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Dependence of Developing Group Ia Afferents on Neurotrophin-3

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ABSTRACT

At birth, group Ia proprioceptive afferents and muscle spindles, whose formation is Ia afferent-dependent, are absent in mice carrying a deletion in the gene for neurotrophin-3 (NT-3 −/−). Whether Ia afferents contact myotubes, resulting in the formation of spindles which subsequently degenerate, or whether Ia afferents and spindles never form was examined in NT-3 −/− mice at embryonic days (E) 10.5–18.5 by light and electron microscopy. Three sets of data indicate that Ia neurons do not develop and spindles do not form in NT-3-deficient mice. First, peripheral projections of Ia afferents did not innervate hindlimbs of NT-3 −/− mice, as reflected by a deficiency of nerve fibers in limb peripheral nerves and an absence of afferent nerve-muscle contacts and spindles in the soleus muscle at E13.5–E18.5. Second, central projections of Ia afferents did not innervate the spinal cord in the absence of NT-3, as shown by an atrophy of the dorsal spinal roots and absence of afferent projections from limb musculature to spinal motor neurons at E13.5 or E15.5. Lastly, the lumbar dorsal root ganglia (DRGs) at E10.5–E14.5, the stages of development that precede or coincide with the innervation of the spinal cord and hindlimbs by Ia afferents, were 20–64% smaller in mutant than in wild-type mice, presumably because the cell bodies of Ia neurons were absent in embryos lacking NT-3.

The failure of Ia neurons to differentiate and/or survive and Ia afferent projections to form in early fetal mice lacking NT-3 suggests that NT-3 may regulate neuronal numbers by mechanisms operating prior to neurite outgrowth to target innervation fields. Thus, developing Ia neurons may be dependent on NT-3 intrinsic to the DRGs before they reach a stage of potential dependence on NT-3 retrogradely derived from skeletal muscles or spinal motor neurons.

Indexing terms: neurotrophins, muscle spindles, sensory neurons, dorsal root ganglia, proprioception

Neurotrophin-3 (NT-3) is a trophic factor essential for he development of proprioceptive neurons that innervate nuscle spindles and Golgi tendon organs (Ernfors et al., 994). Sensory neurons of this class have been shown to equire NT-3 for sustenance in culture and are presumed to xpress the NT-3 receptor, *trkC* (Hohn et al., 1990; Mu et l., 1993; Hory-Lee et al., 1993; McMahon et al., 1994). Moreover, group Ia sensory neurons (primary afferents of pindles) are absent in spinal dorsal root ganglia (DRGs), nd spindles are absent in limb muscles of newborn mice arrying a functional deletion in the NT-3 gene (Ernfors et l., 1994; Fariñas et al., 1994).

Whether proprioceptive neurons form and subsequently egenerate, or never form in the course of fetal develop- ıent of mice lacking NT-3 is uncertain. The population of ensory neurons expressing *trkC*, which presumably includes Ia neurons, is partially depleted or absent in embryonic NT-3 −/− mice, suggesting neuronal abnormalities at early stages of DRG development in the absence of NT-3 (Tessarollo et al., 1994; Tojo et al., 1995). However, the decreased expression of *trkC* in NT-3 −/− DRGs before birth may not necessarily indicate an absence of Ia neurons during fetal development, because the neurons could be present but may not be expressing the *trkC* receptor in the absence of NT-3. Experimental evidence suggests a regulatory effect of tissue levels of neurotrophins on the expression of mRNAs of the *trk* family (Wyatt and Davies, 1993).

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The source of the NT-3 essential for the differentiation and/or survival of NT-3-dependent proprioceptive neurons is likewise uncertain. One possibility is that Ia neurons differentiate from precursor cells independent of NT-3, but their subsequent survival depends on NT-3 generated by target tissues innervated by the neurons. Such dependence on target-derived factors has been demonstrated for other types of sensory neurons. Nociceptive neurons responsive to nerve growth factor (NGF) can differentiate independent of NGF, but become dependent on NGF when their axons innervate the peripheral target tissues (Buchman and Davies, 1993; Buj-Bello et al., 1994).

Group Ia neurons form two processes—a peripheral projection terminating on intrafusal fibers of muscle spindles and a central projection synapsing on spinal neurons including motor neurons of the ventral spinal cord. NT-3 mRNA is present in both the ventral spinal cord and limb buds when Ia neurons are establishing axonal contacts with motor neurons and myotubes in embryonic mice and rats (Hohn et al., 1990; Ernfors and Persson, 1991; Ernfors et al., 1992; Henderson et al., 1993). Thus, muscle-derived as well as cord-derived NT-3 could potentially support developing Ia neurons via retrograde transport. No Ia afferents were observed to innervate the spinal cord of NT-3 −/− embryos at E15.5, when Ia afferents are ordinarily in contact with the target motor neurons (Tessarollo et al., 1994). However, it is not known whether Ia neurons differentiate at all, or form processes that enter skeletal muscles in NT-3-deficient mutants.

If Ia neurons differentiate independent of NT-3, and if the survival of differentiated Ia neurons depends principally on muscle-derived NT-3, then Ia afferents should project into muscles even in mice lacking NT-3. Thus, observation of normal size DRGs and Ia afferent- myotube contacts or muscle spindles (Ia afferent-myotube contacts give rise to spindles) in NT-3 −/− mice at an early developmental stage, followed by the loss of DRG cells and muscle afferent innervation together with spindle degeneration at later developmental stages, would suggest a neurotrophic role for muscle-derived NT-3 in Ia neuron development.

The present study examined the size of DRGs and compared the innervation of fetal hindlimbs between NT-3 −/− and wild-type mice by electron microscopy and tracer dye injection in order to determine whether Ia neurons and muscle spindles form in the absence of NT-3. Data are presented indicating that Ia afferents never innervate limb muscles and spindles do not form in mice lacking NT-3, because Ia neurons never differentiate and/or survive to reach a stage when they can contact myotubes.

MATERIALS AND METHODS

Animals

Progeny of crosses between male and female Balb/c 126 outbred mice (Charles River Laboratories) heterozygous for a deletion in the NT-3 gene (+/−) were used as a source of mice homozygous for the null mutation in the NT-3 gene (−/−) as well as wild-type (+/+) mice. Genotypes were determined by extracting tail DNA and subjecting it to polymerase chain reaction or Southern blot analysis, as described previously (Ernfors et al., 1994). The morning after an overnight mating of an NT-3 +/− female to an NT-3 +/− male was considered as day E0.5. Dams were anesthetized with sodium pentobarbital (35–50 mg/kg b.wt., i.p.), and fetuses were removed in the morning of days E10.5–E18.5 or the evening of day E13.5 (E14.0). Euthanasia was accomplished by an overdose of sodium pentobarbital (200 mg/kg b.wt., i.p.). Hindlimbs of at least two mutant and two wild-type fetuses were obtained at daily intervals from E13.5 to E18.5. The thoracolumbar spinal cords with associated DRGs and dorsal spinal roots (DRs) were removed daily from NT-3 −/− and NT-3 +/+ littermates at E10.5–E14.5. In addition, hindlimbs of two mutant and two wild-type mice were excised under sodium pentobarbital anesthesia at postnatal day (P) 14. All experimental procedures involving mice were approved by the institutional Animal Study Committee.

Tissue processing

Hindlimbs and spinal cords were fixed by immersion in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide, and embedded in Eponate 12. The soleus (SOL) muscles of NT-3 −/− and NT-3 +/+ littermates were examined by electron microscopy daily from E13.5 to E18.5 to determine the extent of muscle innervation by afferents. Transverse sections of plastic-embedded SOL muscles were cut on an ultramicrotome at a thickness of either 1.0 or 0.09 μm. The 1.0-μm-thick sections stained with 1% toluidine blue were used to locate the neuromuscular hilum of the muscles, a region where the motor and sensory nerve fibers course and spindles form (Kozeka and Ontell, 1981). The 0.09-μm sections stained with lead citrate and uranyl acetate were used to examine the features of neuromuscular contacts by electron microscope. Lengths of tissue spanning 50–100 μm were cut in serial ultrathin sections so that the course of intramuscular nerve bundles and innervation of individual muscle fibers could be traced. Electron micrographs of all visible nerve and muscle junctions were obtained at magnifications ranging from ×2,000 to 10,000. Details of these procedures have been published previously (Kucera et al., 1989).

Labeling with DiI

To determine whether Ia afferents innervate the spinal cord, the lipophilic dye DiI (1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate, Molecular Probes) was used as an axonal tracer in two pairs of NT-3 −/− and NT-3 +/+ littermates at E13.5 and E15.5. Embryos were immobilized with insect pins on a Sylgard-coated petri dish and fixed overnight in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer. Crystals of DiI were applied to the thigh muscles bilaterally and the embryos were incubated for 6 days at 42°C submerged in 4% paraformaldehyde. The spinal columns were dissected, embedded in 2% agar, sectioned at 100 μm on a vibratome, and examined under a rhodamine filter by fluorescence microscopy. These procedures have been detailed previously (Ernfors et al., 1994).

