Formation of a Full Complement of Cranial Proprioceptors Requires Multiple Neurotrophins

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ABSTRACT **Inactivation of neurotrophin-3 (NT3) completely blocks the development of limb proprioceptive neurons and their end organs, the muscle spindles. We examined whether cranial proprioceptive neurons of the trigeminal mesencephalic nucleus (TMN) require NT3, brain-derived neurotrophic factor (BDNF) or neurotrophin-4 (NT4) for their development. Complements of TMN neurons and masticatory muscle spindles were decreased by 62% in NT3 null mutants, 33% in BDNF null mutants, and 10% in NT4 null mutant mice at birth. The extent of proprioceptive deficiencies differed among different masticatory muscles, particularly in NT3 null mice. Masticatory muscles of embryonic mice heterozygous for the NT3^{lacZneo} or BDNF^{lacZ} reporter genes expressed both NT3 and BDNF, consistent with target-derived neurotrophin support of TMN neurons. Although more than 90% of TMN neurons expressed TrkB as well as TrkC receptor proteins by immunocytochemistry in wild-type newborns, TrkC or TrkB null mice exhibited only partial proprioceptive deficiencies similar to those present in NT3 or BDNF; NT4 null mice. Thus, in terms of the survival outcome, two main subpopulations of TMN neurons may exist during embryogenesis, one dependent on TrkC/NT3 functioning and the other utilizing TrkB/BDNF signaling. The differential dependence of TMN neurons on neurotrophins may reflect differential accessibility of the neurons to limiting amounts of NT3, BDNF, or NT4 in target tissues, especially if the tissue distribution or levels of BDNF, NT3, and NT4 were dynamically regulated both spatially and temporally. *Dev Dyn 2000;218:359–370.* © 2000 Wiley-Liss, Inc.**

Key words: **neurotrophin-3; brain-derived neurotrophic factor; neurotrophin-4; proprioceptive sensory neurons; Trk receptors; muscle spindles; mutant mice**

INTRODUCTION

Neurotrophins are a family of trophic factors essential for the survival and differentiation of neurons. This family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4 (NT4). NT3 is essential for the development of proprioception in limbs. Proprioceptive neurons and muscle spindles, their end organs, are absent from limb muscles of mice carrying a targeted deletion in the NT3 gene, but are present undisturbed in all other neurotrophin null mutant mice (Ernfors et al., 1994a; Fariñas et al., 1994; Jones et al., 1994). Thus, limb proprioceptors depend solely on NT3, and other neurotrophins cannot prevent cell death of NT3-deprived limb proprioceptive neurons.

Cell bodies of cranial proprioceptive afferents supplying masticatory muscles reside in the mesencephalic nucleus of the trigeminal nerve (TMN), a central nervous system (CNS) locale presumably rich in multiple neurotrophins. Neurotrophins other than NT3 might support developing TMN proprioceptive neurons, in contrast to dorsal root ganglion (DRG) proprioceptive neurons. Fewer proprioceptive afferents and muscle spindles are present in jaw muscles of mice lacking NT3 or its tyrosine kinase (Trk) receptor (Kucera et al., 1998), but they are not entirely absent as in the limbs of NT3 null mutants (Ernfors et al., 1994a). The presence of masticatory spindles in NT3-/- mice suggests that the corresponding proprioceptive TMN neurons depend on a neurotrophin(s) other than NT3. Fifty percent of TMN neurons are absent in BDNF-/- mice, suggestive of a role for BDNF in TMN neuron development (Ernfors et al., 1994b; Jones et al., 1994). However, nonproprioceptive neurons also reside in the TMN nucleus (Jerge, 1963), thus the missing
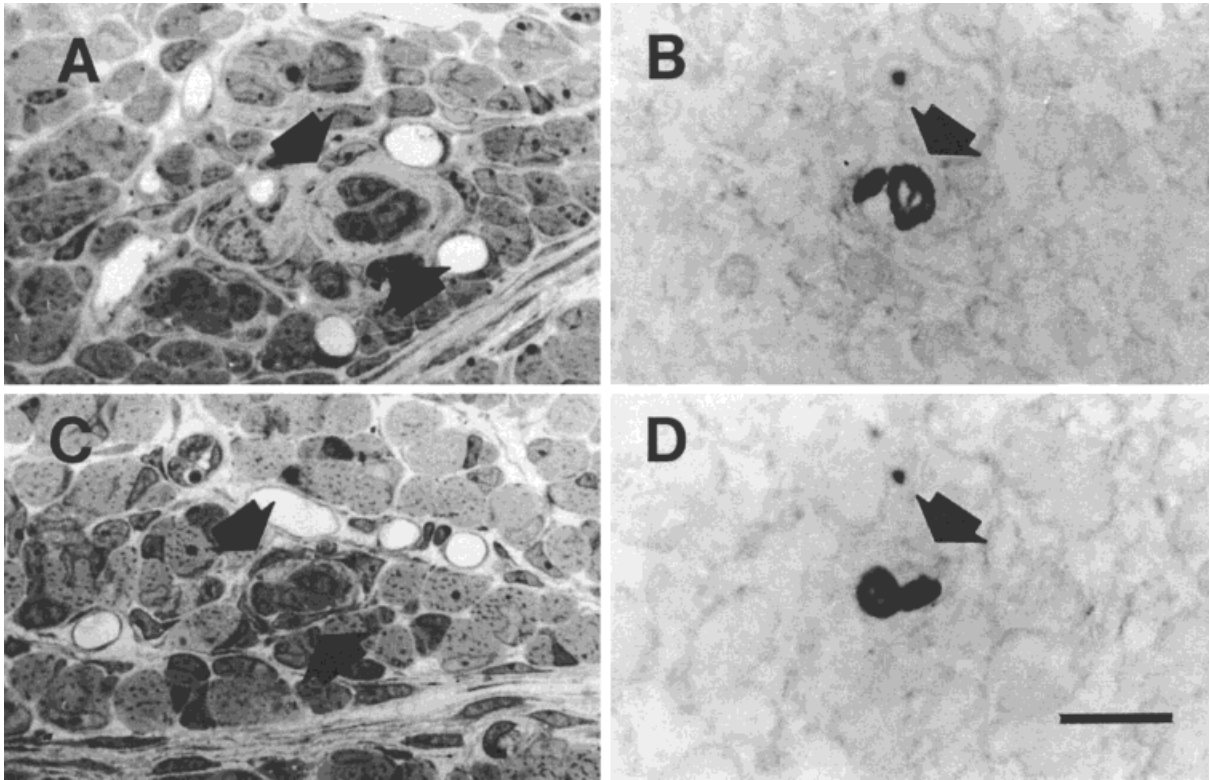
Fig. 1. Transverse sections of the zygomaticomandibular muscle of wild-type (**A, B**) and BDNF-/- (**C, D**) mice taken at P0–P1. Stained with toluidine blue (A, C) or immunoreacted with the spindle-specific S46 antibody (B, D). Note the presence of spindles (arrowheads) in both the mutant and wild-type muscles. That NT3-/- jaw muscles also contain spindles expressing the S46 antigen similar to wild-type and BDNF-/- mice was shown previously (Kucera et al., 1998). Scale bar = 20 μm.

