mice appeared slightly smaller (Figure 2B) compared with control mice (Figure 2A). The size difference was more pronounced at E14 and E17. Quantitation revealed that, at E11, 15% of the dorsal root ganglion neurons were absent, and 1 day later, the deficit was 49%. At E14, a reduction of 58% of the neurons was detected, after which no further significant changes were observed (Figure 1A; Tables 1 and 2). These results show that almost 80% of the excessive loss occurs before E12. In addition to neuronal counts, 50–100 neurons were sampled to measure the neuronal size at each developmental stage and genotype. No significant size difference was detected between control and NT-3$^{-/-}$ mice of the same embryonic stage. However, differences in neuronal size were seen between embryos of different stages. The actual number of neurons can therefore only be compared between control and NT-3$^{-/-}$ mice of the same embryonic age. Despite these limitations, the results suggest that NT-3 is important for development of mouse dorsal root ganglion neurons at early embryonic stages.

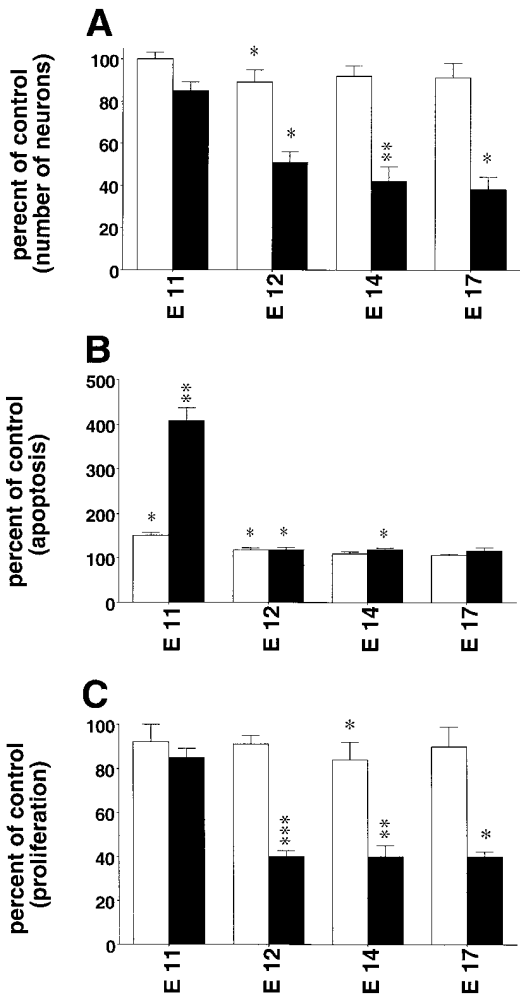

Figure 1. Neuronal Numbers, Proliferation, and Cell Death in the L5 Dorsal Root Ganglion

(A) Number of neurons in the dorsal root ganglion of NT-3$^{+/-}$ and NT-3$^{-/-}$ mice as percent of age-matched control mice. Note the pronounced reduction in neuronal numbers between E11 and E12 of the NT-3$^{-/-}$ mice (closed bars). A slight reduction in neuronal numbers was detected in NT-3$^{+/-}$ mice (open bars).

(B) Cell death in the NT-3$^{+/-}$ and NT-3$^{-/-}$ mice as percent of age-matched control mice detected with the TUNEL method at indicated embryonic stages. Note the pronounced excessive cell death at E11 and almost normal levels at E17.

(C) Number of proliferating cells in the NT-3$^{+/-}$ and NT-3$^{-/-}$ mice as percent of age-matched control mice detected with BrdU immunohistochemistry at indicated embryonic stages.

Student's t tests: single asterisk, $p < 0.05$; double asterisk, $p < 0.01$; triple asterisk, $p < 0.001$.

The Excessive Cell Death Leads to a Decline in the Number of Proliferating Sensory Precursor Cells of the NT-3$^{-/-}$ Mice

Because the neuronal loss in the NT-3$^{-/-}$ mice occurred during dorsal root ganglion formation, we quantified the number of proliferating and degenerating cells at several developmental stages of mouse development. Degenerating dorsal root ganglion cells were detected with the terminal deoxynucleotidyl transferase-mediated dUTP

Table 1. Cell Death in the L5 Dorsal Root Ganglion

	Control		NT-3$^{+/-}$		NT-3$^{-/-}$	
Age	Total Number of Neurons (± SEM)	Number of Apoptotic Cells (± SEM)	Total Number of Neurons (± SEM)	Number of Apoptotic Cells (± SEM)	Total Number of Neurons (± SEM)	Number of Apoptotic Cells (± SEM)
E11	2375 ± 130	90 ± 12	2367 ± 171	136 ± 7	2026 ± 111	367 ± 20
E12	2490 ± 26	356 ± 4	2213 ± 129	422 ± 16	1272 ± 20	422 ± 6
E14	2730 ± 14	130 ± 6	2503 ± 45	142 ± 6	1144 ± 44	154 ± 6
E17	3048 ± 160	32 ± 4	2784 ± 54	34 ± 1	1164 ± 57	37 ± 3

The number of positive cells in the L5 dorsal root ganglion was counted in every fourth section. Differences in neuronal size were seen between embryos of different stages. The actual number of neurons can therefore only be compared between control, NT-3$^{+/-}$, and NT-3$^{-/-}$ mice of the same embryonic stage.

nick end-labeling (TUNEL) method. This staining method visualizes cells containing fragmented DNA, a hallmark of apoptosis. In control mice, an increased number of TUNEL$^+$ cells was detected between E12 and E14, presumably corresponding to the period of programmed cell death in the control dorsal root ganglion (Table 1). The programmed cell death appeared to be restricted to a short developmental period, because only relatively few degenerating cells were detected at E11 and E17 (Table 1). In contrast with control mice, abundant cell death was detected already at E11 in the NT-3$^{-/-}$ mice (Figures 2C and 2D, respectively), and quantitation revealed an increase to 408% of TUNEL$^+$ cells compared with control (Figure 1B; Table 1). The levels remained elevated compared with controls at E12 and E14 (119% and 118% of control; Figure 1B; Table 1). Only rarely were degenerating cells detected at E17 (Figures 2G and 2H). Thus, the onset of excessive cell death in the NT-3$^{-/-}$ mice occurred prior to the developmental period of programmed cell death.