Identification of structures and analyses

Primary myotubes, secondary myotubes, and myoblasts were identified according to conventional ultrastructural criteria (Harris et al., 1989). Muscle spindles form at sites of contact between Ia afferents and primary myotubes (Zelená, 1957, 1994). The single primary myotube of the nascent one-fiber spindles was recognized as the nuclear bag_2 intrafusal fiber (Milburn, 1973; Kucera and Walro, 1995). Sensory (Ia) nerve-muscle contacts lacked interposed basal lamina in the synaptic cleft between the abutting axolemma

and plasmalemma (Landon, 1972). Developing motor nerve-muscle contacts had the essential features of mature motor neuromuscular junctions, including the presence of basal lamina in the synaptic cleft (Landon, 1972). Undifferentiated nerve-muscle contacts involved axons or bundles of axons whose ultrastructural features did not permit classification of the contacts as either sensory or motor.

The number of nerve fibers composing the SOL muscle nerve was determined in NT-3 −/− and +/+ littermates at daily intervals from E14.0 to E18.5. The nerve was sectioned transversely at a point 100–200 μm proximal to its entry into the SOL muscle and electron micrographs were obtained at ×3,000 magnification. The unmyelinated axons comprising the fetal SOL nerve (no myelinated fibers are present at the fetal stages of development) were counted from the micrographs using the Zeiss Videoplan Image Analysis System. In addition, myelinated nerve fibers only were counted in the SOL muscle nerve of three mutant and three wild-type mice at P14, because Ia afferents are myelinated by P14 in the mouse (Kucera et al., 1995). In addition, the cross-sectional area of the tibial nerve at the midcalf level was determined in two pairs of mutant and wild-type mice at E13.5, a developmental stage preceding the formation of the SOL muscle nerve. Values for the right and left nerves were averaged, the counts and area measurements transformed by arc sin transformation, and compared for statistical significance between the age-matched NT-3 −/− and +/+ mice using an analysis of variance (anova). Any statistical differences indicated by anova were compared post hoc using a Student-Newman-Ceuls test.

The size of lumbar DRGs and DRs was determined in pairs of NT-3 +/+ and NT-3 −/− littermates at E10.5–E14.5. The spinal column was cut transversely at 1-μm thickness in serial sections from the last thoracic vertebra (identified by the last thoracic rib) to the fourth lumbar (L) vertebra. In E10.5 and E11.5 embryos the DRGs just proximal to the insertion of the limb bud were considered as L4. The volume of the L1 and L4 DRGs was determined in the following manner: the cross-sectional area of every tenth 1-μm-thick section (i.e., every tenth micron) throughout the length of the ganglion was obtained using the Zeiss Videoplan Image Analysis System and each area multiplied by 10 to represent the volume of the 10 μm DRG segment. Volumes of the 10 μm-thick segments were then summed to obtain the volume of the entire ganglion in cubic microns. To confirm that any differences in DRG volume between NT-3 +/+ and −/− mice were due to differences in the total number of neurons rather than differences in amounts of nonneuronal tissue, the density of neurons and their precursors (all cells other than red blood cells) was determined in the section of each DRG with the maximal cross-sectional area, expressed as number of neurons per cubic micron, and compared between the mutant and wild-type littermates. In addition, the maximal cross-sectional area of the L1 DRs was obtained at a point just cranial to the L1 DRGs in some of the same pairs of mutant and wild-type mice. L1 DRs rather than L4 DRs were examined because the shorter L4 DR frequently run obliquely to the plane of the section, thus precluding reliable measurements of its cross-sectional area. Values for the right and left DRG and DR of a mouse were averaged, and the means of area, volume and density measurements were compared between NT-3 −/− and NT-3 +/+ mice for statistical significance by anova, as detailed above.

RESULTS

Nerve-muscle contacts

In the morning of day E13.5 the SOL muscle had not yet cleaved from the posterior premuscle mass and no SOL muscle nerve was visible. However, by the evening of the same day (E14.0) the SOL constituted a discrete muscle mass consisting of numerous mononucleated cells and clusters of recently assembled primary myotubes with scant myofilaments and patchy basal lamina. The muscle nerve had formed by E14.0 and gave off small intramuscular nerve bundles that were incompletely enveloped by perineurial cells. One or more constituent axons of the bundles occasionally abutted neighboring myotubes and presumed myoblasts in a peripheral bundle region devoid of ensheathing cells. The gap between the apposing axolemma and plasmalemma was narrow and devoid of basal lamina. However, no ultrastructural specializations of the axon or the underlying cell were visible that would permit recognition of these undifferentiated nerve-muscle appositions as either sensory or motor in nature. No ultrastructural differences between the SOL muscles of NT-3 +/+ and NT-3 −/− fetuses were evident at E14.0 except for a seemingly lower density of intramuscular innervation in the muscles devoid of NT-3.

By E14.5 the density of primary myotubes had increased, and individual myotubes contained more myofilaments. Occasional undifferentiated nerve-muscle appositions similar to those of E14.0 muscles were also observed at E14.5. They involved axons abutting myoblasts and myotubes in the peripheral regions of intramuscular nerve bundles devoid of ensheathing perineurial cells (Fig. 1A,B). Although not quantified, the density of muscle innervation, incidence of large axons and frequency of nerve-muscle contacts appeared higher in NT-3 +/+ than −/− SOL muscles. In one of the E14.5 NT-3 +/+ muscles a few relatively large axons arranged in a manner similar to that of the sensory nerve-muscle contacts of E15.5 muscles contacted myotubes (Fig. 2A).

At E15.5, the NT-3 +/+ SOL muscles contained identifiable contacts involving primary myotubes and clusters of sensory and motor axons disengaged from the intramuscular nerve bundles. The E15.5 sensory neuron-myotube contacts represented a stage of muscle spindle development preceding the assembly of the spindle capsule (Fig. 2B). These nascent spindles comprised scattered single myotubes (differentiating bag_2 intrafusal fibers) innervated by one or more axon terminals derived from relatively large axons that had separated from a neighboring nerve bundle. These terminals were closely apposed to the sarcolemma, and sometimes penetrated deeply into the fiber, as is characteristic of sensory axon terminals (Milburn, 1973). Multiple axon terminals of an ending frequently overlapped, and no basal lamina separated the terminals from the sarcolemma (Landon, 1972). The terminals contained numerous neurofilaments, but only a few dense-core or clear vesicles, and had a tendency to either encircle the myotube or to give rise to multiple terminals along most of the fiber perimeter. Definite motor nerve-muscle contacts were also observed in E15.5 NT-3 +/+ muscles. They were derived from axons smaller than those innervating nascent spindles, and occupied a more restricted area of the fiber perimeter as well as a shorter length of the myotube than sensory contacts. The axon terminals contained numerous

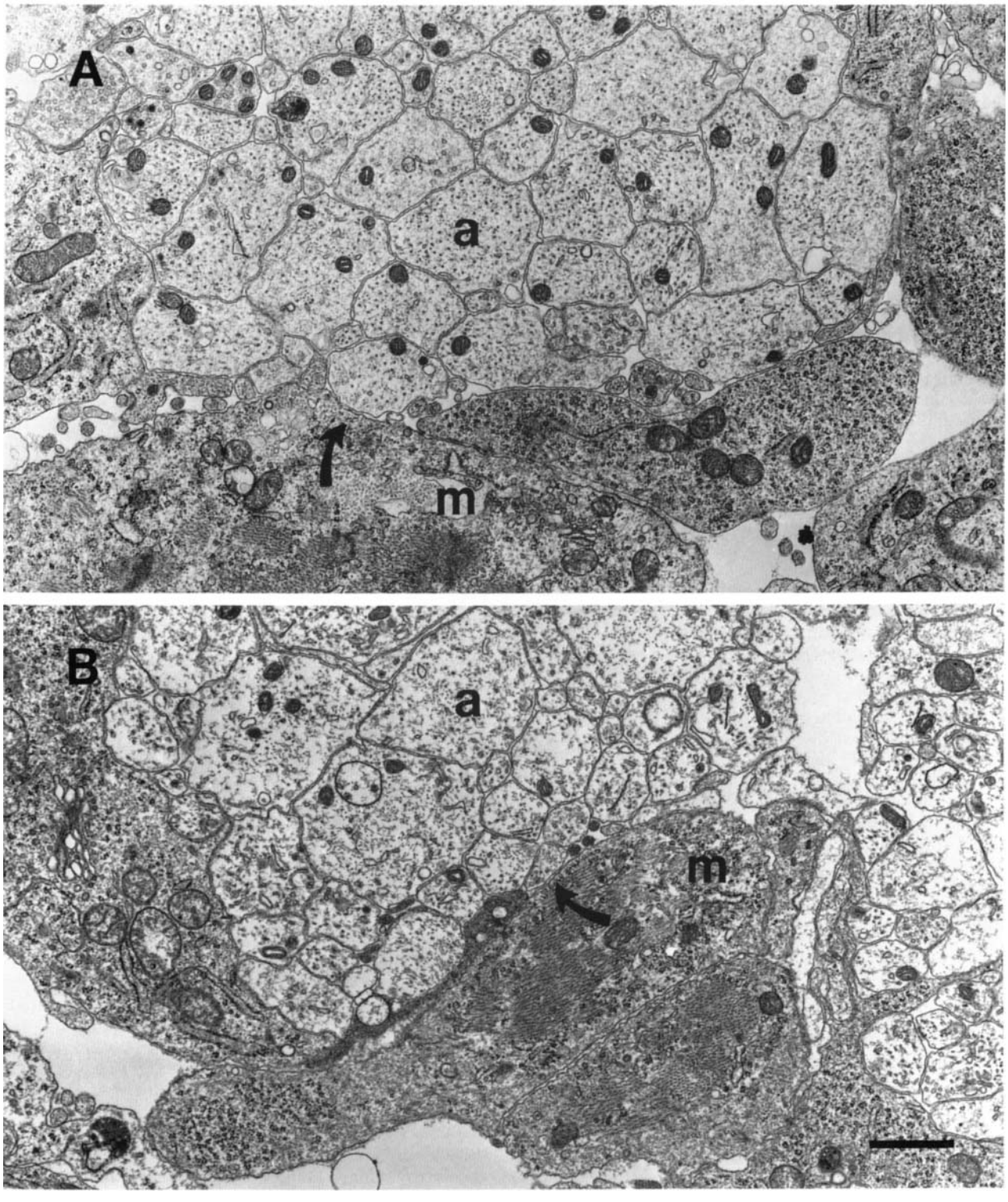

Fig. 1. Electron micrographs of undifferentiated nerve-muscle contacts in E14.5 NT-3 −/− **(A)** and NT-3 +/+ **(B)** soleus (SOL) muscles. The sites of apposition between primary myotubes (m) and axons (a) at the periphery of relatively large nerve bundles are marked with arrows. Note the similarity in structure of these primitive nerve-muscle contacts between mutant and wild-type muscles. Bar = 1 μm.

