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Targeted mutation in the neurotrophin-3 gene results in loss of muscle sensory neurons

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ABSTRACT **Neurotrophin 3 (NT-3) is one of four related polypeptide growth factors that share structural and functional homology to nerve growth factor (NGF). NT-3 and its receptor, called neurotrophic tyrosine kinase receptor type 3 (Ntrk3; also called TrkC), are expressed early and throughout embryogenesis. We have inactivated the NT-3 gene in embryonic stem (ES) cells by homologous recombination. The mutated allele has been transmitted through the mouse germ line, and heterozygote intercrosses have yielded homozygous mutant newborn pups. The NT-3-deficient mutants fail to thrive and exhibit severe neurological dysfunction. Analysis of mutant embryos uncovers loss of *Ntrk3/TrkC*-expressing sensory neurons and abnormalities at early stages of sensory neuronal development. NT-3-deficient mice will permit further study of the role of this neurotrophin in neural development.**

Neurotrophins are soluble polypeptide factors that support the survival of neurons *in vivo* and in culture (1). Nerve growth factor (NGF) is the best and most widely studied of the neurotrophins, largely because of discovery several decades ago (2). Brain-derived neurotrophic factor (BDNF) was identified in the last decade, and neurotrophins 3 (NT-3) and 4/5 (NT-4/5) were isolated by homology to NGF and BDNF more recently (3–5). NT-3 and its neurotrophic tyrosine kinase receptor type 3 (Ntrk3; also called TrkC, which is used here) are expressed widely, although primarily in the nervous system where they are presumed to have multiple functions during development (6–10). In primary cultures, NT-3 promotes the survival and/or differentiation of cells from different neural populations of the peripheral and central nervous systems (PNS and CNS), including neural crest cells and oligodendrocyte precursors (1, 3, 11–15). *In vivo* the function of NT-3 is less well understood, although recent reports indicate that it can prevent the death of adult central noradrenergic neurons, enhance sprouting of corticospinal tracts during development and after spinal cord lesion, and promote oligodendrocyte proliferation in the optic nerve (16–18).

Cell culture experiments aimed at understanding the role of the Trk family of receptors and their interactions with neurotrophins have provided a somewhat controversial picture of the receptor–ligand interactions. Thus, while TrkC appears to bind NT-3 exclusively (19, 20), TrkB (Ntrk2) can bind NT-3 with apparently equivalent affinities to the binding of BDNF and NT-4/5 (21–23), and TrkA (Ntrk1)—the NGF receptor—has also been reported to bind and become activated by NT-3 under certain conditions (24). Recently, homologous recombination techniques have been used to generate mice lacking specific gene functions, including the known *Trk* (*Ntrk*) family of genes and the genes encoding neurotrophins NGF and BDNF (25–30). Mice homozygous for these mutations have severe deficits in multiple populations of peripheral sensory and autonomic neurons (25–30). However, analysis of the CNS in these mutant mice has shown less obvious developmental defects. Motoneuron loss in *TrkB* mutant mice and reduced expression of various neuronal markers in mice lacking BDNF so far represent the only specific CNS deficiencies reported in the neurotrophin and Trk mutations (25–27). These results suggest a possible *in vivo* redundancy of interactions between the neurotrophins and receptors. To understand the physiological role of NT-3 during mouse development, we have targeted the gene for NT-3 in mouse embryonic stem (ES) cells. Newborn pups homozygous for the mutated NT-3 allele exhibit nervous-system dysfunction within hours after birth, and the large majority die during the ensuing 48 hr. Analysis of homozygous mutant embryos reveals developmental defects in a subpopulation of sensory neurons. These defects were observed at early stages of development—prior to and during target innervation.

MATERIALS AND METHODS

Targeting Vector, Electroporation, and Selection. The replacement-type targeting vector consisted of an 8.0-kb 129 SV (Stratagene) mouse genomic fragment containing the NT-3 coding sequence. The neomycin-resistance (*neo*) gene with the phosphoglycerate kinase 1 promoter and the bovine growth hormone polyadenylylation sequence (pGKneobpA) was introduced as a positive selectable marker, and a pGK-thymidine kinase cassette, as a negative selectable marker (31). Electroporation and selection were performed by using the CJ7 ES cell line as described elsewhere (32). DNAs derived by G418/FIAU (fialuridine)-resistant ES clones were screened by using a diagnostic *Sca* I restriction enzyme digestion with the 5′ probe external to the targeting vector sequence indicated in Fig. 1*b*. Recombinant clones containing the predicted 8.2-kb rearranged band were obtained at a frequency of 1/18.