TMN neurons in BDNF-/- mice might be either proprioceptive or nonproprioceptive in function.

We undertook a comprehensive study of the dependence of cranial proprioceptors on NT3, BDNF, and NT4. We compared BDNF-deficient, NT3-deficient, and NT4-deficient mutant mice for numbers of proprioceptive afferents and muscle spindles in masticatory muscles in order to assess the relative importance of the three neurotrophins in supporting the differentiation and/or survival of cranial proprioceptive neurons. To explore the role of neurotrophin receptors, we examined TMN neurons for the expression of members of the Trk family of receptors that specifically bind NT3, BDNF, and/or NT4 and the extent of proprioceptive deficits in mice lacking Trk receptors. Lastly, we examined expression of NT3 and BDNF in target muscles using transgenic mice in which a *lacZ* reporter gene was inserted in place of a NT3 or BDNF gene.

RESULTS
Reduced Complements of Spindles in the Absence of NT3 or BDNF

Numbers of muscle spindles, the end organs of proprioceptive afferents, were used as indicators of proprioceptive neuron survival in mice at P0–P4. Fewer muscle spindles were present in each of four different masticatory muscles examined in NT3-/- and BDNF-/- mutants (Fig. 1). The reduction in spindle numbers was 62% in NT3-/- compared to 33% in BDNF-/- muscles (Table 1). Masticatory muscles differed in the extent of their spindle deficiency in mutant mice, particularly in NT3-deficient mutants. The superficial masseter showed a 75% loss whereas the zygomaticomandibularis showed only a 47% loss of spindles in NT3-/- relative to wild-type mice. Such intermuscular variability in spindle deficits was less apparent in BDNF-/- mutants, in which spindle counts differed by no more than 10% among the four different jaw muscles examined (Table 1). Thus, major proprioceptive deficits result from the developmental absence of single neurotrophins, and different masticatory muscles may differ in the proportion of spindles (and presumably proprioceptive afferents) dependent on NT3 or BDNF.

The morphology and spatial distribution of residual spindles in NT3- or BDNF-deficient mice were similar to wild-type spindles. Spindles in mutants contained one to four intrafusal fibers surrounded by a connective tissue capsule. Bundles of unmyelinated axons, assumed to be proprioceptive afferents and fusimotor efferents, terminated in the equatorial and polar re-

TABLE 1. Deficits of Spindles and Neurons in Mice Deficient in One or More Neurotrophins[a]

Mice	WT	NT4−/−	BDNF−/−	NT4−/−;BDNF−/−	NT3−/−	NT3−/−;NT4−/−	NT3−/−;BDNF−/−	NT3−/−;BDNF−/−;NT4−/−
Spindles								
AM	33.6 ± 3 (5)	30.5 ± 1 (2)	24.0 ± 2 (4)	17.5 ± 2 (4)	13.9 ± 1 (12)	13.3 ± 1 (3)	5.5 ± 0.5 (6)	2.0 ± 0.6 (4)
ZM	11.4 ± 2 (8)	11.6 ± 1 (6)	7.8 ± 0.5 (4)	5.6 ± 0.5 (5)	6.1 ± 2 (13)	4.3 ± 0.6 (3)	1.5 ± 0.9 (6)	1.0 ± 0.0 (4)
SM	19.1 ± 1 (10)	16.2 ± 1 (4)	13.3 ± 1 (4)	9.5 ± 2 (4)	4.8 ± 1 (13)	4.0 ± 1 (2)	2.2 ± 1 (6)	1.2 ± 0.6 (4)
PI	24.0 ± 1 (4)	20.7 ± 1 (3)	14.0 ± 0 (2)	12.0 ± 2 (4)	8.8 ± 2 (4)	8.3 ± 1 (3)	2.5 ± 0.7 (2)	1.5 ± 0.6 (4)
Pooled	88.1	79.0	59.1	44.6	33.6	29.9	11.7	5.7
% Deficit (vs. WT)	0%	10%	33%	49%	62%	66%	87%	94%
Neurons								
TMN	496 ± 16 (7)	456 ± 27 (4)	292 ± 28 (5)	268 ± 11 (2)	213 ± 13 (5)	208 ± 0 (1)	64 ± 9 (3)	25 ± 8 (2)
% Deficit (vs. WT)	0%	8%	41%	46%	57%	58%	88%	95%