clear vesicles, and were separated from the underlying myotube by interposed basal lamina. The plasmalemma underlying the motor axon terminals was electron-dense. In striking contrast to the NT-3 +/+ muscles, only motor nerve-muscle contacts were observed in muscles of NT-3 −/− mutants at E15.5. Moreover, the motor endings of the NT-3 −/− muscles tended to have fewer axon terminals and a lower frequency of synaptic vesicles, as if the onset of

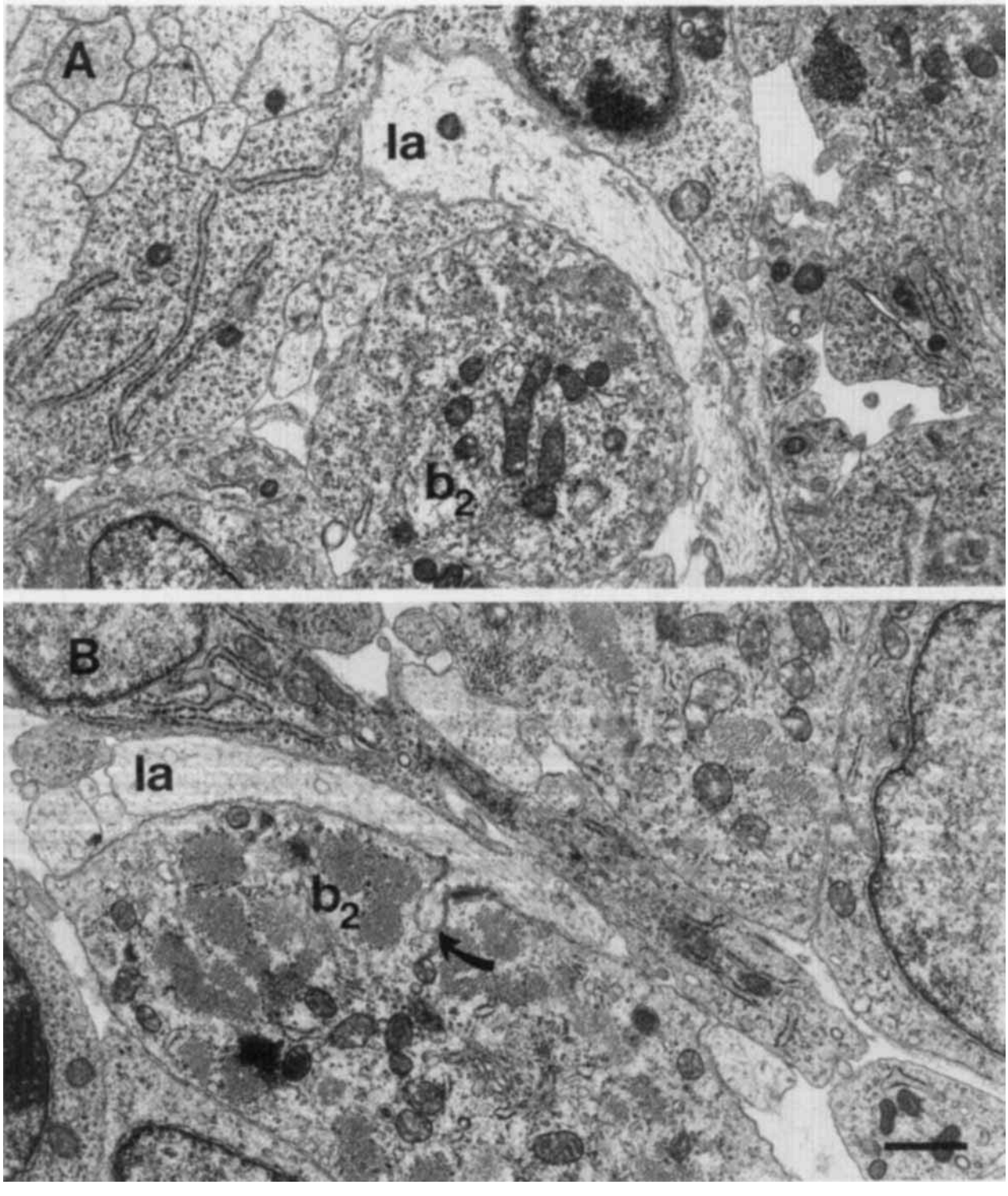

Fig. 2. Electron micrographs of Ia afferent-myotube contacts in E14.5 **(A)** and E15.5 **(B)** NT-3 +/+ SOL muscles. The contacted myotubes represent early nuclear bag_2 intrafusal fibers (b_2). Note that in B, the Ia afferent (Ia) invaginates into the myotube (arrow) and the basal lamina is absent from the synaptic cleft, two features characteristic of sensory endings of nascent muscle spindles. Bar = 1 μm.

development of motor endings was slightly delayed in NT-3 −/− mice relative to their NT-3 +/+ littermates.

SOL muscles of E16.5 NT-3 +/+ mice exhibited both sensory and motor endings, whereas only motor endplates were visible in muscles of mutants lacking NT-3 (Fig. 3A–C). The afferent nerve-muscle contacts of E16.5 NT-3 +/+ muscles were ultrastructurally similar to the sensory endings observed a day earlier, except that the density of

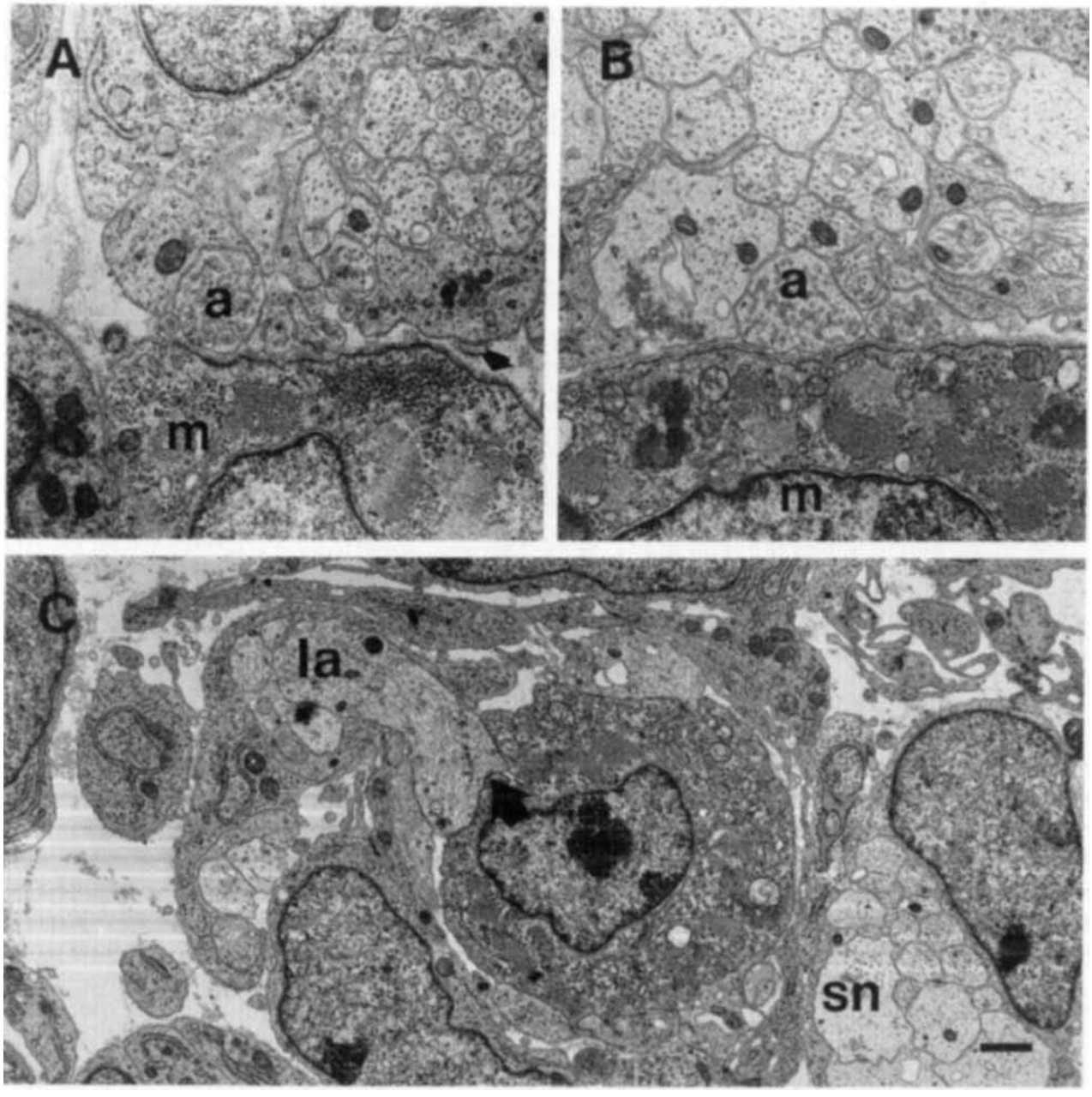

Fig. 3. Electron micrographs of motor **(A,B)** and sensory **(C)** nerve endings in E16.5 NT-3 −/− (A) and NT-3 +/+ (B,C) SOL muscles. The basal lamina (small arrow in A) is present in the synaptic cleft of the motor but not the sensory endings. The Ia afferent invaginates into the nuclear bag_2 myotube of the nascent spindle (large arrow) similar to Figure 2B, and an adjacent spindle nerve (sn) contains relatively large sensory axons (C). Note the similarity between the ultrastructural features of motor endings in mutant and wild-type muscles (A,B). Symbols as in Figures 1 and 2. Bar = 1 μm.