To detect proliferating cells in the dorsal root ganglion by immunohistochemistry, pregnant females were injected with bromodeoxyuridine (BrdU). In control mice, staining for BrdU should detect the period of proliferative generation of dorsal root ganglion cells. Abundant proliferation in the dorsal root ganglion of control mice was detected at E11 and E12 (Table 2). Upon examination of the NT-3$^{-/-}$ mice, a significant deficit in the number of proliferating cells was detected compared with control mice (Figures 2F and 2E, respectively). While proliferation in the control mice reached peak numbers at E12, there was instead a decrease between E11 and E12 in the NT-3$^{-/-}$ mice (Table 2). NT-3$^{-/-}$ mice displayed a decrease to 85% of control already at E11 and 40% of control at E12 (Figure 1C). The reduction in proliferating cells of NT-3$^{-/-}$ mice coincided with the period of excessive cell death as detected by the TUNEL method, indicating that proliferating precursor cells require NT-3 for their survival. Furthermore, these deficits did not lead to a change in the proportion of proliferating cells to total number of cells, compared with age-matched controls (Table 3), showing that the deficit in the NT-3$^{-/-}$ mice is not caused by the lack of a mitogen or differentiation factor.

To determine whether NT-3 is present at limiting concentrations during sensory neuron development, mice that carry a single functional NT-3 gene (NT-3$^{+/-}$ mice) were analyzed. Histological examination of the dorsal root ganglion of NT-3$^{+/-}$ mice did not reveal any reduction in size compared with control mice. However, measurements of neuronal numbers revealed a small decrease in the NT-3$^{+/-}$ mice at E12 (11%), compared with controls (Figure 1A; Tables 1 and 2). This loss coincided with a small, but significant, elevation of cell death at E11 and E12, and quantitative analysis revealed an increase to 151% and 119% of TUNEL$^+$ cells, respectively, compared with control mice (Table 1). The cell loss caused a slight decrease in the number of proliferating cells (Figure 1C; Table 2).

NT-3, a Survival Factor for Proliferating Sensory Precursor Cells

To address directly whether the degenerating cells in the NT-3$^{-/-}$ mice were proliferating precursor cells or

Table 2. Proliferation in the L5 Dorsal Root Ganglion

	Control		NT-3$^{+/-}$		NT-3$^{-/-}$	
Age	Total Number of Neurons (± SEM)	Number of BrdU$^+$ Cells (± SEM)	Total Number of Neurons (± SEM)	Number of BrdU$^+$ Cells (± SEM)	Total Number of Neurons (± SEM)	Number of BrdU$^+$ Cells (± SEM)
E11	2375 ± 130	1120 ± 127	2367 ± 171	1030 ± 89	2026 ± 111	952 ± 56
E12	2490 ± 26	1682 ± 86	2213 ± 129	1531 ± 66	1272 ± 20	672 ± 84
E14	2730 ± 14	100 ± 12	2503 ± 45	84 ± 4	1144 ± 44	40 ± 8
E17	3048 ± 160	82 ± 18	2784 ± 54	74 ± 2	1164 ± 57	33 ± 4

The number of positive cells in the L5 dorsal root ganglion was counted in every fourth section. Differences in neuronal size were seen between embryos of different stages. The actual number of neurons can therefore only be compared between control, NT-3$^{+/-}$, and NT-3$^{-/-}$ mice of the same embryonic stage.

