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Neurotrophin-3 and trkC in muscle are non-essential for the development of mouse muscle spindles

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NEUROTROPHIN -3 (NT3) or TrkC null mutant mice were examined for the presence of muscle spindles. Muscles of mastication, but not limbs, contained spindles in newborn and adolescent mutants. The intramuscular distribution and morphological properties of spindles in mutant masticatory muscles were indistinguishable from those of wild-type spindles. Intrafusal fibers of NT3- or trkC-deficient spindles expressed the slow-tonic isoform of myosin heavy chains, characteristic of wild-type spindles. Sensory nerve endings were observed in spindles of mutants by electron microscopy. Thus, NT3 or trkC, which is expressed in wild-type spindles, may serve functions other than those related to spindle assembly. Presumably, proprioceptive neurons innervating jaw muscles are dependent on factors other than NT3 for survival and maintenance *NeuroReport* 9: 905–909 © 1998 Rapid Science Ltd.

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Introduction

Muscle spindles, the principal sense organs of proprioception, are absent in limbs of mutant mice carrying a deletion in the gene for neurotrophin-3 (NT3).[1–3] Proprioceptive deficits are also present in mice lacking trkC, the high affinity receptor for NT3.[4] No spindles are present in hindlimbs of NT3 null (–/–) mutants at any stage of embryonic development.[5] Thus, the absence of spindles in mice lacking NT-3 is due to the failure of spindles to develop rather than degeneration of existing spindles.

In addition to the absence of muscle spindles in limbs, NT3 –/– mutants also exhibit a 60% reduction in the number of neurons residing in dorsal root ganglia (DRGs), which include the group Ia and II proprioceptive neurons that innervate spindles.[1–6] The failure of proprioceptive neurons to survive and afferents to innervate muscles was considered to be the cause of the absence of spindles in NT3 –/– mutants[1,5] because spindles do not develop in muscles devoid of innervation.[7,8] However, intramuscular NT3/trkC functioning can not be excluded as a factor essential for the assembly of spindles in embryonic muscles. Developing spindles in limbs express both NT3 and trkC,[9,10] and intramuscular sites of NT3 expression might serve as foci for spindle formation. Spindles develop at sites of contacts between group Ia proprioceptive afferents and myotubes.[7] The myotubes that develop into intrafusal fibers were hypothesized to differ from myotubes which form extrafusal fibers.[11] Nuclear bag fibers, the first intrafusal fibers to form, express NT3 whereas extrafusal fibers do not.[9] Thus, Ia afferents might interact only with myotubes expressing NT3. If so, Ia afferents would not be expected to contact myotubes and induce spindle formation in muscles that do not express NT-3.

We now report that spindles are present in jaw muscles of NT3, as well as trkC, null mutant mice. Thus, NT3 and trkC affect the assembly of muscle spindles only indirectly, presumably by promoting the survival and differentiation of proprioceptive neurons that innervate limbs.

Material and Methods

Animals: NT3 and trkC null mutants were analysed in parallel because the extent of sensory neuron loss differs between the two mutants.[1,4,12] Mice carrying the mutated NT3 allele were generated in the balb/c 129 genetic strain background whereas mice carrying the mutated trkC allele were generated in the 129/C57bl/6 background, as described previously.[1,12] All the isoforms encoded by the trkC locus were disrupted in the trkC –/– mutant line of mice.[12] Matings of heterozygous (+/–) siblings gave rise to homozygous mutant mice with the expected frequency of 25%. Wild-type (+/+) mice from the same litters served as controls. No difference was observed in spindle features between the two strains of wild-type mice. Polymerase chain reaction (PCR) was used to genotype individuals of both strains, as described elsewhere.[1,12]

Histology: Semi-serial sections of heads and hindlimbs were surveyed for the presence of spindles. Mutant and wild type littermates were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused with fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer. Heads and hindlimbs were excised, postfixed in OsO_4 and embedded in Eponate 12. Plastic-embedded heads were serially cross-sectioned in the coronal plane at 1 μm, beginning at the anterior boundary of the eye and ending at the posterior angle of the mandibula. Hindlimbs were cross-sectioned at 1 μm from the ankle to the knee. Every 20th section was collected, and stained with toluidine blue. Spindles in muscles were identified as encapsulated bundles of small muscle fibers that exhibited accumulations of central myonuclei at their midportion.[7]