[a]Values are counts of neurons in the TMN nucleus and muscle spindles in the anterior masseter (AM), zygomaticomandibularis (ZM), superficial masseter (SM), and internal pterygoid (PI) muscles of wild-type (WT) and mutant (NT4−/−, BDNF−/−, NT4−/−;BDNF−/−, NT3−/−, NT3−/−;NT4−/−, NT3−/−;BDNF−/−, and NT3−/−;BDNF−/−;NT4−/−) mice at P0–P4. Mutants are listed in the order of increasing spindle deficits. Data are shown as means ± s.d. (N). The spindle counts pooled for all four muscles are also given, and expressed as percent deficits of spindles in mutant versus wild-type mice for each mutation. Underlined groups only are not significantly different at $p < 0.05$.

gions of mutant spindles, similar to wild-type spindles at birth (Kozeka and Ontell, 1981). Normal spindle development was further supported by the expression of the spindle-specific slow-developmental myosin heavy-chain (MyHC) isoform in nuclear bag fibers of the residual spindles of mutants (Fig. 1). Expression of this isoform by intrafusal fibers of spindles is dependent on the presence of Ia afferent innervation. Innervation of residual spindles by afferents in NT3 and TrkC null mice has been confirmed previously by electron microscopy (Kucera et al., 1998). Although numbers of spindles were reduced in mutant NT3 or BDNF mice, the distribution of spindles in individual muscles paralleled that of wild-type mice. Spindles are restricted to specific regions of most jaw muscles (Bredman et al., 1991). This feature was especially conspicuous in the anterior masseter muscle in which spindles were restricted to the medial-inferior aspect of the muscle in wild-type as well as NT3 or BDNF mutant mice. These observations suggest that neurotrophins do not directly regulate spindle morphogenesis and presumably act via proprioceptive afferent neurons.

NT3 and BDNF Support Separate Populations of Spindles

The extent of proprioceptive deficiencies differed among different masticatory muscles, suggesting that NT3 and BDNF support separate subpopulations of spindles and their proprioceptive afferents. To explore this issue further, we compared the number of spindles in NT3-/-;BDNF-/- double gene mutants with numbers of spindles in NT3-/- and BDNF-/- single gene mutants (Table 1). If a subpopulation of proprioceptive neurons utilized equivalently either NT3 or BDNF, then the deficit of spindles in NT3-/-;BDNF-/- mice would be expected to be greater than the sum of deficits in NT3-/- and BDNF-/- mice because the absence of one of the neurotrophins would be compensated by the other neurotrophin in single gene mutants. However, the 87% spindle loss in NT3-/-; BDNF-/- mutants was approximately equivalent to the sum of spindle deficits in single gene mutants (94% spindle loss). This observation suggests that distinct populations of afferents utilize either NT3 or BDNF and that these two neurotrophins cannot effectively compensate for each other in mediating the survival and/or differentiation of cranial proprioceptors. NT4 can also support cranial proprioceptors (see below). Similar to NT3-/-;BDNF-/- mutants, the overall spindle deficit (66%) in NT3-/-;NT4-/- mutants was approximately equal to the sum of deficits (72%) in NT3 and NT4 null mutants, suggesting that the populations of NT3- and NT4-dependent proprioceptors do not overlap (Table 1).

Numbers of Spindles Correlate With NT3 or BDNF Gene Dosage

In the limb, numbers of proprioceptive afferents and spindles correlate with NT3 gene dose and hence pre-

TABLE 2. Comparison of Pooled Spindle Deficits in Mutants Lacking One or Both Copies of Either NT3 or BDNF Genes[a]

	WT	NT3−/−	NT3+/−	BDNF−/−	BDNF+/−
No. of spindles	88.1	33.6	60.6	59.1	72.5
% Deficit (vs. WT)	—	61.9%	31.2%	32.9%	17.7%
% Deficit (vs. Null)	—	100%	50.4%	100%	53.8%

[a]Note that spindle deficits in homozygous mutants are twice as severe as in heterozygous mutants. Symbols as in Table 1.

sumably with tissue level of NT3 (Ernfors et al., 1994a). Deletion of one copy of the NT3 gene, which presumably decreases the level of NT3 in tissues by 50%, results in a 50% loss of spindles, whereas deletion of two NT3 gene copies results in a 100% loss of spindles (Ernfors et al., 1994a). Similarly, the overall deficit of spindles in the masticatory muscles of NT3+/− mice was one half of the deficit observed in NT3-/- mice (Table 2). Thus, within the population of NT3-dependent spindles (and presumably proprioceptive afferents), decreasing levels of NT3 had the same effect on cranial proprioceptors as on limb proprioceptors.