Generation of Mutant Mice. Injection of two independent recombinant clones carrying the targeted ES cell NT-3 gene into C57BL/6 blastocysts generated chimeras that transmitted the mutated NT-3 allele to the progeny. Breeding of two NT-3$^{BRP/+}$ mice (shown as −/+ in Fig. 1) gave rise to homozygous mutant mice (NT-3$^{BRP/BRP}$) at a frequency of 20%; BRP (Basic Research Program) represents our mutant NT-3 allele/phenotype, shown as −/− in Figs. 1–5.

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Abbreviations: BRP, Basic Research Program; BDNF, brain-derived neurotrophic factor; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DRG, dorsal root ganglia; NGF, nerve growth factor; NT-3, neurotrophin 3; NT-4/5, neurotrophin 4/5; ES cells, embryonic stem cells; PNS and CNS, peripheral and central nervous systems; E12.5-E15.5, embryonic days 12.5 to 15.5.

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Histologic Preparation and *in Situ* Hybridization. Gravid uteri were removed from timed pregnant females, washed in phosphate-buffered saline (PBS), and dissected in ice-cold PBS. The embryos were fixed immediately in >20 volumes of ice-cold 4% paraformaldehyde in PBS. After fixation, the samples were dehydrated through a graded series of alcohols and xylenes and embedded in paraffin. Embryos were sectioned at 5-μm thickness, mounted on gelatin-treated slides, and stained with hematoxylin and eosin or processed for *in situ* hybridization analysis. *In situ* hybridization protocols with *TrkA*-, *TrkB*-, and *TrkC*-specific probes were performed as described (8). Sections were photographed with a Zeiss Axiophot microscope equipped with an integral camera.

Primary Neuronal Cultures. Cervical and thoracic dorsal root ganglia (DRGs) were dissected from individual embryonic day 12.5 (E12.5) mutant and wild-type littermates and were dissociated to a single-cell suspension by gentle trituration after a 20-min incubation in 0.125% trypsin (GIBCO). The ganglion cells from each embryo were distributed evenly among 4 or 5 35-mm tissue culture plastic dishes (Nunc) that had been coated previously with polyornithine (Sigma) and laminin (GIBCO) and were cultured in Ham's F-14 medium (Imperial Chemical Industries) supplemented with 10% (vol/vol) heat-inactivated horse serum (JRH Biosciences, Lenexa, KS), 5% (vol/vol) heat-inactivated fetal calf serum (HyClone), 50 units of penicillin and 50 μg of streptomycin per ml (GIBCO), and 2 mM glutamine (GIBCO). Four to five hours after plating, recombinant purified neurotrophins NGF, BDNF, and NT-3 (Genentech) were added to the cultures at a concentration of 2 ng/ml. Note that the experimenter was unaware of the individual genotypes at the time neuron counts were made.

Labeling with 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine Perchlorate (DiI). Embryonic day 15.5 (E15.5) mutant and normal littermates were decapitated and perfused with PBS and 4% paraformaldehyde through the left ventricle, eviscerated, and incubated overnight at 4°C in 4% paraformaldehyde. The skin was removed, and crystals of DiI (Molecular Probes) were inserted into axial muscles. After 7–11 days of incubation in 10% formalin (37°C), the spinal cords were removed, embedded in 3.5% agar/sucrose, and sectioned transversely at 100 μm on a vibratome. Sections were viewed on a Zeiss Axiophot microscope with the rhodamine filter set to visualize DiI-labeled afferents.