sensory axon terminals was higher and a developing spindle capsule partially enclosed the contacts. The nerve bundle innervating the developing spindles also contained small-diameter axons presumed to be motor axons (Fig. 3C), but no motor endings were observed on the bag_2 myotubes contacted by afferents in the muscle regions examined. Motor endings at various stages of development were observed on the extrafusal fibers in both NT-3 +/+ and −/− muscles. Motor endings of both wild-type and mutant muscles had similar ultrastructure at E16.5 (Fig. 3A,B).

Spindles never assembled in the SOL muscles lacking NT-3. No afferent nerve-muscle contacts and no spindles or their precursors were observed in E17.5 or E18.5 NT-3 −/− muscles even though numerous one-fiber and two-fiber encapsulated spindles were observed in all NT-3 +/+ muscles surveyed at E17.5-E18.5. No fetal mice older than E18.5 were examined. However, spindles are known to be absent at P0 or P14 in SOL muscles of NT-3 −/− mice (Ernfors et al., 1994; Fariñas et al., 1994; Kucera et al., 1995). Thus, afferent nerve-muscle contacts and muscle

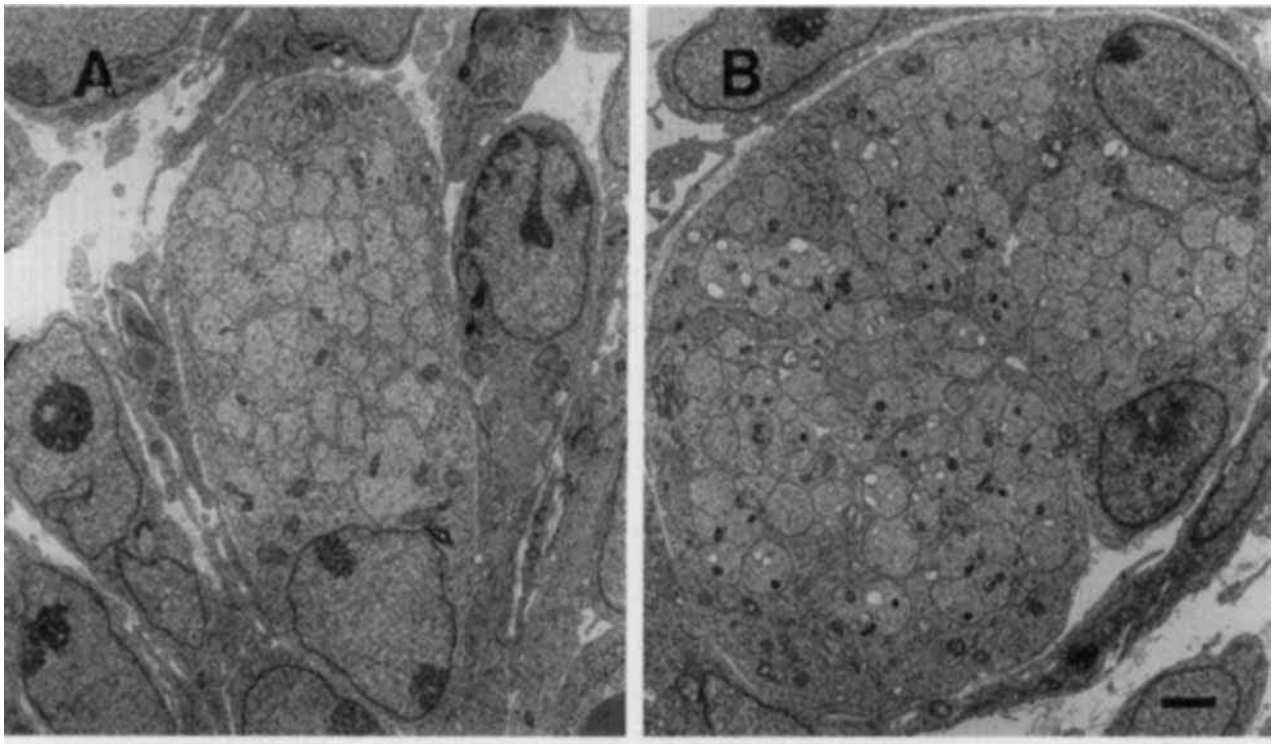

Fig. 4. Low-magnification electron micrographs of E14.5 NT-3 −/− **(A)** and NT-3 +/+ **(B)** SOL muscle nerves. Note that the muscle nerve contains fewer axons in the mutant (53) than in the wild-type mouse (126), consistent with a paucity of Ia afferents. Bar = 2 μm.

spindles probably never form in muscles of mice lacking NT-3.

Limb innervation

The absence of sensory nerve-muscle contacts and spindles in NT-3 −/− fetal SOL muscles could have resulted either from a failure of Ia afferents to project into the hindlimb, or from a failure of Ia afferents which enter the muscles to contact myotubes in the absence of NT-3. To address whether or not Ia afferents enter limb muscles, we compared the number of nerve fibers comprising the SOL muscle nerve between NT-3 −/− and NT-3 +/+ mice at daily intervals from E14.0 to E18.5. Mutant nerves were invariably smaller and contained 38% of the axons observed in nerves of wild-type mice (Fig. 4). The mean number of axonal processes (all unmyelinated) was 59 ± 15 (N = 22) in NT-3 −/− and 154 ± 31 (N = 19) in NT-3 +/+ mice in the pooled sample E14.0–E18.5 SOL nerves (the difference was statistically significant at $P \leq .01$). The deficiency in the number of axons entering the SOL muscle was present as early as E14.0, the earliest stage at which the SOL muscle and its nerve could be identified, and persisted throughout fetal development (Fig. 5). Moreover, the deficiency persisted into the postnatal period when most of the fibers of SOL muscle nerves became myelinated. The mean number of myelinated fibers in the SOL nerve was 22 ± 2 (N = 4) in NT-3 −/− mice and 82 ± 3 (N = 4) in NT-3 +/+ mice at P14 ($P \leq .01$). Although not quantified, unmyelinated axons also appeared to be less numerous in NT-3 −/− than +/+ muscle nerves at P14.

To address the possibility that innervation of the distal limb was deficient in the absence of NT-3 prior to the cleavage of the SOL muscle, we compared the size of the tibial nerve in mutant and wild-type mice at E13.5. The tibial nerve was thinner and contained fewer axons in mutant than wild-type mice. The cross-sectional area of the tibial nerve, as measured at the midcalf level, was 351 μm^2 (±36 SD, N = 3) in NT-3 −/− and 651 μm^2 (±75 SD, N = 5) in NT-3 +/+ mice ($P \leq .01$). Direct nerve fiber count in one of the three pairs of E13.5 NT-3 −/− and +/+ mice showed that the smaller cross-sectional area reflected a

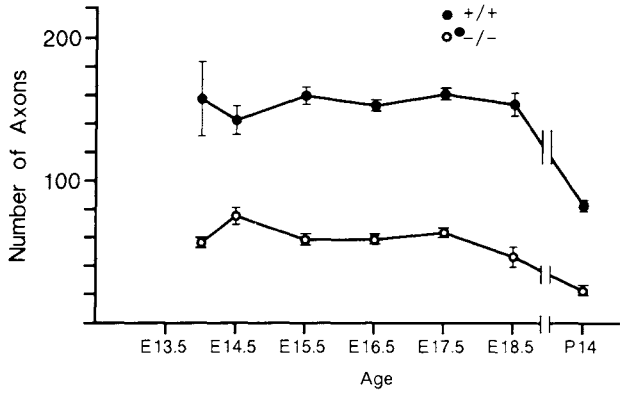

Fig. 5. Comparison of the number of nerve fibers in the SOL muscle nerve between 19 NT-3 +/+ and 22 NT-3 −/− mice from E14.0 to E18.5 and at P14. No SOL muscle nerve was present prior to E14.0. Note that the muscle nerves contain fewer nerve fibers in mutant than wild-type mice during both the fetal and the postnatal period. Means and standard errors are given.