In limb muscles, deletion of one or two copies of the BDNF gene has no effect on numbers of spindles because DRG proprioceptive neurons do not utilize BDNF (Hory-Lee et al., 1993; Jones et al., 1994). However, in the masticatory system, spindle deficits in BDNF-deficient mice correlated with the number of BDNF gene copies deleted, and twice as many spindles were missing in BDNF-/- than in BDNF+/- mice (Table 2). Thus, the population of BDNF-dependent spindles (and proprioceptive afferents) is regulated by limiting concentrations of BDNF, similar to and independent of the regulation of NT3-dependent spindles by levels of NT3. BDNF shows a gene dosage effect on neurons of the vestibular and nodose ganglia (Ernfors et al., 1994b).

Trophic Factors Other Than NT3 or BDNF Support Spindles

As much as 13% of the normal complement of spindles formed in the combined absence of NT3 and BDNF (Table 1). The presence of residual spindles in NT3-/-; BDNF-/- double gene mutants suggests that a trophic factor(s) other that NT3 and BDNF also support cranial proprioceptors. TMN neurons express TrkB receptor proteins (Yan et al., 1997), thus NT4 is a plausible candidate for supporting the population of TMN neurons. We deleted the NT4 gene alone or in combination with the BDNF gene. Spindle deficits were observed in NT4-/- mutants, indicating that a subset of proprioceptive afferents requires NT4 for survival in spite of the presence of BDNF (Table 1). However, more spindles were missing in NT4-/-;BDNF-/- double mutants than in NT4-/- and BDNF-/- single mutants, suggesting a partial overlap of the subpopulations of proprioceptive afferents dependent on the two TrkB receptor ligands (Table 1). Thus, NT4 can support cranial propriocep-

tors, and at least some of the residual spindles (and proprioceptive afferents) in NT3-/-;BDNF-/- mutants may be dependent on NT4. Residual spindles were present even in NT3-/-;BDNF-/-;NT4-/- triple gene mutants (Table 1), thus trophic factors other than NT3, BDNF, and NT4 may support some proprioceptive afferents in the absence of the three neurotrophins. We observed no jaw spindle deficits in NGF-/- neonates (data not shown). NGF has little effect on the survival of cultured chick TMN neurons (Davey and Davies, 1998).

Deficits of TMN Neurons Parallel Deficits of Spindles

Deficits of spindles in the NT3 and BDNF mutants were assumed to result from a decreased number of proprioceptive neurons in the TMN nucleus where the neurons reside (Jerge, 1963; Shigenaga et al., 1988a). To determine the extent of neuron loss, we compared numbers of TMN neurons stained with cresyl violet in mutant and wild-type mice (Fig. 2). As anticipated, deficits in numbers of TMN neurons paralleled the deficits of jaw spindles. The TMN neuron deficits were greater in NT3 than BDNF mutants, greater in double mutants than single mutants, and greatest in NT3-/-; BDNF-/-;NT4 triple mutants (Table 1). Nonproprioceptive neurons of the TMN nucleus might also be dependent on neurotrophins because only a few TMN neurons survived in the triple mutants.

TrkC- and TrkB-Deficient Mutants Exhibit Proprioceptive Deficits

Complements of masticatory spindles and TMN neurons were also decreased in newborns lacking either TrkC or TrkB receptors (Table 3). The overall spindle deficit in TrkC null mutants was comparable to the spindle deficit in mice lacking NT3, and the spindle deficit in TrkB-/- mice was comparable to that of BDNF-/-;NT4-/- mice, consistent with Trk mediation of neurotrophin actions (Table 3). Thus, NT3-dependent or BDNF/NT4-dependent proprioceptive neurons overlap with neuron populations dependent on TrkC or TrkB. Furthermore, spindle populations in the four masticatory muscles showed differential dependence on the presence of TrkC or TrkB. The anterior masseter and zygomaticomandibularis, two anterior muscles, had spindles equally dependent on TrkB or TrkC
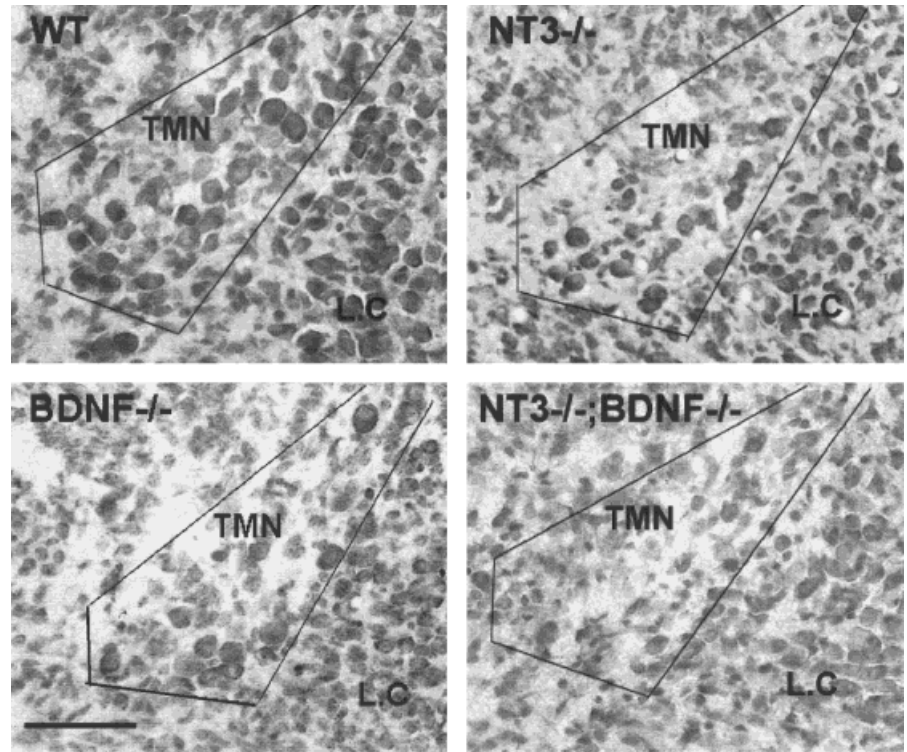
Fig. 2. Photomicrographs of the ventro-caudal portion of the TMN nucleus in WT, NT3-/-, BDNF-/-, and NT3-/-;BDNF-/- mice. Stained with cresyl violet. The triangular area containing most of the typical large, round unipolar TMN neurons is located adjacent to the locus coeruleus (LC) and is demarcated by thin lines. Scale bar = 100 μm.