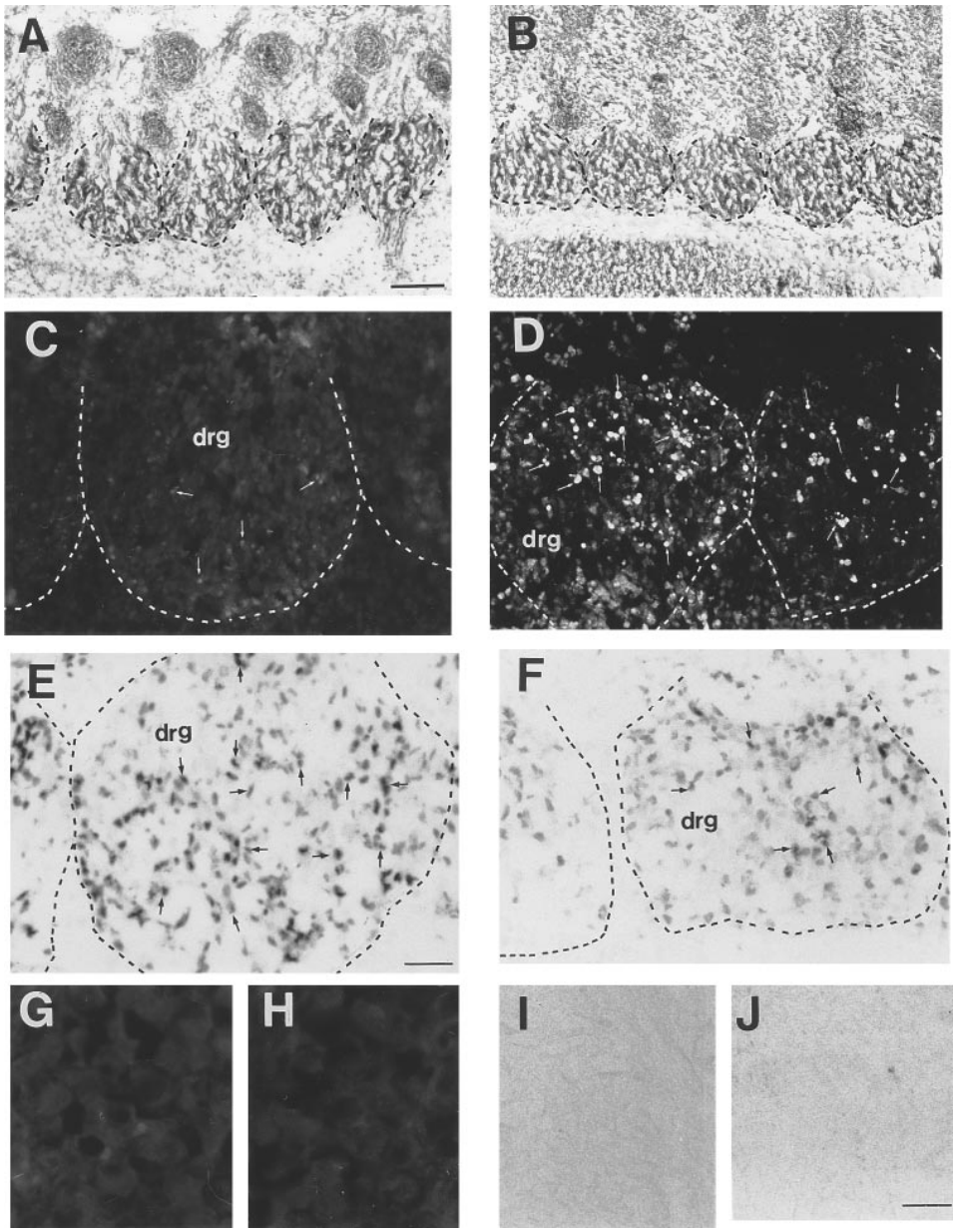

Figure 2. Cell Death in the Dorsal Root Ganglion

(A and B) Histology of the dorsal root ganglion. Photomicrographs of E11 control (A) and NT-3$^{-/-}$ (B) lumbar dorsal root ganglia stained with cresyl violet. Note the reduction in ganglion size already at E11 of NT-3$^{-/-}$ mice.

(C, D, G, and H) Cell death in the lumbar dorsal root ganglia. Photomicrographs of E11 (C) and E17 (G) control and E11 (D) and E17 (H) NT-3$^{-/-}$ lumbar dorsal root ganglia stained with the TUNEL method. Note numerous apoptotic cells at E11 in the NT-3$^{-/-}$ ganglia (D; arrows).

(E, F, I, and J) Proliferation in the lumbar dorsal root ganglion. BrdU immunohistochemistry of E11 (E) and E17 (I) control, and E11 (F) and E17 (J) NT-3$^{-/-}$ lumbar dorsal root ganglia. Arrows indicate some BrdU^{+} cells. Note the reduction in the number of stained cells in the E11 NT-3$^{-/-}$ lumbar dorsal root ganglion (F).

Scale bar in (A) and (B) is 80 μm and in (C)–(J) is 40 μm.

postmitotic neurons, we processed sections from the E11 dorsal root ganglion with the TUNEL method combined with immunohistochemistry for the detection of BrdU incorporation, or immunohistochemistry for the detection of postmitotic neuronal cells. Combining the TUNEL method and anti-BrdU immunohistochemistry revealed that the majority of BrdU^{+} cells also were TUNEL^{+} (Figure 3A), directly showing that a population of proliferating cells require NT-3 to prevent cell death. However, many TUNEL^{+} cells were BrdU^{-}, which at least partly could be caused by the short pulse of BrdU administered. Sections were also double stained with the TUNEL method and for peripherin, a marker of differentiated postmitotic neurons. Although several large peripherin^{+} neurons were identified in every section, only in a few instances were peripherin^{+} neurons also TUNEL^{+} (Figure 3B). These data are consistent with a requirement of NT-3 for the survival of proliferating dorsal root ganglion precursor cells during early embryonic stages.

Table 3. The Proportion of Dying and Proliferating Cells in the L5 Dorsal Root Ganglion Compared with the Total Neuronal Numbers

	Cell Death			Proflieration		
Age	Control	NT-3$^{+/-}$	NT-3$^{-/-}$	Control	NT-3$^{+/-}$	NT-3$^{-/-}$
E11	0.04	0.06	0.18[a]	0.47	0.44	0.47
E12	0.14	0.19[a]	0.33[b]	0.68	0.69	0.53
E14	0.05	0.06	0.13[a]	0.04	0.03	0.03
E17	0.01	0.01	0.03	0.03	0.03	0.03

Note a significant difference in the proportion of TUNEL$^+$ cells of NT-3$^{-/-}$ mice, and to a lesser extent, of NT-3$^{+/-}$ mice. There was no significant difference in the proportion of proliferating cells compared with total neuronal numbers in NT-3$^{-/-}$ or NT-3$^{+/-}$ mice. Student's t tests: [a] $p < 0.05$; [b] $p < 0.01$.