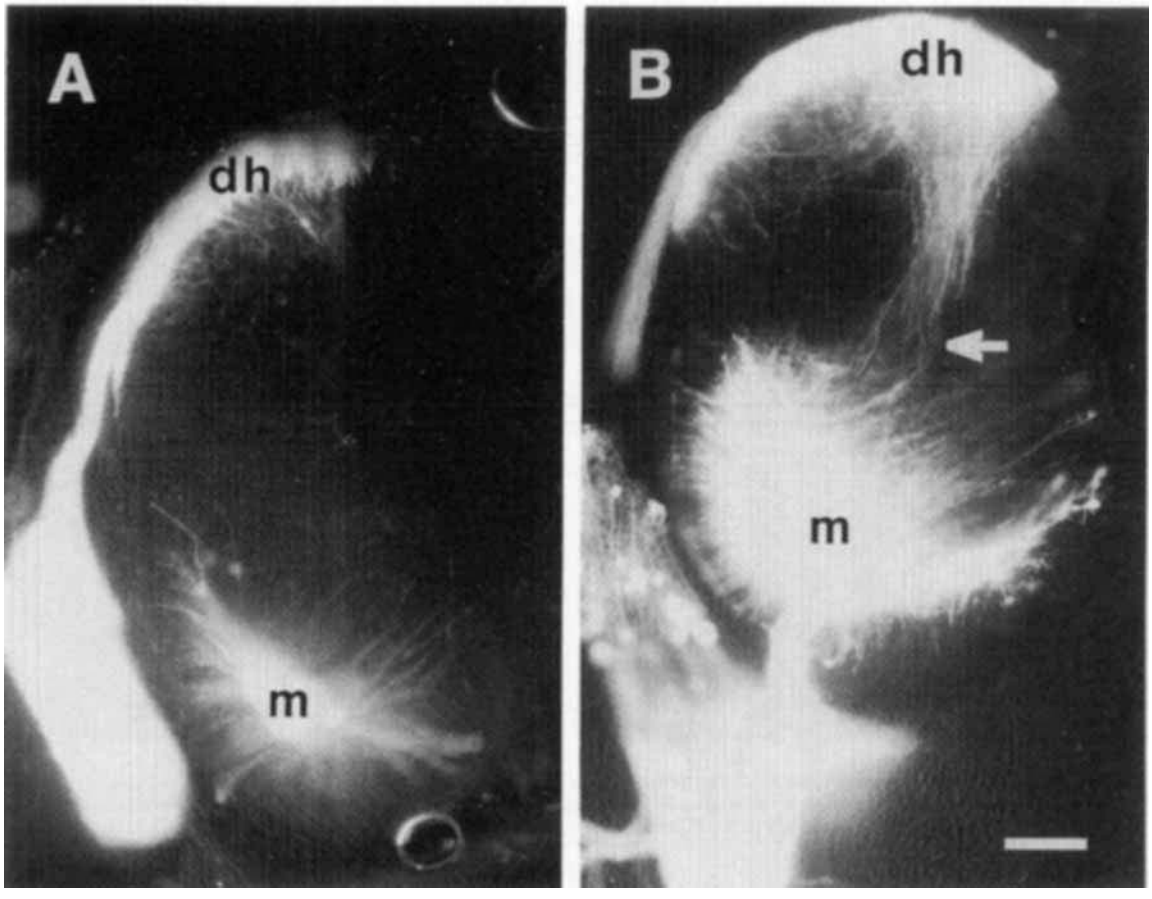

Fig. 6. Low-magnification photomicrographs of afferent projections of dorsal root ganglia (DRG) neurons to the spinal cord in E13.5 NT-3 −/− **(A)** and NT-3 +/+ **(B)** mice after injection of DiI into the hindlimbs. Note that the intensity of labeling of dorsal spinal horns (dh) is less in the mutant than wild-type mice, reflective of a decreased number of afferents entering the spinal cord in the absence of NT-3. Also note that DiI-labeled fascicles of Ia axons project into the ventral spinal motor neuron pool (m) in the wild-type (arrow) but not in the mutant mice, consistent with the absence of Ia afferents in mice lacking NT-3. Bar = 100 μm.

deficiency of nerve fibers in mutant relative to wild-type tibial nerves (data not shown). Thus, fewer nerve fibers coursed in the main nerve trunks of NT-3-deficient hindlimbs prior to the cleavage of individual calf muscles from the premuscle mass.

Cord innervation

To further characterize the extent of Ia afferent development in NT-3 −/− embryos, we compared the patterns of spinal projections of DRG neurons between NT-3 +/+ and NT-3 −/− mice. Muscle afferents were retrogradely labeled by introducing the tracer dye DiI into thigh musculature at E13.5 or E15.5. Labeled afferents originating in the hindlimb were observed to traverse the DRGs and enter the dorsal spinal roots in both groups of mice. In addition, labeled muscle afferents entered the spinal cord and projected ventrally into layer IX in both E13.5 and E15.5 wild-type mice, terminating in the region of retrogradely labeled motor neurons. Such afferent projections extending into the ventral spinal cord are considered to be diagnostic for Ia afferents, which are the only afferents forming monosynaptic connections with motor neurons in the spinal cord (Snider et al., 1992). In contrast, only few afferents entered the spinal cord in NT-3 −/− mice at E13.5, and they terminated in superficial layers I and II and a few in layers III and IV of the dorsal spinal cord, as is characteristic of nociceptive and mechanoceptive fibers (Snider et al., 1992). More labeled afferents entered the spinal cord at E15.5 than at E13.5 in NT-3 −/− mice, but none projected into the ventral layer IX of the spinal cord where motor neurons reside. The absence of intraspinal axons with the distribution pattern of Ia afferents was assumed to be independent of the absence of Ia afferents in the hindlimbs because similar results are obtained when DiI is injected directly into the DRG in newborn NT-3 −/− mice (Ernfors et al., 1994). Thus, the central projections of Ia afferents to spinal motor neurons were absent in mice lacking NT-3 at both E13.5 and E15.5 (Fig. 6).

Dorsal root ganglia

The absence of muscle spindles which require Ia afferents for assembly, coupled with the absence of Ia afferent projections in spinal cords, raised the possibility that Ia sensory neurons were absent in the NT-3 −/− mice. A paucity of Ia neurons would be expected to be reflected by smaller DRGs and smaller DRs in mutant relative to wild-type mice because the cell bodies of Ia neurons reside

in the DRGs and the central axons of Ia neurons course in the DRs on their way to spinal motor neurons.

The L4 DRGs were not yet assembled in one of two litters examined at E10.5. In embryos of the other E10.5 litter, nascent DRGs were represented by clusters of neuronal precursor cells (Fig. 7A,B). No DRs were visible and no sensory axons projected from the DRGs into the spinal cord or limb buds at E10.5, even though a few pioneering motor axons were observed to exit from the ventrolateral spinal cord at this stage. At E11.5 DRG afferents entered the spinal cord and peripheral projections of DRG neurons reached the base of the limb bud in both the mutant and wild-type mice (Fig. 8A,B). By E12.5 numerous afferents projected into the spinal cord and hindlimbs, and axons coursed from the DR entry zone to the ventrolateral cluster of motor nuclei in wild-type mice.

We compared the size of lumbar DRGs between mutant and wild-type mice at E10.5–E14.5, the period of development coinciding with or preceding neurite outgrowth (Fig. 7). The L1 and L4 DRGs were 20–64% smaller in the NT-3 −/− than NT-3 +/+ mice at each of the several fetal ages studied, including E10.5 (Fig. 7A,B). The mean volume of mutant L4 DRGs was $6.3 \pm 3.4 \times 10^6$ μm^3 (N = 20) compared to $14.4 \pm 9.4 \times 10^6$ μm^3 (N = 18) in wild-type mice in the pooled sample of all ages studied ($P \leq .01$). The deficiency in size of mutant relative to wild-type DRGs was greater at the L4 than at the L1 spinal level, and increased with increasing fetal age (Table 1). The smaller size of DRGs in mutant mice was taken to reflect a deficiency of sensory neurons because the density of neurons (number of neurons per μm^3 of DRG tissue) was comparable between the mutant and wild-type mice ($6.8 \pm 0.5 \times 10^{-3}$, N = 6 in NT-3 −/− and $6.5 \pm 0.6 \times 10^{-3}$, N = 6 in NT-3 +/+ DRGs of a random sample of E11.5–E14.5 mice, $P \leq 0.01$).

Similarly, lumbar DRs were thinner and dorsal spinal horns where DRs enter the spinal cord were smaller in mutant than wild-type mice at E11.5-E14.5, reflective of a deficiency of nerve fibers projecting from the DRGs into the spinal cord at the earliest stages of DR development (Fig. 7E,F). Owing to the small size and oblique course of most DRs, reliable determination of the cross-sectional area was feasible for the L1 DRs at E14.0 and E14.5 only. These DRs had a smaller cross-sectional area in mutant than wild-type mice ($2{,}310 \pm 464$ μm^3, N = 4 in NT-3 +/+ and $1{,}043 \pm 266$ μm^3, N = 4 in NT-3 −/−, $P \leq 0.01$). Thus, the deficiency in size of both the DRGs and DRs was consistent with a paucity of Ia neurons in NT-3 −/− mice at E10.5–E14.5, similar to the absence of Ia neurons in NT-3 −/− mice at birth (Ernfors et al., 1994; Fariñas et al., 1994).