TABLE 3. Counts of Muscle Spindles and TMN Neurons in Mice Lacking Neurotrophin Receptors[a]

	WT	TrkC−/−	TrkB−/−
Spindle counts			
AM	33.6 ± 3 (5)	16.3 ± 1 (4)	14.0 ± 2 (7)
ZM	11.4 ± 2 (8)	6.8 ± 1 (7)	4.4 ± 1 (7)
SM	19.1 ± 1 (10)	4.0 ± 0.8 (5)	12.8 ± 2 (6)
PI	24.0 ± 1 (4)	10.4 ± 1 (6)	12.5 ± 3 (6)
Pooled counts	88.1	37.5	43.7
Deficit relative to NT3−/−	—	93%	—
Deficit relative to BDNF−/−; NT4−/−	—	—	102%
Neuron counts			
TMN	496 ± 16 (5)	272 ± 52 (5)	307 ± 25 (4)
Deficits relative to NT3−/−	—	79%	—
Deficit relative to BDNF−/−; NT4−/−	—	—	83%

[a]Symbols for the four masticatory muscles are as in Table 1. Data are shown as means ± s.d. (N). The spindle counts pooled for all four muscles are also given, and expressed as percent deficits of spindles in trk receptor-versus neurotrophin-deficient mice for each mutation. Note that spindle deficits are comparable between mice lacking Trk receptors and their corresponding neurotrophin ligands. Underlined groups are not significantly different at p = 0.05.

receptors. In contrast, the superficial masseter and internal pterygoid, the two posterior muscles, had more spindles dependent on TrkC than TrkB receptors (Table 3).

Postnatal TMN Neurons Co-Express TrkC and TrkB Receptors

Developmental dependence on single neurotrophins suggests functional expression of single Trk receptors in TMN neurons before birth. We examined whether Trk receptors are expressed differentially by TMN neurons at birth when proprioceptive deficits were also determined. Using a specific antiserum (Yan et al., 1997), we detected expression of TrkB protein in TMN neurons of neonatal wild-type mice. The mean number of neurons reactive for TrkB was 458 ± 20 (mean±s.e.m.; N=2) in TMN nuclei that contained 464 ± 11 (mean±s.e.m.; N=2) neurons when examined in serial sections processed alternately for Nissl staining or TrkB immunocytochemistry. Thus, 98% of TMN neurons expressed detectable amounts of the TrkB protein. To determine whether TrkB-
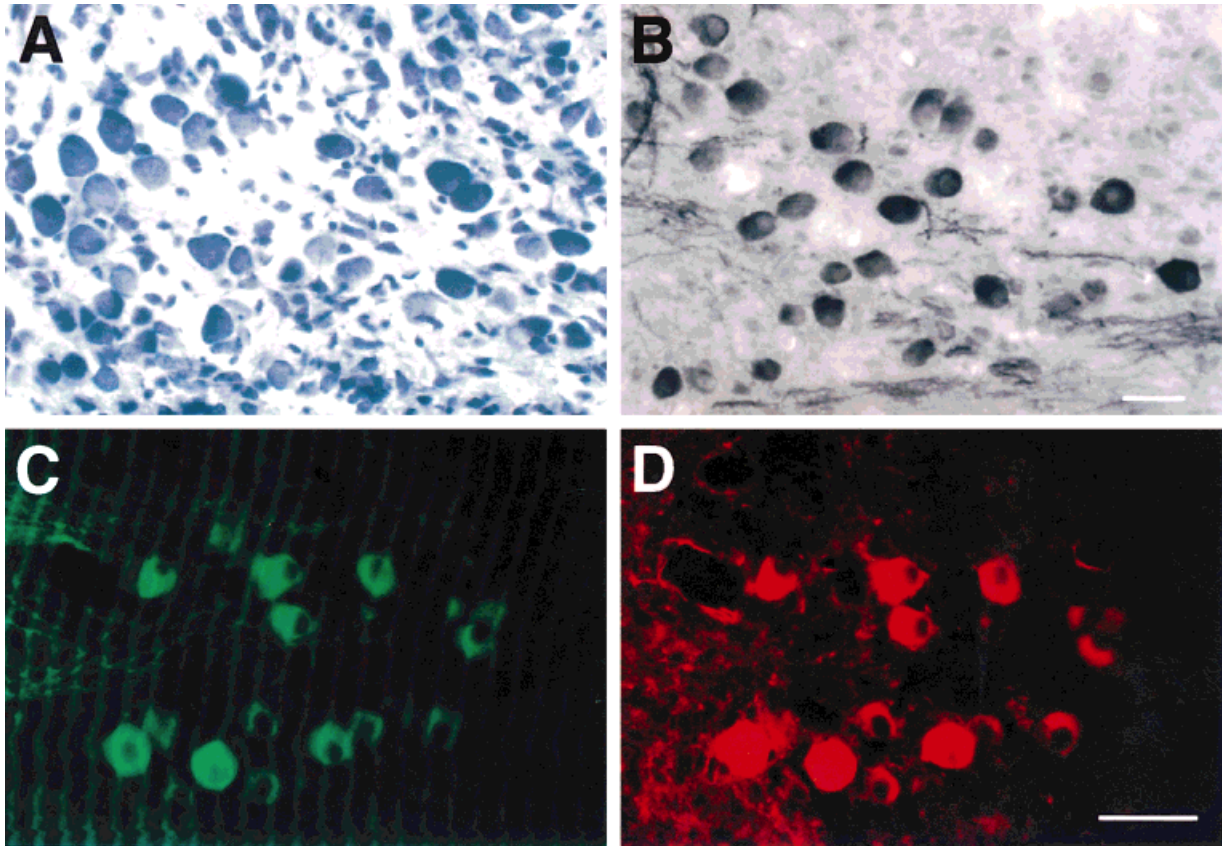
expressing TMN neurons also express the TrkC receptor protein, we used double labeling with antisera specific for TrkB or TrkC (Yan et al., 1997; Fariñas et al., 1998; Huang et al., 1999). In a random sample of neonatal TMN neurons, 122 of 135 (90%) neurons were positive for both TrkB and TrkC (Fig. 3), whereas 12 neurons were positive for TrkB only and one neuron was positive for TrkC only. Extensive colabeling of TrkB and TrkC was also detected in an adult TMN nucleus, in which 50 out of 54 (93%) neurons were positive for both TrkB and TrkC. The remaining four neurons were TrkB-positive and TrkC-negative. In both neonatal and adult TMN nuclei, a minority of the colabeled neurons appeared to be more intensely stained for TrkB than TrkC, suggesting heterogeneity in TrkB and TrkC expression by individual neurons. Thus, most postnatal wild-type TMN neurons express detectable amounts of both TrkB and TrkC proteins, although some TMN neurons may express a greater amount of the TrkB than TrkC protein by immunocytochemistry.