Expression of NT-3 mRNA in the Developing Dorsal Root Ganglion

NT-3 has been shown to be expressed in the target fields of dorsal root ganglion neurons (the spinal cord and the muscle) during embryogenesis (Ernfors and Persson, 1991; Schecterson and Bothwell, 1992; Copray and Brouwer, 1994). However, precursor cells of the dorsal root ganglion have no contacts with the targets of innervation. It therefore appeared likely that NT-3 is produced locally within the ganglion. To examine this possibility, dorsal root ganglia of E11, E17, postnatal day 15 (P15), and adult mice were dissected and RNA was purified for RNase protection assay. Low, but clearly detectable signals for NT-3 mRNA were seen at E11 and E17 (Figure 4). NT-3 mRNA expression was below the detection limit at P15 and in the adult, suggesting a down-regulation of NT-3 mRNA expression postnatally.

A Local Action of NT-3 Prevents the Death of Early Embryonic Dorsal Root Ganglion Neurons

The previous results suggested that early dorsal root ganglion neurons when cultured survive in the absence of exogenously added NT-3 because NT-3 is provided locally. To test this hypothesis, we cultured E11 dorsal root ganglion neurons from wild-type mice and NT-3$^{-/-}$ mice with or without NT-3 supplemented to the medium.

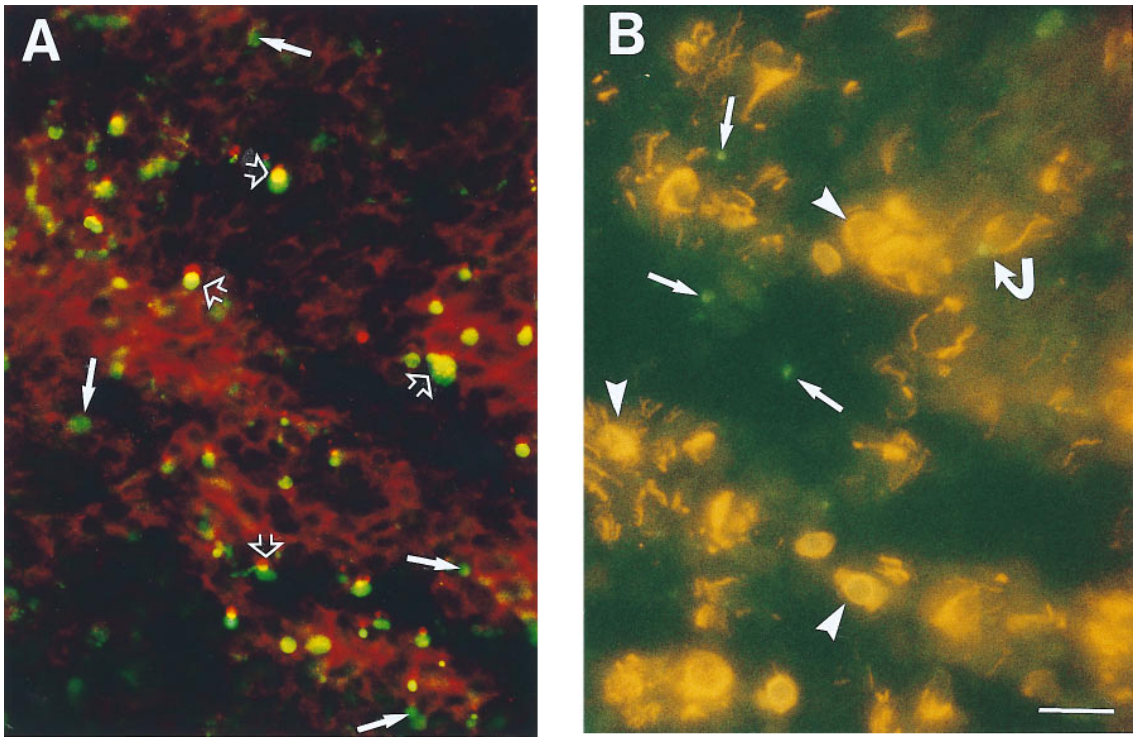

Figure 3. Dying Dorsal Root Ganglion Cells of the NT-3$^{-/-}$ Mice Incorporate BrdU, but Do Not Express Peripherin

Photomicrographs are from E11 NT-3$^{-/-}$ mice.

(A) Staining with the TUNEL method and for incorporation of BrdU, marking proliferating cells. TUNEL staining is green and BrdU is red and both stain the nucleus. Note the presence of numerous double stained cells (light yellow) indicated by open arrows and the staining of some cells with only the TUNEL method (arrows).

(B) Staining with the TUNEL method and for expression of peripherin, a marker for postmitotic neurons. TUNEL staining is green and localized to the nucleus (arrows), and peripherin is red and localized to the cytoplasm (arrowheads). Only rarely was peripherin present in dying cells. One such cell is indicated by a curved arrow.

Scale bar in (A) and (B) are 20 μm.