Immunocytochemistry: Immunocytochemistry was performed to determine whether the three types of intrafusal fiber (bag_2, bag_1, and chain) normally present in spindles of rodents were present in muscles of mutant mice. Heads and hindlimbs of mutant and wild-type littermates were excised and fresh frozen in isopentane cooled to –160°C with liquid nitrogen. Tissues were cut transversely into serial 8 μm sections in a cryostat. Sets of serial sections were reacted for 75 min at 37°C with three monoclonal antibodies (Abs) known to bind different isoforms of myosin heavy chains (MHC).[13] The Abs included the S46 Ab reactive to the spindle specific slow-tonic MHC isoform, MY32 Ab reactive to neonatal/fast MHC and WBMHC reactive to the slow-twitch MHC isoform. Binding of the primary Abs was detected using the ABC (avidin-biotin complex) peroxidase method.[13]

Electron microscopy: Electron microscopy was performed to determine whether spindles in mutant muscles were innervated by sensory and motor neurons. Plastic embedded transverse sections of the masseter muscle from two NT3 –/– or trkC –/– newborn mutants each and one 14 day old (P14) NT-3 –/– mouse were cut on an ultramicrotome at either 1 μm or 90 nm. The 1 μm sections were stained with 1% toluidine blue and used to locate spindles in the muscle. The 90 nm sections were stained with lead citrate and uranyl acetate, and used to examine nerve endings in the spindles by electron microscope at magnifications ranging from ×2000 to ×10 000.

Results

Heads of five NT3 –/–, five trkC –/– and six wild-type (three of each strain) newborn (12–24 h after birth) mice were cut in the coronal plane and surveyed for the presence of muscle spindles in plastic-embedded sections stained with toluidine blue. Spindles were observed in several jaw muscles of both the mutant and wild-type newborns (Figs 1, 2). In contrast, the hindlimbs of NT-3 or trkC null mutants did not contained muscle spindles, consistent with the reported proprioceptive deficits.[1,4,12] One NT-3 –/– mouse was examined at P14. It contained spindles in the jaw but not limb musculature. Thus, the absence of spindles in NT3 –/– limbs at P0–P1 did not result from a delay in spindle development in limb relative to head muscles in the absence of NT3. Most of the jaw spindles in the P14 NT3 null mutant contained four muscle fibers, indicating that the full complement of mouse intrafusal fibers (two bag and two chain fibers)[14] can develop in NT3-deficient spindles.

Three jaw muscles were selected for a comparative study of spindle distribution and features between the mutant and wild-type newborns: the anterior deep masseter, zygomaticomandibularis, and medial ptelygoideus. No difference in the appearance, distribution or immunocytochemical properties was observed among spindles of the three groups of mice upon examination in serial transverse sections. However, the density of spindles appeared to be less in NT-3 and trkC null mutant than the corresponding wild-type muscles.

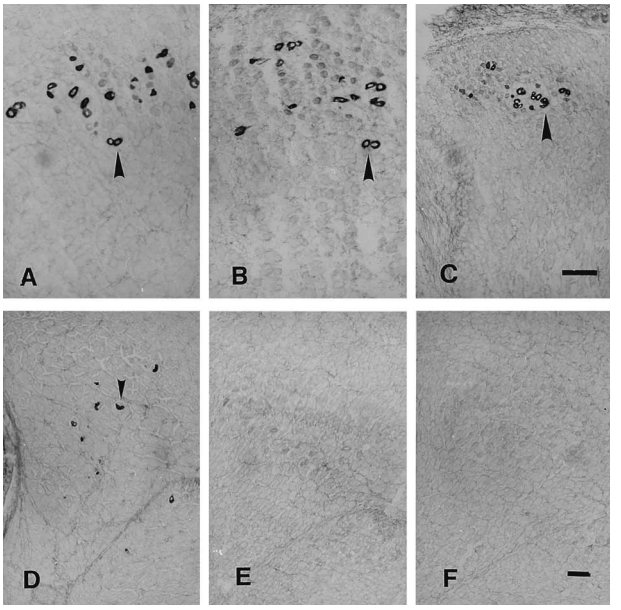

FIG. 1. Transverse sections of the deep anterior masseter muscle (**A**–**C**) and tibialis anterior muscle (**D**– **F**) of wild-type (A,D), trkC –/– (B,E) and NT3 –/– (C,F) mice taken at P0–P1. Immunoreacted with the spindle-specific Ab S46. Note the presence of spindles (arrowheads) in the masseter (B,C) but not tibialis anterior muscle (E,F) of mutant mice. Both jaw and limb muscles (A,D) contained spindles in wild-type mice. Bars = 50 μm.