Differential Expression NT3 and BDNF in Masticatory Muscles and Spindles

Neurotrophins that support developing TMN neurons might be derived retrogradely from target muscles. Mice heterozygous for the NT3^{lacZneo} or BDNF^{lacZ} allele were therefore used to compare the expression pattern of endogenous NT3 and BDNF in the cranial musculature. Cranial whole mounts and transverse cranial sections were stained for X-gal to detect expression of the *lacZ* marker gene at E14.5, E15.5, and P0, corresponding to stages of development just before and after spindle assembly (Fig. 4). TMN afferents begin to innervate masticatory muscles at E11.5–E12.5 (Widmer et al., 1998). In our wild-type embryos, intramuscular nerve bundles but no encapsulated spindles were present in masticatory muscles examined in semi-thin sections at E15.5.

The E14.5 or E15.5 superficial masseter diffusely stained for X-gal in its medial-inferior region in both NT3^{lacZneo} and BDNF^{lacZ} embryos, suggesting that NT3
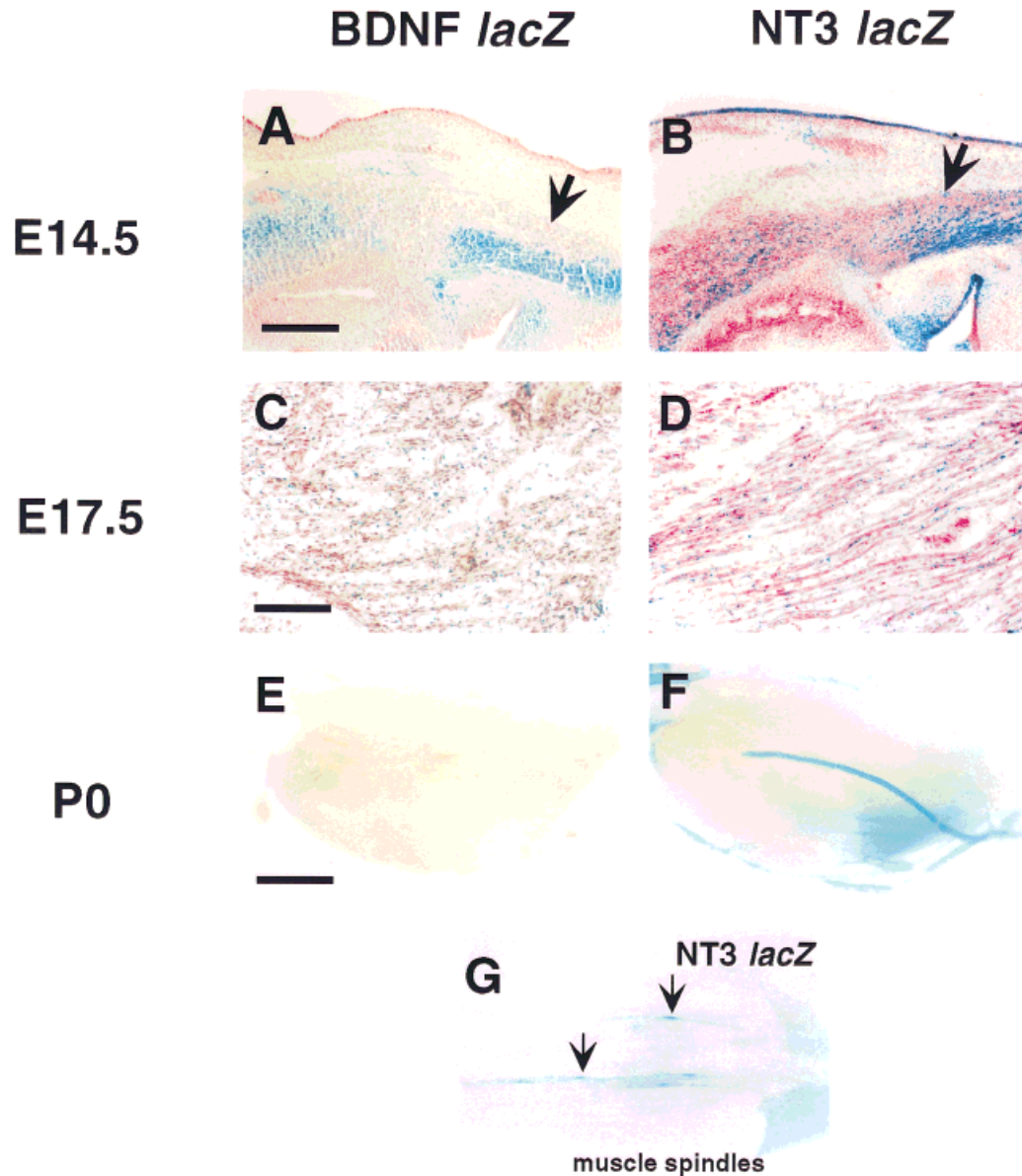
Fig. 4. Expression of BDNF and NT3 in the masseter muscle as assessed by the expression of the reporter gene *LacZ* in embryonic (**A–D**) and neonatal (**E–G**) mice heterozygous for the NT3^{lacZneo} or BD-NF^{lacZ} allele. Muscle sections or whole mounts were stained histochemically for β-galactosidase. Counterstained with neutral red (A,C) or eosin (B,D). Note that the E14.5 masseter (arrows) expresses *LacZ* both in NT3^{lacZneo} and BDNF^{lacZ} embryos (A,B), and the expression is less at E17.5 than at E14.5 (A–D). The P0 NT3^{lacZneo} but not BDNF^{lacZ} masseter muscle expresses *LacZ* diffusely in its inferomedial part (E–F). Equatorial regions of spindles (arrows in G) strongly express *LacZ* in NT3^{lacZneo} masseter. The strongly X-gal stained streaks overlying the NT3^{lacZneo} but not BDNF^{lacZ} masseter muscle are blood vessels (E–F). Scale bar = 10 μm (A–D) and 50 μm (E–F).