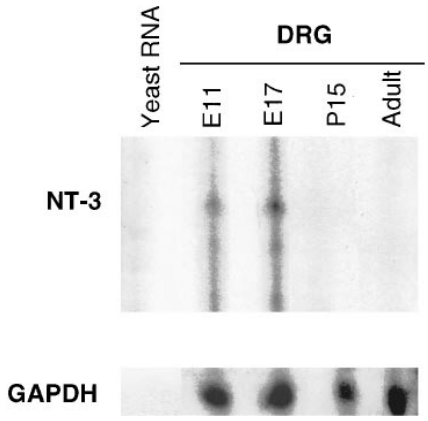

Figure 4. RNase Protection Assay for the Detection of NT-3 mRNA Expression in Freshly Dissected Dorsal Root Ganglia at Embryonic and Postnatal Stages

Yeast RNA was used as a negative control for the protection. Note NT-3 mRNA expression in the dorsal root ganglion at E11 and E17, but not postnatally. A GAPDH probe was used to assess mRNA integrity and to compare loading among the lanes.

The number of surviving neurons was determined 24 hr later. Because many dorsal root ganglion neurons of the E11 NT-3$^{-/-}$ mice were committed to apoptosis already at the time of dissociation of the ganglia for the cultures, as detected with the TUNEL method, survival was compared between cultures maintained in the presence or absence of supplemented NT-3; 46% of the cultured wild-type dorsal root ganglion neurons survived independently of NT-3 protein in the medium, compared to cultures where NT-3 was added (10 ng/ml). Neurons from NT-3$^{-/-}$ mice displayed marked reduced survival in cultures without NT-3 supplemented to the medium, and only 16% survived (Figure 5A). Thus, 66% of the neurons died that normally would have survived for 24 hr independently of exogenously added NT-3.

Because the above result indicated that NT-3 produced within the dorsal root ganglion sustain the survival of early sensory neurons, we next examined the consequence of blocking NT-3 function with NT-3 neutralizing antibodies in cultures of E11 wild-type dorsal root ganglion neurons. The antibodies used were a turkey anti-NT-3 antibody (Ghosh and Greenberg, 1995) and a rabbit anti-NT-3 antibody (Zhou and Rush, 1994). The specificity of these antibodies to NT-3 have been described previously (Ghosh and Greenberg, 1995; Zhou and Rush, 1994), and the potency of the turkey anti-NT-3 in blocking NT-3 function in culture has been examined (Ghosh and Greenberg, 1995). Furthermore, in our experiment, the turkey anti-NT-3 efficiently blocked NT-3 elicited survival, but had no effects on the survival of neurons cultured with BDNF (Figure 5B); 38% of the plated wild-type neurons survived in the absence of NT-3 supplemented to the medium. Blocking endogenous NT-3 with the turkey or rabbit anti-NT-3 antibodies led to a reduction in the number of surviving neurons to 11% $\pm$ 1.3% and 14% $\pm$ 3.7%, respectively. Whereas the turkey anti-NT-3 completely blocked exogenously added NT-3 (8% $\pm$ 1.6% survival), the rabbit anti-NT-3 insufficiently blocked NT-3 added to the medium (35% $\pm$ 3.1% survival; Figure 5B). Thus, together these findings suggest a requirement for locally produced NT-3 to prevent cell death in the early sensory ganglion.

Discussion

We have investigated the role of NT-3 in early development of the dorsal root ganglion and have found that it is crucial during the period of sensory ganglion formation in the mouse. An early role of NT-3 has previously been shown in the chick, where the administration of neutralizing NT-3 antibodies during gangliogenesis leads to neuronal loss (Gaese et al., 1994). Furthermore, mitosis of cultured chick neural crest cells and early rat dorsal root ganglion neurons can be stimulated by NT-3 (Kalcheim et al., 1992; Memberg and Hall, 1995), and the morphological maturation of isolated chick dorsal root ganglion neurons in culture can be accelerated by the addition of NT-3 to the medium (Wright et al., 1992). Thus, the neuronal deficit in the NT-3$^{-/-}$ mice could be caused by a deficit in the differentiation of precursor cells, the proliferation of precursor cells, and/or the survival of the proliferating precursor cells. We found excessive cell death in the dorsal root ganglion leading to

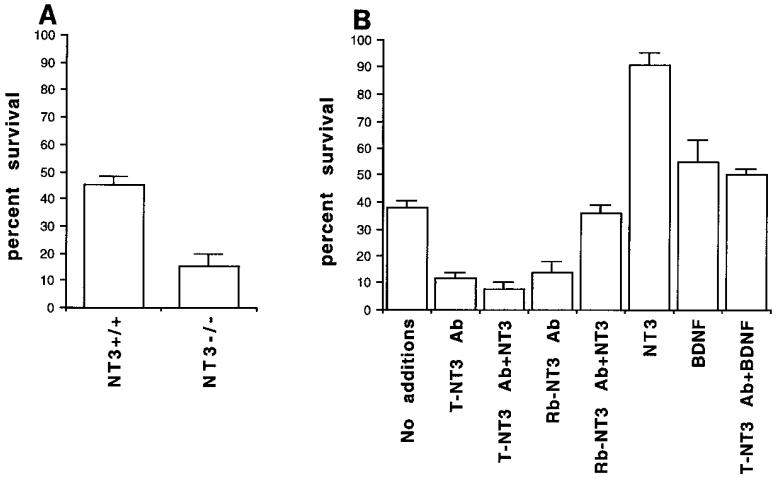

Figure 5. A Local Source of NT-3 Prevents the Cell Death of Cultured Early Sensory Neurons

(A) The survival of E11 dorsal root ganglion neurons from control and NT-3$^{-/-}$ mice cultured in the presence or absence of recombinant NT-3 (10 ng/ml). The percent survival of neurons in the absence of NT-3 compared with in the presence of NT-3 is shown. Note the pronounced loss of NT-3$^{-/-}$ neurons compared with control neurons.