RESULTS AND DISCUSSION

To undertake analysis of the role of NT-3 during embryogenesis, a targeting cassette was constructed to disrupt the locus in ES cells by homologous recombination. Since the entire NT-3 polypeptide is encoded by one exon, disruption of the coding region by insertion of a pGKneo recombination cassette was used (Fig. 1 *a–c*). (31, 32). Chimeric mice generated with two independent recombinant clones of mouse ES cells transmitted the mutated allele to their offspring. Heterozygous mice for the NT-3 locus (NT-3$^{BRP/+}$) appeared normal, and intercrossing gave rise to live born homozygotes (NT-3$^{BRP/BRP}$) at a frequency of 20% (n = 226). At birth, surviving homozygous mutant pups are indistinguishable from their normal littermates and respond to tail-pinch stimuli, indicating functional nociceptive innervation. However, within hours after birth, all NT-3$^{BRP/BRP}$ pups exhibit radically abnormal movements and postures indicative of neurological dysfunction (Fig. 1*d*). While the surviving newborn mutant pups feed successfully as evidenced by milk in their stomachs, a small percentage (≈10%) die at birth, and the remaining majority (80%) perish within 24–48 hr. The few surviving mutant pups have lived for up to 16 days, although they fail to thrive compared with their littermates (25–50% of control body weight for the 4 of 46 mutants

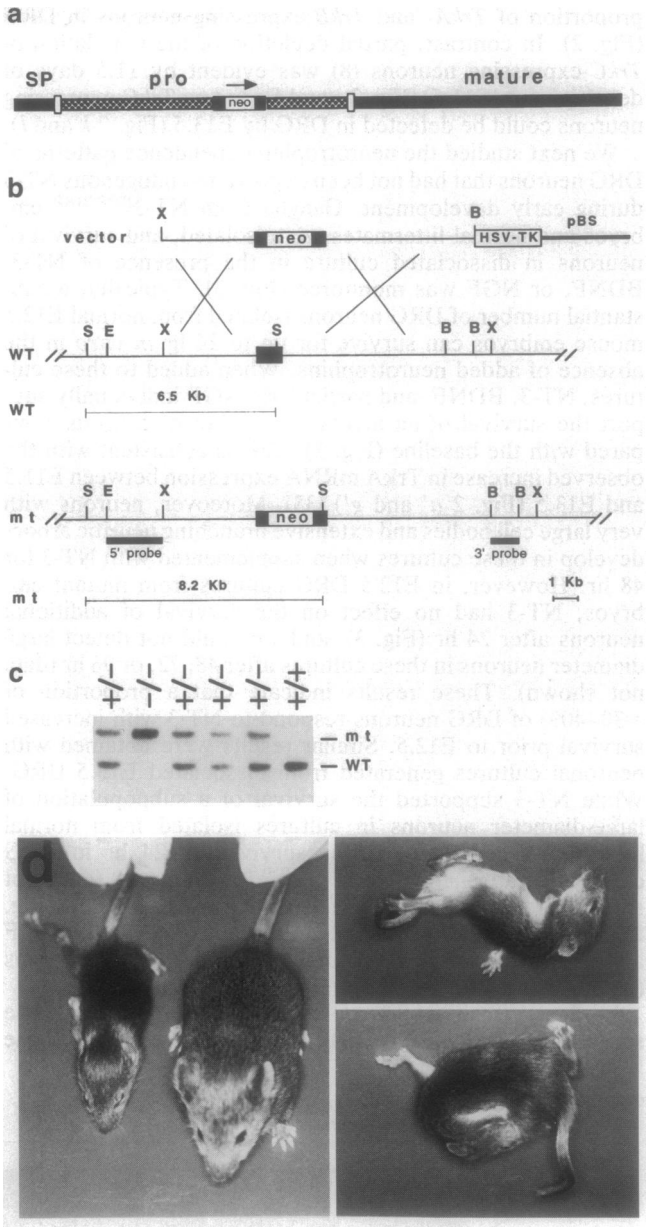

FIG. 1. Generation of NT-3$^{BRP/BRP}$ mutant mice, indicated in *c* as −/−. (*a*) Schematic showing the site of insertion of the *neo* selectable marker into the NT-3 gene. The coding regions for the signal peptide (SP) and mature peptide are indicated by closed bars; the coding region for the processed amino-terminal (pro) region is stippled. The arrow indicates the direction of transcription. (*b*) Schematic showing the replacement vector and strategy used to inactivate the NT-3 locus. (*c*) Southern blot analysis of tail DNA from a litter obtained intercrossing two NT-3$^{BRP/+}$ mice, shown as −/+. *Sca* I (S) restriction enzyme digestion and the 5' probe indicated in *b* were used to detect rearrangement in the mouse NT-3 locus. The 6.5-kb wild-type (wt) and 8.2-kb rearranged (mt) DNA bands are indicated. (*d*) Postpartum day 16 NT-3$^{BRP/BRP}$ mutant mouse (*Right*) compared with a normal littermate (*Left*).

surviving >10 days; Fig. 1*d*) and exhibit behavioral abnormalities.