DISCUSSION

Development of Ia afferent projections in the absence of NT-3

Muscle spindles, defined as encapsulated myotubes innervated by Ia afferents, were recognizable by electron microscopy in SOL muscles of wild-type mice at E16.5, consistent with that reported by Kozeka and Ontell (1981). Unencapsulated precursors of spindles were observed one day earlier, and a few afferent nerve-muscle contacts were encountered as early as E14.5. The early innervation of spindles is derived exclusively from Ia afferents (Zelená, 1957; Milburn, 1973), thus Ia neuron-myotube contacts are established by E15.5 in wild-type mice. However most, if not all, Ia afferents destined for the SOL may have already reached the muscle by E14.0 because the wild-type muscle nerve contained the same or higher number of axons at E14.0 than at subsequent stages of development.

No muscle spindles, precursors of spindles or identifiable afferent nerve-muscle contacts were observed in NT-3 −/− SOL muscles. The nonspecific appositions observed on days E14.0–E14.5 between myotubes and axons at the periphery of relatively large, but incompletely ensheathed, nerve bundles in NT-3 −/− muscles may have been random, or may have represented sites of future motor endplate formations. The failure of Ia afferents to contact myotubes and of spindles to form in muscles of mice lacking NT-3 is unlikely to result from an aberrant myogenic factor, such as the absence of myotubes capable of interacting with Ia afferents. The population of primary myotubes that are contacted by Ia afferents is bipotential in rodents and gives rise to both nuclear bag_2 intrafusal and type I extrafusal fibers (Kucera and Walro, 1995). Type I fibers are present in hindlimbs of newborn NT-3 −/− mice (Ernfors et al., 1994; Kucera et al., unpublished), indicating that the precursors of slow extrafusal fibers, which develop simultaneously with and are presently indistinguishable from the precursors of intrafusal fibers, are present in NT-3 −/− muscles. Rather, the absence of spindles in NT-3 −/− fetal muscles is both an indicator and a consequence of the failure of Ia afferents to innervate muscle. In rats, no spindles assemble in hindlimb muscles devoid of afferent innervation either by treatment with β-bungarotoxin or following transection of the sciatic nerve before birth (Zelená, 1957; Kucera and Walro, 1990). Moreover, supernumerary spindles assemble in rat neonatal muscles innervated by extra collaterals of Ia afferents (Sekiya et al., 1986; Kucera and Walro, 1992). Thus, factors associated with Ia afferents rather than those inherent in muscle cells are the principal force driving the formation of spindles, and in their absence no spindles form in NT-3 −/− mice.

The absence of spindles in fetal NT-3 −/− mice suggested that either no Ia afferents enter SOL muscles or Ia afferents innervate the muscles, but do not establish contacts with myotubes. Our observations favor the former possibility. Group Ia afferents are among the largest axons of developing nerve bundles (Milburn, 1973), and few large axons were present in the intramuscular nerve bundles of fetuses lacking NT-3. Moreover, fewer axons entered the SOL muscle in NT-3 −/− than in wild-type mice regardless of the stage of development, consistent with the hypothesis that Ia afferents never innervate hindlimb muscles of the mutant mice.

Nerve fibers other than Ia afferents may also fail to innervate hindlimb muscles in NT-3 −/− mice. The deficiency of axons in the SOL muscle nerve was greater than that which could be attributed solely to an absence of spindle primary afferents. The mouse SOL muscle contains 10–12 spindles (Ernfors et al., 1994) that are innervated by 10–12 Ia afferents, given the 1:1 ratio between spindles and Ia afferents in adults (Barker, 1974). However, approximately 100 fewer axons innervated fetal SOL muscles of the mutant than the wild-type mouse. Other sensory nerve fibers, such as Ib axons of Golgi tendon organs and group II afferents of spindles, may also be deficient in muscles developing without NT-3 (Ernfors et al., 1994). Similarly, fusimotor efferents destined to contact intrafusal fibers might not innervate developing NT-3 −/− muscles because they are absent in postnatal NT-3 −/− mice devoid of spindles (Kucera et al., 1995). The number of myelinated

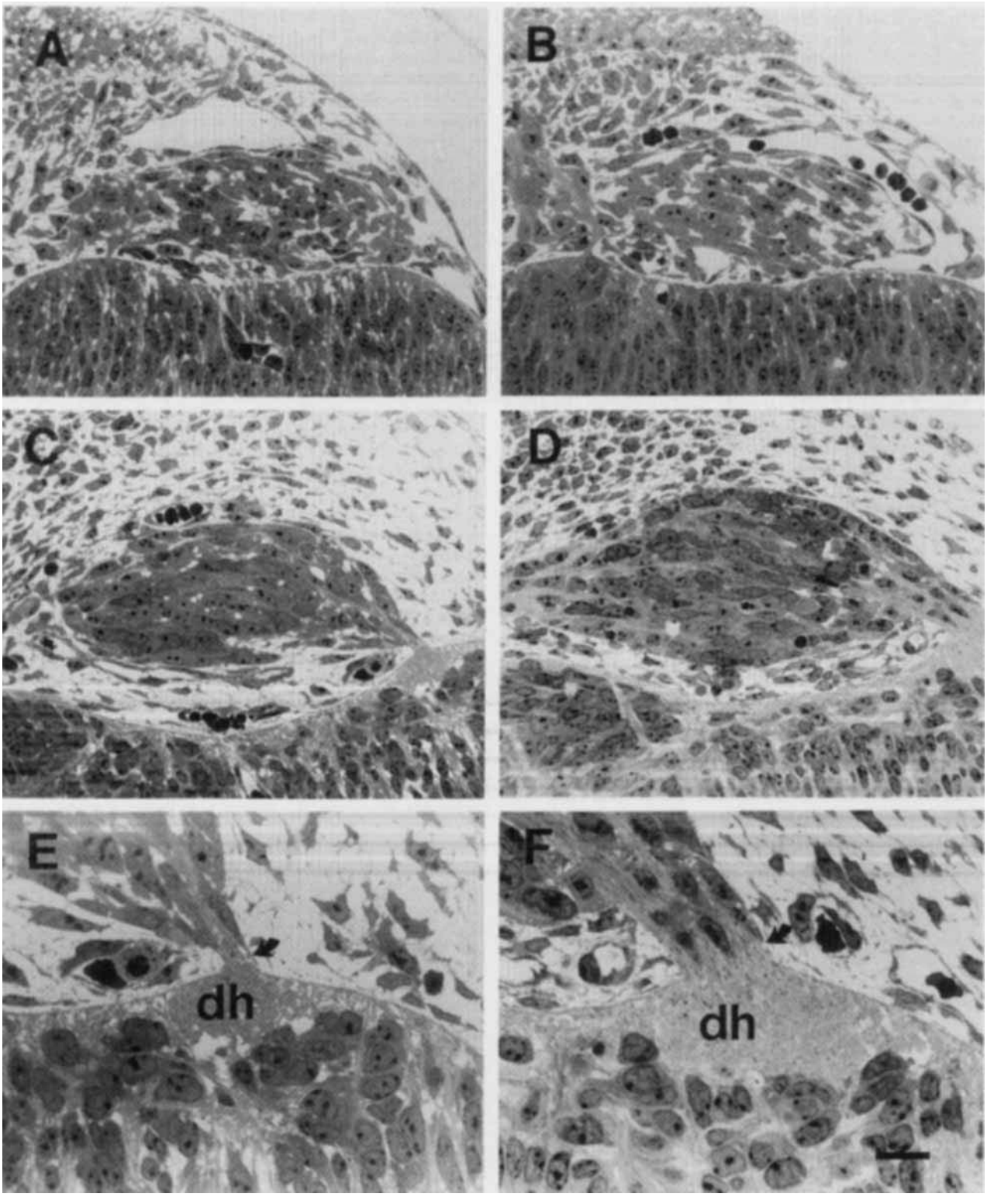

Fig. 7. Light micrographs of L4 DRGs from pairs of NT-3 −/− **(A,C,E)** and NT-3 +/+ **(B,D,F)** littermates at E10.5 (A,B) and E11.5 (C–F). E and F represent details of DRGs shown in C and D, respectively. Differences in cross-sectional area reflect differences in volumes of DRGs, which were 15% and 22% smaller in the mutant than the wild-type mouse for the DRGs shown at E10.5 and E11.5, respectively. Note that fewer afferents (arrows) enter the dorsal horn (dh) of the spinal cord in the mutant than the wild-type mouse at E11.5 (E,F). No afferents projected from DRGs to the spinal cord at E10.5 (A,B). Bar = 30 μm (A–D); 15 μm (E,F).