as well as BDNF are expressed in comparable regions of embryonic masticatory muscles. However, no spindles were identified in the X-gal stained muscles at E14.5 or E15.5. In contrast, clusters of spindles could be identified in X-gal stained inferomedial region of the masseter muscle of NT3^{lacZneo} mutants at P0. The spindles exhibited strong X-gal reactivity in the equatorial region where the afferents terminate and weak X-gal reactivity in the polar

region where the motor innervation resides, suggesting that NT3 expression is principally associated with sensory innervation. In contrast, no P0 spindles expressed detectable BDNF, as indicated by the absence of any X-gal staining in the spindle-containing masseter region of BDNF^{lacZ} newborns. However, the lower jawbone and masseter regions adjacent to it stained faintly for X-gal in BDNF^{lacZ} mice. Downregulation of BDNF expression in

masticatory muscles may take place at late prenatal stages because X-gal staining was less intense in E17.5 and P0 than E14.5-E15.5 masseter muscles of BDNF^{lacZ} mice (Fig. 4).

To determine whether all or only some masticatory spindles express NT3, we counted the number of X-gal stained spindles in NT3^{lacZneo} mutants at P0. The number of X-gal reactive spindles in the zygomandibularis (9.5±0.5; N=2) and superficial masseter (10±0.5; N=2) muscles of heterozygous NT3^{lacZneo} mutants was similar to that observed in NT3+/- mutants (9.7±0.5; N=13 and 10.7±0.5; N=11). Since about one third of spindles that survive in mice heterozygous for the NT3^{lacZneo} allele are innervated by afferents dependent on BDNF, we concluded that spindles innervated by BDNF-dependent proprioceptive neurons express NT3 similar to spindles innervated by NT3-dependent proprioceptive neurons.

DISCUSSION

Our study shows that the development of a full complement of cranial proprioceptors requires the presence of NT3, BDNF, and NT4. Mice carrying a targeted mutation in genes for any of the three neurotrophins, or their cognate Trk receptors, developed with measurable deficits in TMN neurons and masticatory muscle spindles. In contrast, limb proprioceptive neurons depend only on NT3/TrkC signaling for their survival and/or differentiation during development (Ernfors et al., 1994a; Farinas et al., 1994). Thus, developmental regulation by neurotrophins may differ between proprioceptive neurons located in the brain stem (CNS) and dorsal root ganglia (PNS), even though neurons in both locations are considered to be of neural crest origin (Stainier and Gilbert, 1991) and mediate the same functional modality.