To determine the time of onset of defects that lead to the observed phenotype at birth, we have investigated the state of the peripheral nervous system in the homozygous mutants at various stages of embryogenesis. Since the Trk family of receptors is thought to mediate the biological effects of neurotrophins, we analyzed the expression pattern of *TrkA*, *TrkB*, and *TrkC* by RNA *in situ* hybridization in mutant embryos. These data revealed no apparent changes in the

proportion of *TrkA*- and *TrkB*-expressing neurons in DRG (Fig. 2). In contrast, partial depletion of the population of *TrkC*-expressing neurons (8) was evident by 11.5 days of development (E11.5) (Fig. 2 *e* and *f*), and no *TrkC*-expressing neurons could be detected in DRG by E13.5 (Fig. 2 *k* and *l*).

We next studied the neurotrophin dependence patterns of DRG neurons that had not been exposed to endogenous NT-3 during early development. Ganglia from NT-3$^{BRP/BRP}$ embryos and normal littermates were isolated, and survival of neurons in dissociated culture in the presence of NT-3, BDNF, or NGF was monitored (Fig. 3). Typically, a substantial number of DRG neurons isolated from normal E12.5 mouse embryos can survive for up to 24 hr *in vitro* in the absence of added neurotrophins. When added to these cultures, NT-3, BDNF, and particularly NGF individually support the survival of an increased number of neurons compared with the baseline (Fig. 3). This is consistent with the observed increase in TrkA mRNA expression between E11.5 and E13.5 (Fig. 2 *a'* and *g'*) (33). Moreover, neurons with very large cell bodies and extensive branching neuritic arbors develop in these cultures when supplemented with NT-3 for 48 hr. However, in E12.5 DRG cultures from mutant embryos, NT-3 had no effect on the survival of additional neurons after 24 hr (Fig. 3), and we could not detect large diameter neurons in these cultures after 48, 72, or 96 hr (data not shown). These results indicate that a proportion of ≈30–40% of DRG neurons respond to NT-3 with increased survival prior to E12.5. Similar results were obtained with neuronal cultures generated from dissociated E13.5 DRG. While NT-3 supported the survival of a subpopulation of large-diameter neurons in cultures isolated from normal littermates, no such neurons survived to 24 hr in DRG cultures established from NT-3$^{BRP/BRP}$ embryos (data not shown). Consistent with the TrkA and TrkB *in situ* data (Fig. 2), the subpopulations of DRG neurons supported by NGF and BDNF appear to be unaffected in the mutant embryos (data not shown).

In the chicken, NT-3 promotes the survival of muscle sensory neurons *in vitro* (34). To characterize further the

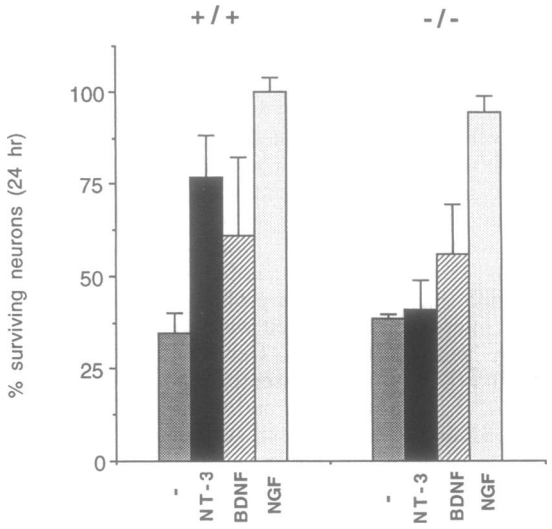

FIG. 3. Neurotrophin dependence of E12.5 wild-type (shown as +/+) and NT-3$^{BRP/BRP}$ (shown as −/−) DRG neurons in culture. Survival data from three independent culture experiments from DRG-dissected neurons upon addition of NGF, BDNF, NT-3 or no neurotrophin are expressed as a percentage of neurons surviving upon addition of the three combined neurotrophins.