(B) Percent survival of wild-type neurons in the presence or absence of BDNF, NT-3, anti-NT-3 antibodies, or a combination of antibody with BDNF or NT-3. Note the pronounced loss of neurons cultured with anti-NT-3 antibodies compared with cultures maintained in the absence of antibodies (no additions). Abbreviations: Rb-NT-3 Ab, rabbit anti-NT-3 antibody; T-NT-3 Ab, turkey anti-NT-3 antibody.

a decreased number of proliferating precursor cells and a reduced complement of neurons, without an alteration in the proportion of proliferating cells to total number of neurons in the NT-3$^{-/-}$ mice, as compared with control mice. An exclusive role of NT-3 during early stages affecting differentiation or proliferation would not be expected to cause excessive cell death and should lead to a change in the proportion of proliferating cells to total number of neurons. Instead, our data are consistent with excessive cell death of proliferating dorsal root ganglion cells in the NT-3$^{-/-}$ mice. Furthermore, this conclusion agrees with the finding that most BrdU^{+} cells were TUNEL^{+} in the NT-3$^{-/-}$ mice, which directly addresses whether endogenous NT-3 prevents the death of proliferating neuronal precursor cells. However, our results do not exclude the possibility that NT-3 also affects differentiation of the NT-3-dependent precursor cells that may be eliminated prior or during differentiating into neurons in the NT-3$^{-/-}$ mice.

The restricted expression of NT-3 in many target tissues of innervation (i.e., spinal cord motor neurons, muscle spindles, sensory epithelia of the inner ear, and the whisker pad) (Ernfors et al., 1990b, 1992; Phillips et al., 1990; Pirvola et al., 1992; Ibáñez et al., 1993; Copray and Brouwer, 1994) and the loss of particular functional subsets of sensory neurons in mice lacking NT-3 (Ernfors et al., 1994a; Fariñas et al., 1994; Tessarollo et al., 1994) is consistent with a target-derived role of NT-3 for postmitotic neurons. It has been unclear why postnatal NT-3$^{-/-}$ mice display a loss of 60% of the lumbar dorsal root ganglion neurons but show a deficit of only limb proprioceptive and Merkel sensory neurons (Arvidsson et al., 1995, Soc. Neurosci., abstract; Ernfors et al., 1994a; Fariñas et al., 1994; Tessarollo et al., 1994). Peripheral sensory neurons isolated at stages prior to target innervation survive independently of neurotrophic factors in culture (Vogel and Davies, 1991). However, these cells have been shown to express trkB (Klein et al., 1990; Ernfors et al., 1992) and trkC mRNAs (Ernfors et al., 1992). In fact, many more dorsal root ganglion cells express trkC during ganglion formation than during target innervation, suggesting that more cells depend on NT-3 at early embryonic stages than at later stages (Ernfors et al., 1992). The inability of cultured early NT-3$^{-/-}$ neurons as well as wild-type neurons when cultured with neutralizing NT-3 antibodies to survive in the absence of NT-3, and the finding that NT-3 mRNA is expressed in the early dorsal root ganglion despite the lack of any afferent input to peripheral sensory neurons, suggest that this factor acts locally to prevent cell death during the period of ganglion formation. The loss of proliferating sensory precursor cells in the NT-3$^{-/-}$ mice would be expected to cause a reduction in postmitotic neurons of many functional classes, since these cells may differentiate only after terminal mitosis (Rohrer and Thoenen, 1987).

The locally derived NT-3 preventing the death of early dorsal root ganglion neurons may represent a more general phenomenon, occurring in many ganglia of the peripheral nervous system during embryogenesis and including several members of the neurotrophin family. For instance, BDNF and trkB mRNAs are expressed in the dorsal root ganglion, the nodose ganglion, and the trigeminal ganglion at the time these neurons respond to BDNF (Ernfors et al., 1992; Schecterson and Bothwell, 1992), and NT-3 and trkC mRNAs are expressed in the geniculate ganglion (Ernfors et al., 1992). A local source of neurotrophins preventing the death of proliferating peripheral precursor cells may also be present in the sympathetic nervous system, since proliferating sympathetic cells have been shown both in culture (Birren et al., 1993; Dechant et al., 1993; DiCicco-Bloom et al., 1993) and in vivo (ElShamy et al., 1996) to require NT-3 for their survival.

Neurotrophic factors have for a long time been considered to play a critical role in neuronal survival during the developmental period of programmed cell death in the peripheral nervous system. The basic concept of the neurotrophic factor hypothesis has been that limiting amounts of neurotrophic factors assures target innervation by the appropriate number of neurons, eliminating the excessive neurons by programmed cell death owing to the failure to attain sufficient amounts of survival factors produced in the targets of innervation (Thoenen and Barde, 1980; Levi-Montalcini, 1987; Barde, 1989). Whereas this hypothesis has more recently been strengthened by many studies on neurotrophins in the peripheral nervous system, these factors have been shown to act locally and have diverse functions in development of the central nervous system. Interestingly, one early activity of NT-3 in the central nervous system is its ability to promote the differentiation of cultured neuroectodermal cells into neurons (Ghosh and Greenberg, 1995; Vicario-Abejon et al., 1995). The observation of an early local role of NT-3 in preventing the death of peripheral precursor cells suggests a broad role of neurotrophins also in development of the peripheral nervous system, acting on proliferating precursor cells and postmitotic neurons in a local and target-derived fashion at different stages of embryogenesis.