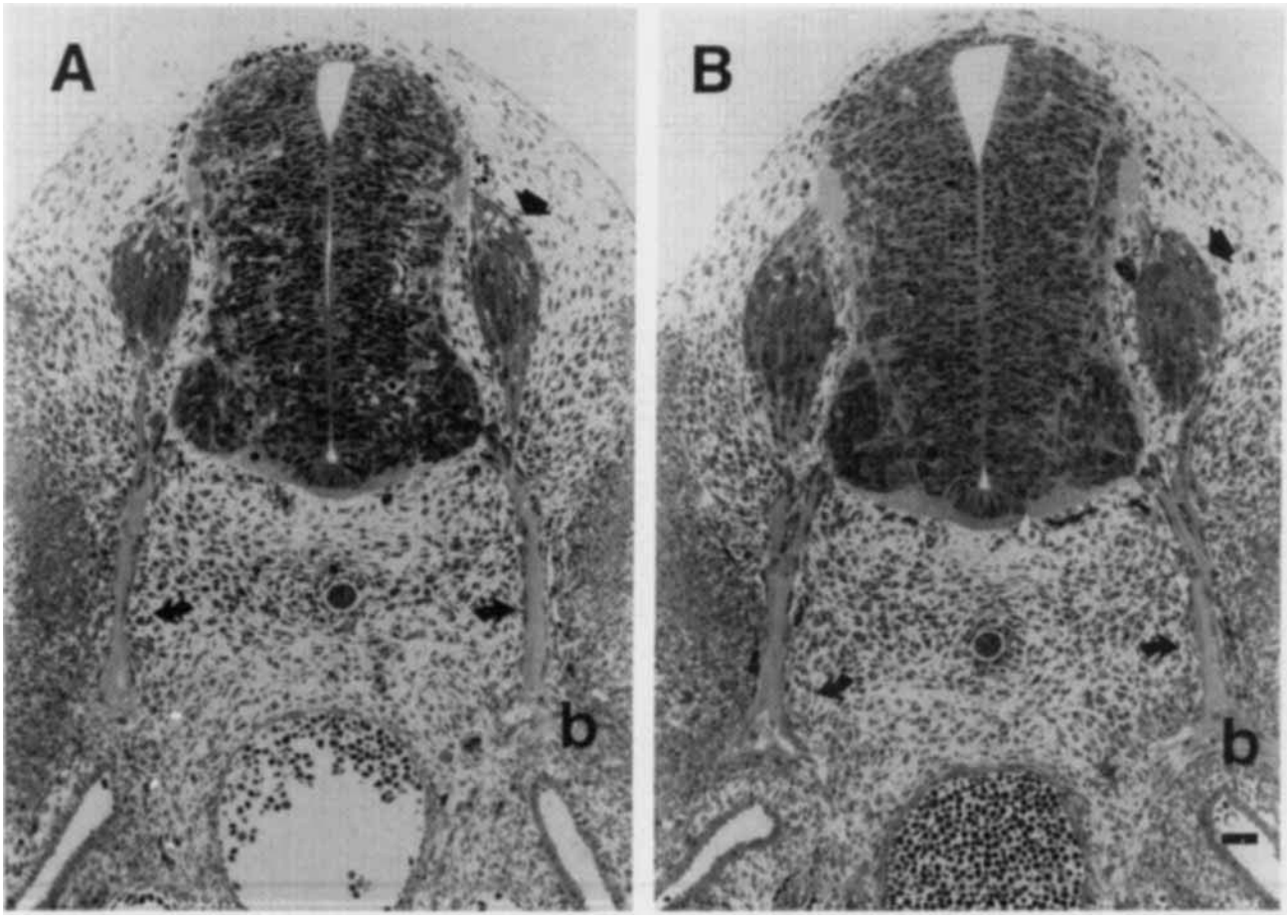

Fig. 8. Cross section of the spinal cord and adjacent L4 DRGs (arrowheads) in NT-3 −/− **(A)** and NT-3 +/+ **(B)** mice at E11.5. The central projections of DRG neurons enter the spinal cord at this stage. Note that the peripheral projections (arrows) of sensory neurons, together with motor axons, reach the base of the limb bud (b) in both mutant and wild-type mice. Bar = 20 μm.

TABLE 1. Comparison of the Volume of L1 and L4 DRGs in NT-3 −/− and NT-3 +/+ Mice at Several Embryonic Stages

	E10.5		E11.5		E12.5		E13.5		E14.0		E14.5	
	−/−	+/+	−/−	+/+	−/−	+/+	−/−	+/+	−/−	+/+	−/−	+/+
L^4 DRG volume ($\times 10^6$ μm^3)	1.7 ± 0.2 N = 4 89%	1.9 ± 0.1 N = 2 100%	2.3 ± 0.2 N = 2 72%	3.2 ± 0.0 N = 1 100%	6.0 ± 0.4 N = 2 68%	8.8 ± 1.6 N = 3 100%	8.8 ± 1.6 N = 7 55%	15.9 ± 2.3 N = 7 100%	8.4 ± 0.3 N = 2 36%	23.1 ± 0.8 N = 3 100%	8.3 ± 1.2 N = 3 37%	22.7 ± 0.9 N = 2 100%
L_1 DRG volume ($\times 10^6$ μm^3)							7.0 ± 1.9 N = 7 63%	11.1 ± 1.2 N = 7 100%	5.8 ± 0.9 N = 2 49%	11.8 ± 1.6 N = 3 100%	7.0 ± 0.9 N = 3 56%	12.5 ± 0.9 N = 2 100%

L1 dorsal root ganglia (DRG)s were studied only at the three oldest stages. Percentages shown indicate the relative DRG volumes in mutants compared to wild type mice in each age group. A single litter was studied at each age from E10.5 to E12.5. N stands for the number of mice studied, and mean values of the two L4 or L1 DRGs in each embryo were used to generate the group means and standard deviations. Note that DRGs are smaller in the mutant than wild type mice, and the extent of the size deficiency increases with increasing embryonic age.

axons in the SOL nerve at P14 (mean = 22) was similar to the number of extrafusal motor units (mean = 24), and hence to the number of skeletomotor fibers reported to be present in wild-type SOL muscle based on physiological and histological criteria (Parry et al., 1982; Habgood et al., 1984). The population of spinal skeletomotor neurons is preserved in NT-3 −/− mice (Kucera et al., 1995). Thus, nerve fibers other than the skeletomotor axons to extrafusal fibers are deficient in limb muscles of mutant mice.

The number of axons innervating developing muscles was greater than the number of myelinated axons in the SOL nerve at P14 in both mutant and wild-type mice. Some of the difference may be due to autonomic nerve fibers that remain unmyelinated even after birth, and hence were not counted at P14. In addition, the decreasing number of axons in the SOL nerve with increasing age might also reflect elimination of sensory or motor neurons due to programmed cell death in the late fetal and perinatal periods. This process involving NT-3-independent neurons is presumably preserved in the mutant mice.

The absence of Ia afferents in muscles was paralleled by the absence of Ia afferents in the spinal cord, as determined by retrograde labeling of afferents from limb musculature by DiI. Intraspinal Ia afferents were absent in NT-3 −/−

mice not only at E15.5 (Tessarollo et al., 1994) but also at E13.5. No Ia afferents were observed to innervate the ventral spinal motor neuron pools at either E13.5 or E15.5, whereas innervation of the spinal motor region by Ia neurons was established in wild-type mice by E13.5. Thus, Ia afferent innervation of motor neurons and myotubes, the two principal targets of Ia neurons, is absent in NT-3 −/− mice.

Interestingly, innervation of motor neurons by Ia afferents preceded the establishment of afferent-muscle contacts and spindle formation by approximately 1 day in wild-type mice (E13.5 vs. E14.5). A similar situation occurs in the rat where Ia afferents contact lumbar motor neurons at E16–E17 and SOL spindles form a day later at E17–E18 (Snider et al., 1992; Kucera and Walro, 1995). The difference may reflect the greater proximity of DRGs to the spinal cord relative to limb muscles rather than a difference in the rate of growth of two axonal projections of Ia neurons. Not all NT-3-dependent neurons of DRGs develop into Ia neurons (Ernfors et al., 1994). Thus, the asynchronous onset of innervation of motor neurons and myotubes by afferents might relate to the process whereby only some of the NT-3-dependent neurons become Ia neurons. Conceivably, only those sensory neurons that survive the normal period of sensory neuron death and succeed in contacting spinal motor neurons become capable of inducing the formation of muscle spindles, thereby acquiring the Ia phenotype and a capacity to mediate the monosynaptic stretch reflex.

Ia neuron development in the absence of NT-3

Group Ia neurons, defined as neurons capable of forming monosynaptic connections with both myofibers and motor neurons, may never develop in the absence of NT-3. In newborn NT-3 −/− mice, the absence of spindles in muscles, of intraspinal projections of Ia afferents to motor neurons, and of DRG neurons reactive to parvalbumin were assumed to reflect an absence of Ia neurons (Ernfors et al., 1994). The deficiency of DRG neurons and absence of peripheral as well as central Ia afferent projections at E10.5–E18.5 indicate that Ia neurons are also absent in fetal NT-3 −/− mice. The most parsimonious explanations for the observed abnormalities in the development of DRGs and afferent innervation in NT-3 −/− embryos are that the cell bodies of Ia neurons either do not differentiate from precursor cells during gangliogenesis, or differentiate, but do not survive long enough for Ia afferents to contact the target tissues in the absence of NT-3 −/−. The former seems likely because the size deficiency of mutant relative to wild-type DRGs was present as early as E10.5–E12.5, as if neurons were failing to be generated in the absence of NT-3. However, that at least some NT-3-receptive neurons may form but fail to survive is indicated by the observation that DRG neurons expressing *trkC* receptors, a neuronal population which presumably includes Ia neurons, are present in greater numbers at E11.5 than E13.5 in NT-3 −/− mice (Tessarollo et al., 1994).

The greater loss of neurons from L4 than L1 DRGs in NT-3 −/− mice also suggests that Ia neurons are absent in fetal mice lacking NT-3. The L4 DRGs exhibited a relatively greater decrease in size than L1 DRGs in mutants because Ia neurons represent a higher proportion of sensory neurons in the L4 than L1 DRG (Hory-Lee et al., 1993). More L4 than L1 DRG neurons also survive in cultures treated with NT-3, reflective of a higher proportion of Ia neurons in the L4 DRG (Hory-Lee et al., 1993). Spindle density is also higher in limb muscles innervated by the L4 DRG than in axial muscles innervated by the L1 DRG (Botterman et al., 1978).