affected DRG subpopulation in NT-3$^{BRP/BRP}$ mice, we retrogradely labeled sensory and motor neurons from axial muscle at the cervical, thoracic, and lumbar levels by using the lipid-soluble fluorescent tracer DiI in fixed mutant and normal E15.5 embryos (35). The central axons of muscle sensory neurons terminate in deeper laminae of the spinal cord than do cutaneous afferents (36); specifically, the central axon collaterals of Ia proprioceptive neurons terminate in lamina IX, where they contact motor neurons to form reflex arcs (Fig. 4*a*) (37). We observed no Ia afferent projections to the motor neurons in spinal cords from E15.5 NT-3$^{BRP/BRP}$ mice at any axial level examined (0 of 7 embryos; Fig. 4 *b* and *c* and Fig.

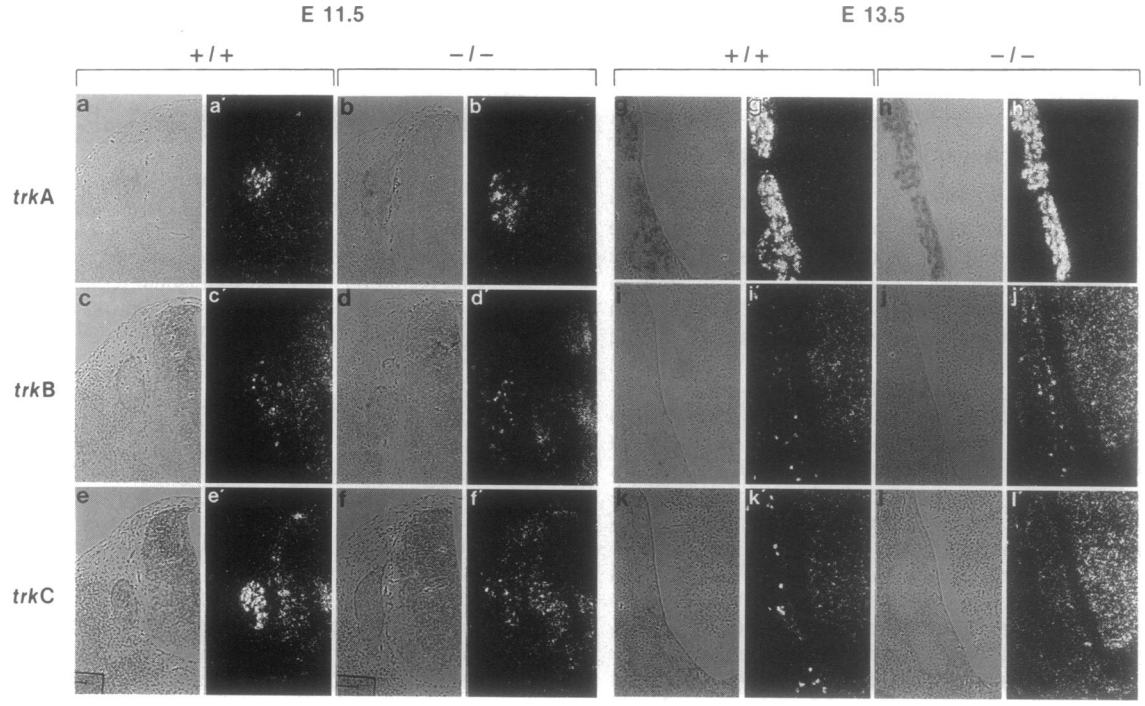

FIG. 2. Trk family receptor expression and neurotrophin-dependence analysis of DRG in NT-3$^{BRP/BRP}$ mutant mice, shown as −/−. Comparative *in situ* hybridization analysis of the *Trk* family of genes in E11.5 and E13.5 midlumbar DRGs from wild-type (+/+) and NT-3$^{BRP/BRP}$ mutant mice, employing *TrkA*-, *TrkB*-, and *TrkC*-specific probes. (*a–l*) Lightfield views. (*a'–l'*) Darkfield views.