The role of neurotrophins preventing the death of precursor cells in early development is unclear. However, neurotrophins may be present at limiting concentrations during this period, as have previously been described for NT-3 and BDNF at later developmental stages (Ernfors et al., 1994a, 1995). This raises the possibility that NT-3 could inflict constraints on the size of the pool of proliferating precursor cells. The difference in the size of sensory ganglia and the number of motor neurons at the rostrocaudal axis may be established prior to programmed cell death. Proliferation in the dorsal root ganglion and of motor neurons have been shown to vary at different rostrocaudal levels, and more neurons are generated in limb regions than in the trunk. This difference corresponds to the later difference in neuronal numbers (Hamburger and Levi-Montalcini, 1949; McMillan-Carr and Simpson, 1978; Oppenheim et al., 1989). Replacing the caudal half of the somite by grafts of rostral half-somites in the E4.5 chicken leads to an increase of thymidine-labeled sensory cells (Goldstein et al., 1990). Thus, proliferation of sensory precursor cells could be controlled by mitogenic activities from the mesodermally derived somites (Goldstein et al., 1990). Because locally expressed NT-3 may limit the number of proliferating precursor cells, this intrinsic constraint could act in concert with extrinsic mitogenic factors to control the final complement of neurons generated in

a ganglion. The molecular mechanisms controlling cell proliferation and apoptosis may coincide to some extent (Evan et al., 1995), and recent results have indicated that the death of cultured sympathetic cells and PC12 cells in the absence of trophic support is caused by an abortive attempt to proliferate (Farinelli and Greene, 1996). It is intriguing that the excessive cell death was relatively minor prior to E11 in the NT-$3^{-/-}$ mice, the time when many neurons are beginning to be born as detected by peripherin immunohistochemistry. This suggests that there could be a requirement of NT-3 to prevent cell death only during the last cell-division(s), in which case NT-3 also could be linked to withdrawal from the cell cycle.

Experimental Procedures

Animals

Embryos were obtained from overnight mating, and the day of the vaginal plug was considered as E0. Pregnant females were injected interaperitoneally with 0.6 ml of a 10 mg/ml aqueous solution of 5-bromodeoxyuridine (Sigma), and embryos (days 11, 12, 14, and 17 of gestation) were collected 5–6 hr later, immersion fixed in 4% paraformaldehyde overnight, sucrose embedded, frozen on dry ice, and sectioned at 10 μm on a cryostat. The mice were genotyped for the inactive allele of the NT-3 gene by the polymerase chain reaction (PCR), as previously described (Ernfors et al., 1995).

Neuronal Counts

Embryos were serial sectioned on a cryostat at 10 μm, and the sections were stained with cresyl violet. The number of neurons in the dorsal root ganglion (L5) with a clear nucleus and nucleoli was counted in every fourth section. The total number of cells was estimated by multiplying the counted number with a factor derived by comparing the thickness of the counted sections to the total size of the ganglion: at E11, NT-$3^{+/+}$ (n = 7), NT-$3^{+/-}$ (n = 4), NT-$3^{-/-}$ (n = 6); at E12, NT-$3^{+/+}$ (n = 5), NT-$3^{+/-}$ (n = 4), NT-$3^{-/-}$ (n = 5); at E14, NT-$3^{+/+}$ (n = 4), NT-$3^{+/-}$ (n = 4), NT-$3^{-/-}$ (n = 4); at E17, NT-$3^{+/+}$ (n = 4), NT-$3^{+/-}$ (n = 4), NT-$3^{-/-}$ (n = 4).

TUNEL Staining

To detect cell death in the dorsal root ganglion (L5), sections were stained with the TUNEL method using the ApopTag in situ apoptosis detection kit (Oncor) according to the instructions of the manufacturer. The number of apoptotic cells was counted in every fourth section for the entire ganglion: at E11, NT-$3^{+/+}$ (n = 4), NT-$3^{+/-}$ (n = 4), NT-$3^{-/-}$ (n = 4); at E12, NT-$3^{+/+}$ (n = 4), NT-$3^{+/-}$ (n = 4), NT-$3^{-/-}$ (n = 4); at E14, NT-$3^{+/+}$ (n = 4), NT-$3^{+/-}$ (n = 4), NT-$3^{-/-}$ (n = 4); at E17, NT-$3^{+/+}$ (n = 2), NT-$3^{+/-}$ (n = 2), NT-$3^{-/-}$ (n = 2).

BrdU Staining

For analysis of proliferation, the cells that had incorporated BrdU were stained by immunohistochemistry. The sections were postfixed for 15 min in 4% paraformaldehyde, washed three times for 5 min in phosphate-buffered saline (PBS), incubated in 2 M HCl in 70% ethanol at −20°C for 10 min, and drained, and endogenous peroxidase activity was blocked in 2% hydrogen peroxide in PBS for 5 min at room temperature and rinsed in PBS (three times for 5 min). The sections were then deproteinized in ice-cold 0.1 M HCl for 20 min, denatured with 2 M HCl in PBS for 30 min, then neutralized in 0.1 M borate buffer (pH 8.5) for 10 min, rinsed twice in PBS for 10 min, and blocked for 1 hr in blocking solution (10% goat serum, 0.1% Tween in PBS). The sections were then incubated overnight with a mouse anti-BrdU antibody (Sigma) diluted 1:500 in blocking solution. The sections were washed four times for 15 min in PBS containing 0.1% Tween and incubated for 4 hr with a peroxidase-conjugated goat anti-mouse secondary antibody (1:200; DAKO). Followed by washes (four times for 15 min) in PBS, the sections were developed with 3-3′-diaminobenzidine (DAB) (Sigma). The DAB (10 mg) was dissolved in 50 ml of 100 mM Tris (pH 7.5) containing 0.05% nickel chloride. The number of proliferating cells was counted in every fourth section for the entire ganglion: at E11, NT-$3^{+/+}$ (n = 4), NT-$3^{+/-}$ (n = 4), NT-$3^{-/-}$ (n = 4); at E12, NT-$3^{+/+}$ (n = 4), NT-$3^{+/-}$ (n = 4), NT-$3^{-/-}$ (n = 4); at E14, NT-$3^{+/+}$ (n = 4), NT-$3^{+/-}$ (n = 4), NT-$3^{-/-}$ (n = 4); at E17, NT-$3^{+/+}$ (n = 2), NT-$3^{+/-}$ (n = 2), NT-$3^{-/-}$ (n = 2).