Spindles frequently occurred in clusters, and exhibited a restricted distribution in the three jaw muscles of both mutant and wild-type mice examined at PO-P1 (Fig. 1). Spindle clusters were present only in muscle regions that contained slow-twitch extrafusal fibers. In addition, the three types of intrafusal fiber normally present in rodent limb spindles were present in the jaw muscles of both the mutant and wild-type mice. Spindles contained one or two fibers expressing the spindle-specific, slow-tonic MHC isoform, as identified by reactivity to the S46 Ab. The S46-reactive fibers of jaw muscles were assumed to correspond to the bag_1 and bag_2 intrafusal fiber types of limb spindles.[13] In addition, some of the mutant and wild-type jaw spindles contained one intrafusal fibers unreactive to S46 Ab, but reactive to an Ab specific for the neonatal/fast MHC. These fibers were assumed to correspond to the chain fibers of limb muscles.[13] The second chain fiber of jaw spindles presumably assembles postnatally, similar to limb spindles.[13,14] No myofibers bound the anti-slow tonic Ab in limb muscles of NT3 or trkC null mutants (Fig. 1).

To determine whether afferents innervated spindles in mutant mice, spindles of the masseter muscle were examined by electron microscopy in NT3 –/– or trkC –/– newborns and the P14 NT3 –/– mouse (Fig.2). The equatorial region of NT3- or trkC-deficient spindles contained sensory nerve endings, recognized by the absence of interposed basal lamina

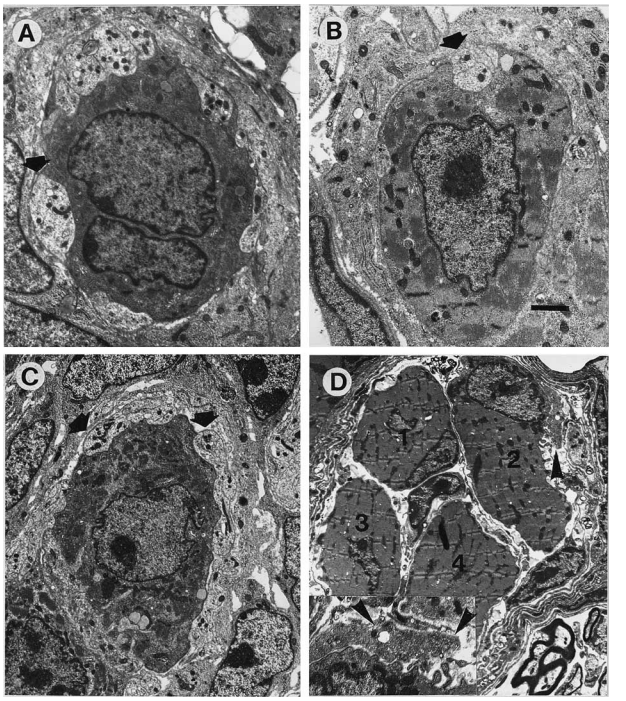

FIG. 2. Electron micrographs of spindles in the masseter muscle of wild-type (**A**), trkC –/– (**B**) and NT3 –/– (**C**,**D**) mice taken at P0–P1 (A–C) and P14 (D). Intrafusal fibers of the P0–P1 spindles are sectioned through the equatorial region and display sensory nerve endings (thick arrows). Intrafusal fibers of the P14 spindle are sectioned through the polar region and fiber 2 (upper right, D) displays a motor nerve ending (arrowhead), which is detailed in the insert (lower left, D). Sensory endings in (A) and (B) are characteristically embedded into the fibers whereas the motor ending in (D) lies superficially. Also note that the P14 NT3 –/– spindle contains the full complement of four intrafusal fibers (numbered 1–4) and is innervated by large caliber (sensory) and small caliber (motor) nerve fibers (lower right, D). Bar in B = 1 μm (A–C) and 4 μm (D).

in the synaptic cleft between the axon terminal and plasmalemma of the underlaying muscle fiber.[14] In addition, motor endings were encountered in the more polar regions of P14 NT3 –/– spindles. They were recognized by the presence of basal lamina in the cleft separating the axon terminal and myofiber.[14] Both large-caliber and small-caliber nerve fibers innervated P14 NT3 –/– intrafusal fibers (Fig. 2D), consistent with the presence of sensory and motor innervation in NT3-deficient spindles.

Discussion

Masticatory muscles of mutant mice lacking NT3 or trkC genes contained spindles that were morphologically and immunocytochemically indistinguishable from spindles of wild-type mice. Thus, expression of NT-3 or trkC in muscle is not essential for the development and maintenance of spindles. Rather, the principal factor that correlates with the presence or absence of spindles is the presence of intramuscular afferents. Limb muscles are devoid of afferents and lack spindles in NT-3 or trkC null mutants.[1,2,5] In

contrast, jaw muscles in both transgenic lines contained intramuscular afferents as well as spindles, despite the absence of NT3 or trkC.