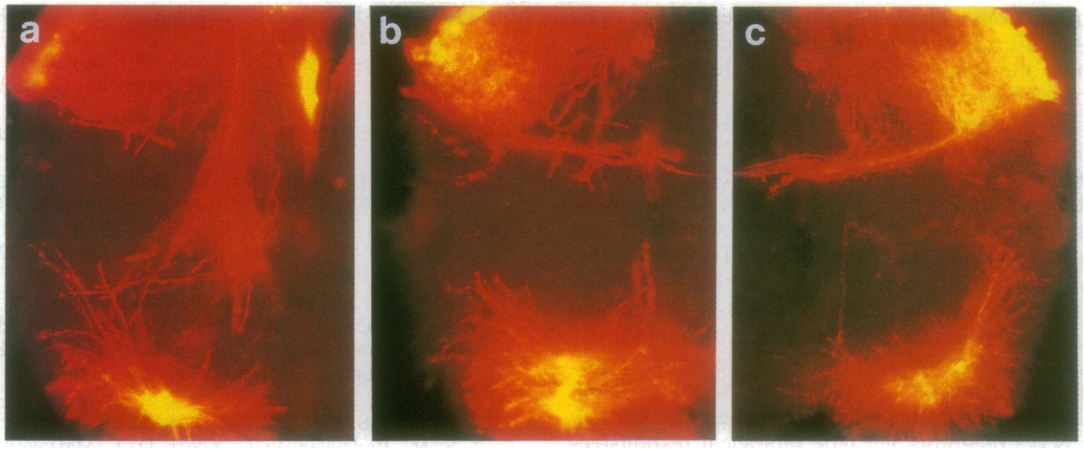

FIG. 4. Fluorescence photomicrographs of spinal cords showing central projections of DRG neurons retrogradely labeled with the lipid-soluble dye DiI from axial muscles in normal (*a*) and NT-3$^{BRP/BRP}$ mutant (*b* and *c*) E15.5 fetuses (dorsal, up; ventral, down). The Ia proprioceptive afferent projections shown in the wild-type mouse connecting the dorsal roots with the ventrally located motor neurons are absent in the mutant spinal cords. Motoneurons (ventrally located) are retrogradely labeled from muscle in both mutant and normal embryos.

5). In contrast, projections to motoneurons were present in the spinal cords of all normal, age-matched littermates analyzed (6 of 6 embryos), and afferent endings intermingled with the dendrites of motoneurons that were also retrogradely labeled from muscle (Figs. 4*a* and 5). Since the elaboration of Ia afferent projections is absent in NT-3$^{BRP/BRP}$ mutant spinal cords at this early stage when central connections are first established, it is unlikely that proprioceptive neurons project axons into the spinal cord followed by precocious death. Rather, these data suggest that the proprioceptive neurons fail to project afferents into the cord. Cutaneous afferents, labeled by placing DiI in the dorsal roots, appeared to be unaffected by the loss of NT-3 in mutant embryos (2 of 2 embryos; data not shown). Taken together, the *in situ*, DRG neuron culture, muscle retrograde labeling, and behavioral data are consistent with a specific loss of proprioceptive sensory neurons in NT-3$^{BRP/BRP}$ mice and with the existence of developmental defects by E12.5. Moreover, this NT-3-deficient phenotype is similar to that observed in infant mice homozygous for a TrkC deficiency. Mice reported to lack the full-length TrkC receptor also exhibit defects in posture and movement and have no Ia proprioceptive afferents (30).

In general, the CNS and PNS of the NT-3$^{BRP/BRP}$ embryos appear morphologically normal. One notable exception is the reduced spinal cord diameter of the newborn mutants when compared with littermates of equal size and weight (Fig. 6). The difference observed indicates additional deficiencies to those described here in the development or survival of cell populations of the spinal cord. Proprioceptive afferents can have trophic influence on motor neurons (38), thus absence of these projections may adversely affect neuron survival (39). We can observe motor neurons in the mutant spinal cord (Fig. 6*d*) and at present lack compelling evidence to indicate a reduction of motor neurons *per se*. More detailed studies to investigate absolute numbers, survival in culture, and response to stress of NT-3$^{BRP/BRP}$ mutant motor neurons may be required to reveal a potential deficiency (40).