Double Staining with the TUNEL Method and for BrdU, or Peripherin

Sections were stained with the TUNEL method. The sections were then preincubated in dilution buffer (0.5 M NaCl, 0.1 M phosphate buffer [pH 7.3], 3% bovine serum albumin, and 0.3% Triton X-100) for 1 hr followed by overnight incubation with the indicated concentration of antisera in dilution buffer with 10% goat serum. After four washes in PBS, sections were incubated for 2 hr with the appropriate conjugated secondary antiserum, washed three times for 10 min, and covered with glycerol:PBS (9:1) for viewing. The mouse anti-BrdU (Sigma) and the rabbit anti-peripherin (Chemicon) were diluted 1:500. Rhodamine-conjugated goat anti-mouse and anti-rabbit antisera were used as secondary antisera.

RNA Purification and RNase Protection Assay

Embryos were obtained from overnight mating of BALB/c mice. Pregnant females were killed by cervical dislocation. Dorsal root ganglia were dissected from E11, E17, P15, and adult mice and were frozen at −70°C until use. Total RNA was purified from 75–100 embryonic ganglia per stage and 25–30 postnatal or adult ganglia by the guanidine-isothiocyanate/phenol-chloroform extraction according to standard procedures (Schomczynski and Sacci, 1987) and was quantified using a spectrophotometer. The RNase protection assay was performed with the RPAII Ribonuclease Protection Assay Kit (Ambion, Austin, TX). The 440 bp NT-3 cRNA probe was synthesized by in vitro transcription from cDNA of the mouse NT-3 between the HindIII and SmaI restriction enzyme sites. The probes were labeled with [α-^{32}P]CTP to a specific activity of 200 Ci/mmol (1–2 × 10^9 cpm/μg) by transcription with T3 RNA polymerase. The RNA protection assay was carried out as described by the manufacturer. Protected cRNA fragment was separated on 4% polyacrylamide gels under denaturing condition, and gels were exposed to X-ray films for 8 days. A GAPDH probe was used to assess mRNA integrity and to compare loading among the lanes. Sequence reactions of unrelated DNA with known sequences were used as size markers.

Tissue Culture

Embryos were obtained from overnight matings of NT-$3^{+/-}$ mice. The embryos were processed for culture individually. Electrolytically sharpened tungsten needles were used to dissect cervical dorsal root ganglia from E11 embryos, the remaining tissue was used for DNA purification to determine the genotype. Ganglia were incubated for 5 min at 37°C with 0.05% trypsin (GIBCO/BRL) in calcium- and magnesium-free Hanks' balanced salt solution. After removal of the trypsin solution, the ganglia were washed once with 10 ml of DMEM containing 10% heat-inactivated horse serum and were gently triturated with a fire-polished Pasteur pipette to give a single cell suspension. The cells were plated on 24-well plates (Nunc) that were precoated with polyornithine (0.5 mg/ml, overnight) and laminine (20 μg/ml for 4 hr; GIBCO/BRL). The neurons were incubated at 37°C in a humidified 5% CO_2 incubator in a defined medium consisting of Hams' F14 supplemented with 2 mM glutamine, 0.35% bovine serum albumin, 60 ng/ml progesterone, 16 mg/ml putrescine, 400 ng/ml L-thyroxine, 38 ng/ml sodium selenite, 340 ng/ml triiodo-thyronine, 60 μg/ml penicillin, and 100 μg/ml streptomycin. After 24 hr incubation, neurons were clearly recognized by their bipolar morphology under phase-contrast optics. The percentage neuronal survival in the absence or presence of purified recombinant NT-3 protein (added to the culture medium at the time of plating the neurons at 10 ng/ml) was assessed after 24 hr by counting the neurons in the wells. Neurons from each embryo were plated in triplicate with and without NT-3. The data illustrated are compiled from the results of several experiments (NT-$3^{+/+}$ [n = 5]; NT-$3^{-/-}$ [n = 3]).

For cultures of wild-type mice (BALB/c), 60–70 cervical dorsal root ganglia (12–14 ganglia/embryo) were dissected, dissociated, and cultured as described above. Cultures were maintained in the

presence or absence of NT-3 or BDNF at a final concentration of 1 ng/ml and antibodies (10 μl/ml of the turkey anti-NT-3 [102] antibody and 0.4 μg/ml of the rabbit anti-NT-3 antibody) added at the time of plating. The survival at 24 hr is presented as percentage of the cells present at 5 hr. All cultures were set up in triplicate, and the entire experiment was performed twice with similar results.

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