Spindles of NT3-deficient jaw muscles contained motor nerve endings, in contrast to the absence of fusimotor innervation in NT3-deficient limb muscles.[15] Thus, the presence of fusimotor innervation in muscle may correlate with the presence of afferents or spindles, rather than intramuscular NT3. The survival of motor neurons innervating jaw spindles may depend on an afferent- or spindle-associated factor other than NT3.

Myotubes which differentiate into intrafusal fibers were postulated to be intrinsically different from extrafusal myotubes.[11] Spindles form only at sites of contacts between proprioceptive afferents and a small fraction of the population of myotubes available for innervation.[7] Thus some myotubes have been considered to carry a marker which serves as a chemoattractant or termination signal to incoming afferents.[11,13] Nuclear bag_2 intrafusal fibers, the first intrafusal fibers to form, are the only myofibers to express NT3 in developing rats.[9] As muscle afferents are known to be dependent for survival on NT3 retrogradely transported from muscle,[1,16] NT3 released from NT3-expressing myotubes might be the signal for sites of Ia afferent termination and spindle formation. Our data refute this possibility for jaw muscles. Moreover, our study suggests that muscle-generated NT3 is not essential for the maintenance of spindle structural integrity. Spindles in mutant jaw muscles appeared to be at the same stage of development as wild-type spindles, and spindles of the P14 NT3 –/– mouse had the same number of intrafusal fibers as wild-type spindles of the same age. However, the functional properties of spindles or central synapses of Ia afferents in NT-3- or trkC-deficient mice were not examined. NT3 is known to modulate the amplitude of synaptic potentials evoked in limb motor neurons by spindle afferent fibers.[17]

The clustering of spindles to regions where slow (type 1) fibers are located was a striking feature of jaw muscles in wild type mice. This spindle distribution was retained in both NT-3 and trkC null mice. Selective overexpression of NT3 in muscles results in the formation of clusters of supernumerary spindles in limb muscles,[18] as does exogenous administration of nerve growth factor (NGF) after nerve crush in neonatal rats.[19] In both experimental situations, excessive branching of intramuscular afferents due to excess of either NT3 or NGF has been proposed to be the factor partially or wholly responsible for the clustering of spindles. However, the nonuniform intramuscular distribution of spindles in jaw muscles is independent of NT-3, thus another factor is probably responsible for this distribution.

The ability of intrafusal fibers to express spindle-specific MHC isoforms is a function of afferent innervation. Removal of afferent, but not efferent, innervation results in the loss of slow-tonic MHC expression in newborn rats.[20] Similarly, the absence of slow tonic MHC expression in limb muscles of NT-3 –/– or trkC –/– mice reflects the lack of myofibers innervated by afferents. Our study shows that the ability to induce and maintain spindle-specific MHC isoforms in intrafusal fibers is not limited to NT-3-dependent limb afferents. Rather, this ability must be intrinsic to the proprioceptive neuronal phenotype, regardless of a neurotrophin supporting the neuron.

Presumably, trophic factors other than NT-3 can support proprioceptive neurons of the mesencephalic nucleus of the trigeminal nerve that innervate spindles of jaw muscles in NT-3 and trkC null mutants.[21,22] Indeed, about 50% of cells in the mesencephalic trigeminal neurons, presumably including proprioceptive neurons, survive in NT3 null mutants.[1] The cell bodies of NT-3-dependent limb afferents are located in the relatively neurotrophin-poor environment of DRGs whereas the cell bodies of NT-3-independent jaw afferents are located in the neurotrophin-rich environment of the brain stem. It would be of interest to determine whether proprioceptive neurons of the brain stem can switch their neurotrophin dependence during embryonic development according to neurotrophin availability.

Conclusion

Our study indicates that expression of NT3 or trkC in muscle is not essential for the development of muscle spindles. Thus, the NT3 or trkC that is expressed in limb spindles[9,10] may serve functions other than those related to spindle assembly. Our study validates the assumption that the absence of spindles in limb muscles of NT3 or trkC null mutants is due to the absence of NT3-dependent proprioceptive neurons in DRGs rather than intramuscular NT3,[1,5] but leaves unanswered the nature of the factor(s) responsible for the maintenance of NT3-independent proprioceptive neurons in brain stem nuclei. Brain-derived neurotrophic factor might be involved because it can support embryonic chick trigeminal mesencephalic neurons in tissue culture.[23]

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