The TrkC receptor tyrosine kinase is thought to be the functional high-affinity receptor for NT-3 (7, 9, 10). However, the phenotype of the recently described *TrkC* mutant mice differs from that of the NT-3$^{BRP/BRP}$ mutant mice reported here (30). In contrast to our two independently derived NT-3-deficient strains of mice, which adopt altered behavior within hours after birth and the majority of which perish within the first 48 hr [postpartum day 2 (P2)] after birth, the TrkC-deficient mice appear to behave normally until P2 and can survive until P30 (30). To date, only 1 NT-3-deficient mouse of 46 liveborn has survived to P16 (Fig. 1*d*). In addition, we have noted a consistent and dramatic (≤50%) reduction in spinal cord diameter in the NT-3$^{BRP/BRP}$ mutants, while the *TrkC* mutants were reported to exhibit

NT-3 -/-		WILDTYPE	
Mouse	Sections with Ia af. fibers	Mouse	Sections with Ia af. fibers
1	0/58	1a	35/48
2	0/34	2a	19/42
3	0/33	3a	31/39
4	0/47	4a	10/13
5	0/14		
6*	0/28	6a*	30/30
7*	0/19	7a*	30/31

FIG. 5. Quantitation of spinal cord sections [from wild-type and NT-3-deficient (−/−) mice] containing afferent (af.) Ia projections to the ventral horn motoneurons were determined for each embryo. *, Afferents were labeled from the dorsal roots rather than from the muscle.

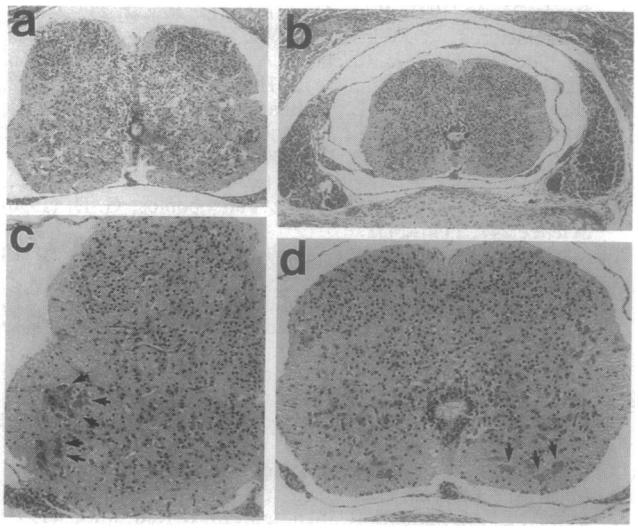

FIG. 6. Spinal cord in NT-3$^{BRP/BRP}$ mutant mice. Transverse lumbar spinal cord sections through a wild-type (+/+) (*a* and *c*) and a mutant NT-3-deficient (−/−) (*b* and *d*) newborn mouse. Sections (5 μm) were prepared from paraffin-embedded material, stained with hematoxylin-eosin, and photographed. Arrows indicate motor neurons. [×75 (*a* and *b*) and ×150 (*c* and *d*).]

only minor reductions (30). While this discrepancy may be attributable to differences in genetic background or in vivarium conditions, alternative explanations for the more severe phenotype of the NT-3$^{BRP/BRP}$ mice include the possibility that *in vivo*, NT-3 may employ other Trk family receptors, as has been noted in cell culture systems (21–23), or that the reported *TrkC* mutation may not affect all TrkC isoforms, thus presenting a reduced phenotype (10, 30, 41). We have recently generated a different *TrkC* mutant mouse line that was designed to exclude translation of all TrkC isoforms. Intercross of this *TrkC* mutant line with the NT-3-deficient mutant mouse lines and comparative analysis could help clarify this and other issues of receptor/neurotrophin use.

Finally, it must be noted that NT-3 and its receptor, TrkC, are widely expressed within and outside the nervous system (6, 8). In chicken, neurons have been identified that contain high-affinity NT-3 receptors yet lack biological responsiveness to this neurotrophin (42). The present mice may provide a means for developing assays toward identifying and distinguishing TrkC-expressing cells that exhibit a functional requirement for NT-3.

After this manuscript was completed and submitted for review two reports appeared describing mutations of the NT-3 gene similar to that described herein (43, 44). Our results are in agreement with and complementary to those described, which focused their analysis on mutants after birth (43, 44). All three studies indicate neurological dysfunction that is attributable to loss of proprioceptive neurons. Ernfors *et al.* (43) further demonstrate the absence of muscle spindles and Golgi tendon organs in newborn homozygous mutants and a 50% reduction in heterozygotes, suggesting the existence of limiting amounts of NT-3 (43). The present study demonstrates the appearance of the proprioceptive neuron deficiency in early embryogenesis.

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