More neurons were absent in DRGs of NT-3 −/− mice than could be accounted for solely by the absence of Ia neurons. Mutant DRGs were 63% smaller than wild-type DRGs at E14.5 and lack 53–78% of the normal complement of neurons at birth (Ernfors et al., 1994; Fariñas et al., 1994). In contrast, Ia neurons represent fewer than 14–18% of DRG neurons in adult wild-type rats, based on binding of the proprioceptive markers parvalbumin or calretinin in DRGs (Carr et al., 1989; Duc et al., 1994). Moreover, the neuronal deficiency in mutant DRGs was greater than the population of DRG neurons expressing the *trkC* receptor in adults, which has been estimated at 10–19% (Mu et al., 1993; Klein et al., 1994; Wright and Snider, 1995). Thus, absence of NT-3 in mutant mice may result in the elimination during gangliogenesis of neuronal subpopulations other than those that become Ia neurons or express *trkC* after birth. Mouse trigeminal ganglia contain neurons that require NT-3 prior to neurite outgrowth, but express *trkA* and depend on NGF after they innervate targets in the skin (Buchman and Davies, 1993; Buj-Bello et al., 1994). Lumbar DRGs may similarly contain subpopulations of neurons that depend on NT-3 for generation from precursor cells (Kalcheim et al., 1992) or for survival at an early stage, but switch neurotrophic dependence to another factor (or factors) when fully differentiated.

The hypothesis that Ia neurons require NT-3 for differentiation and/or survival prior to the stage of target innervation in rodents is consistent with data derived from birds. Avian neural crest cells express NT-3 mRNA as well as *trkC* mRNA from E2, and spinal sensory ganglia express the two mRNAs from E3, suggesting that NT-3 is available to sensory neurons and the neurons have a capacity to respond to it when DRGs begin to differentiate (Yao et al., 1994; Williams et al., 1995). That NT-3 may play a critical role during avian gangliogenesis is shown by the 30% reduction in the number of neurons in the lumbar DRGs of quail embryos treated with an NT-3 antibody from E3 to E6 (Gaese et al., 1994). Moreover, these early-differentiating NT-3-dependent DRG neurons (presumably including Ia neurons) are apparently eliminated by the anti-NT-3 treatment prior to projecting into muscles or the ventral spinal cord (Gaese et al., 1994). Similarly, no central or peripheral axonal projections of Ia neurons develop in chick embryos treated with an anti NT-3 Ab during E3-E12 (Eide et al., 1994; Oakley et al., 1994). Thus, NT-3-dependent proprioceptive neurons in both birds and mammals may require NT-3 for differentiation and/or survival prior to contacting target cells in muscles or the spinal cord.

Sources of NT-3 support

Ia neurons are known to be dependent on NT-3, and NT-3 mRNA is present in the dorsal root ganglia, spinal cord, and limb buds at the time when Ia neurons differentiate and grow neurites (Schecterson and Bothwell, 1992; Scarisbrick et al., 1994). Thus, all three sites could be the sources of NT-3 necessary to sustain Ia neurons. The present study, taken in conjunction with data from the literature, is consistent with the existence of a sequential dependence of Ia neurons on DRG-derived, limb-derived, and spindle-derived NT-3 (Fig. 9).

The paucity of DRG neurons as early as E10.5-E11.5 in NT-3 −/− mice supports the hypothesis that NT-3 present in the DRG milieu or inherent to Ia neurons themselves or their precursors (Ernfors et al., 1992) is the principal

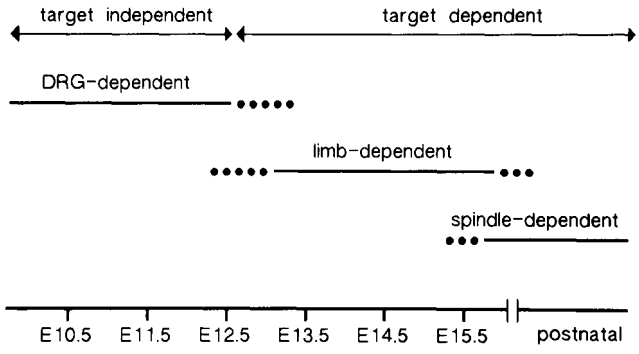

Fig. 9. Changing dependence of Ia neurons on neurotrophin-3 during development. Ia neurons project to spinal motor neurons and skeletal muscles. The neurons are dependent on NT-3 intrinsic to the DRGs prior to axon outgrowth into the target innervation fields. As the axon reaches its target, the Ia neurons become dependent on NT-3 generated by limb buds and subsequently by muscle spindles. The extent of NT-3 dependence of postnatal Ia neurons is uncertain. Age is shown at bottom.

source of the neurotrophic support required by potential Ia neurons prior to formation of their projections. This hypothesis assumes that Ia afferents do not contact targets in limbs or spinal cord prior to E11.5. Thus, paracrine or autocrine sources of NT-3 probably support Ia neurons at the earliest stages of their existence, similar to the in vitro effect of NT-3 on neurite outgrowth and survival of NT-3-dependent sensory neurons (Hory-Lee et al., 1993; Tessarollo et al., 1994). Indeed, NT-3 does not enhance the survival of DRG neurons obtained from E12.5 or E13.5 NT-3 −/− mice, suggesting that NT-3-responsive neurons (presumably including Ia neurons) are already absent by this time (Tessarollo et al., 1994). Whether or not Ia neurons can derive any NT-3 support from DRGs past the initial stages of their existence is not known.

Cord-derived NT-3 could potentially support Ia neurons after Ia afferents innervate the motor neurons. Relatively high amounts of NT-3 mRNA are expressed in association with spinal motor neurons when Ia afferents normally innervate the ventral spinal cord in the mouse (Ernfors and Persson, 1991; Schecterson and Bothwell, 1992). However, cord-derived NT-3 may not be important for the survival of Ia neurons because injection of an NT-3-specific antiserum into the spinal cord to deplete NT-3 has no demonstrable effect on Ia afferents in chicken embryos (Oakley et al., 1995). Moreover, cord-derived NT-3 cannot be essential for the sustenance of Ia neurons at birth because muscle spindles and hence Ia neurons survive neonatal extirpation of the lumbosacral spinal cord and dorsal root transection (Zelená and Soukup, 1973; Zelená, 1994).

Limb-derived NT-3 may support Ia neurons in the second phase of their dependence on NT-3. NT-3 mRNA has been detected in limb muscles prior to the stage when spindles begin to form, and prior to the assembly of myotubes from myoblasts (Schecterson and Bothwell, 1992; Scarisbrick et al., 1994). Spindle afferents may require limb-derived NT-3 to survive the normal period of sensory neurons death because limb injections of an anti-NT-3 antibody reduce the projections of muscle spindle afferents into the spinal cord (Oakley et al., 1995). In addition, NT-3 present in limb buds might provide a chemotactic signal mediating the process of matching Ia afferents with myogenic precursors of intrafusal fibers. That only some primary myotubes can transform into the intrafusal fibers has been suggested by studies of myosin heavy chain expression in developing rat muscles (Pedrosa and Thornell, 1990; Kucera and Walro, 1995).

Spindle-derived NT-3 may support Ia neurons in the third and final phase of their dependence on NT-3. NT-3 mRNA has been detected in the equatorial region of intrafusal fibers, where the peripheral Ia afferents terminate, in late fetal and postnatal rat spindles (Copray and Brouwer, 1994). We have observed immunoreactivity to an anti-NT-3 Ab in the corresponding region of neonatal spindles in the mouse (Kucera et al., unpublished). Moreover, the gradual buildup of NT-3 mRNA in the sensory region of developing spindles parallels the restriction of limb NT-3 mRNA to spindles (Copray and Brouwer, 1994). Conceivably, maturing Ia neurons become less dependent on DRG- or limb-derived NT-3 and more dependent on spindle-derived NT-3 as the full complement of intrafusal fibers innervated by Ia afferents assembles in the perinatal period, and the intrafusal fibers begin to generate NT-3 (Kozeka and Ontell, 1981; Copray and Brouwer, 1994). Moreover, many Ia neurons die after being disconnected from target muscles by sciatic nerve section in newborn rats (Miyata et al., 1986), perhaps due to the loss of the spindle-derived NT-3 support. Established Ia neurons may continue to depend on spindle-derived NT-3 for maintenance of their structural and functional integrity throughout adulthood. Indeed, muscle afferents can transport NT-3 retrogradely from muscles to the DRGs in adult rats (DiStefano et al., 1992).

Neuronal competition for target-derived neurotrophins is considered to play a role in regulating the naturally occurring cell death of developing neurons. Limb-derived rather than cord-derived NT-3 may play this role (Oakley et al., 1995). However, spindle-derived NT-3 is unlikely to regulate the numbers of Ia neurons. The period of cell death in chicken DRGs precedes the stage of spindle formation (Hamburger et al., 1981). Moreover, the numbers of spindles and hence Ia neurons are stable in late fetal rats when NT-3 mRNA begins to be expressed in spindles (Kucera et al., 1989; Copray and Brouwer, 1994).

Conclusions

The absence of NT-3 precludes the development of Ia neurons and growth of Ia afferent projections into the spinal cord and hindlimbs of NT-3 −/− embryos, resulting in the absence of spindles in skeletal muscles. Thus, Ia neurons either do not differentiate from precursor cells, or less likely differentiate but do not survive long enough to innervate the central or peripheral targets. The source of NT-3 required by Ia neurons and the ways in which the factor is presented to the neurons may change in the course of development, reflecting successive stages of differentiation of Ia neurons as well as changing levels of NT-3 in relevant embryonic tissues.

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