Subpopulations of NT3-, BDNF-, and NT4-dependent neurons are assumed to coexist within the confines of the TMN nucleus. Retrograde labeling from masticatory muscles shows that all cell bodies of labeled proprioceptive afferents reside in the ipsilateral TMN nucleus (Shigenaga et al., 1988a). Similarly, each of the four masticatory muscles harbored subpopulations of spindles innervated by NT3-, BDNF-, or NT4-dependent afferents. Deficits of masticatory spindles were associated with deficits of TMN neurons, reflective of the tight developmental link between spindles and proprioceptive neurons. However, numbers of spindles may provide a better index of numbers of cranial proprioceptive neurons than numbers of TMN neurons because the TMN nucleus contains not only proprioceptive but also nonproprioceptive neurons innervating periodontal mechanoreceptors (Shigenaga et al., 1988b). Although mouse data are not available, nonproprioceptive neurons may represent up to 30% of the TMN population in some mammals (Jerge, 1963). Both proprioceptive and nonproprioceptive neurons are dependent on neurotrophins because few TMN neurons

survived in NT3-/-;BDNF-/-;NT4-/- mice. However, most of the nonproprioceptive TMN neurons might depend on BDNF because deficits of TMN neurons were greater than deficits of spindles in BDNF-/- mutants.

Nonoverlapping Neurotrophin Requirements of Subpopulations of TMN Neurons

In vitro studies suggest the existence of one population of postnatal TMN neurons with dual neurotrophin dependence rather than multiple populations of neurons each dependent on one neurotrophin. Avian and mammalian TMN neurons can utilize both NT3 and BDNF in tissue culture. The survival of chicken TMN neurons is unaffected by the absence of NGF, but is promoted by BDNF and a muscle-derived factor assumed to be NT3 (Davies et al., 1986a,b). No additional survival effect is observed in the presence of both factors, but at subsaturating concentrations, the combined effects of BDNF and the muscle-derived factor are additive. BDNF and NT3 can also support cultured embryonic TMN neurons in rat, although only two thirds of the neurons are rescued by saturating concentrations of both NT3 and BDNF, suggesting the requirement for an additional survival factor (Copray and Liem, 1993).

Patterns of Trk receptor expression were consistent with the ability of postnatal TMN neurons to respond to more than one neurotrophin. We detected neuronal expression of both the TrkB and TrkC proteins in postnatal TMN neurons of wild-type mice, attesting to the potential of postnatal proprioceptive neurons to respond to NT3, BDNF, and/or NT4. We assume that the TrkB and TrkC receptors detected by our antibodies were functional because TMN neurons and spindles were deficient in both TrkB-/- and TrkC-/- mice. Similarly, both TrkB and TrkC mRNA are expressed in the chick TMN nucleus, and dual expression of TrkB and TrkC mRNA probably occurs within a subpopulation of TMN neurons in chickens (Williams et al., 1995). Interestingly, co-expression of TrkB and TrkC receptors is not a feature of cutaneous sensory neurons located outside the CNS, in the main trigeminal ganglion (Huang et al., 1999).

Although postnatal TMN neurons may have the ability to respond to both BDNF and NT3, proprioceptive deficits in mice lacking single neurotrophins suggest that NT3 and BDNF cannot substitute for each other as factors supporting the survival of prenatal TMN neurons. Rather, subpopulations of TMN neurons may exist at an early stage of development, whose neurotrophin requirements do not overlap. The evidence supporting the existence of separate and nonoverlapping populations of NT3- and BDNF-dependent TMN neurons at a prenatal stage is twofold. First, the sum of surviving spindles or TMN neurons in NT3- and BDNF-deficient mice was approximately equal to the number of spindles or TMN neurons in wild-type mutants. If BDNF and NT3 could substitute for each

other, then the sum of surviving spindles (and proprioceptive neurons) in NT3- and BDNF-deficient mice would be expected to exceed the wild-type complements. Similarly, the deficit of spindles in NT3-/-; BDNF-/- mice was not greater than the sum of deficits in NT3-/- and BDNF-/- mice. Second, the overall spindle deficit in NT3+/- or BDNF+/- mice was one half of the deficit in NT3-/- or BDNF-/- mice, further indicating that BDNF does not compensate for NT3 and vice versa. The loss of proprioceptive afferents in NT3+/- and BDNF+/- mutants suggests rigid neurotrophin requirements of developing TMN neurons with marginal access to the limiting neurotrophin.

Differential Distribution of NT3 and BDNF in Target Muscle Tissues

Target-derived neurotrophins play a major role in supporting developing neurons. In limbs, proprioceptive afferents retrogradely transport NT3 from NT3-expressing muscles and/or spindles to NT3-dependent DRG neurons (Fariñas et al., 1996; Oakley et al., 1997; Wright et al., 1997). An analogous situation may occur in the cranial proprioceptive system. Using $lacZ$ expression as a reporter for the endogenous expression of NT3, we showed that NT3 is expressed in masticatory muscles already at E14.5-E15.5, prior to spindle assembly. This expression of NT3 in cranial muscles may coincide with the period of naturally occurring death of TMN neurons, although this issue remains to be examined in mice. Approximately 50% of TMN neurons in the hamster are eliminated between E13.5 and E15.5 (Alley, 1974). In addition, postnatal masticatory muscles expressed NT3 in the sensory region of spindles, similar to limb muscles (Copray and Brouwer, 1994). Thus, extensive parallels exist between the organization of cranial and limb proprioceptive systems with respect to sites of expression (and potentially modes of utilization) of NT3.

Similar to NT3, BDNF was detected in embryonic muscles, as indicated by the $lacZ$ reporter gene in BDNF^{lacZ} mutants. Thus, BDNF-dependent TMN neurons could derive BDNF retrogradely from muscles. The concomitant expression of both NT3 and BDNF in masticatory muscles at the time of their innervation by afferents is consistent with the existence of cranial proprioceptive neurons dependent on muscle-derived NT3 or BDNF. In contrast to NT3, BDNF expression declines in late prenatal stages, and little or no BDNF is detectable in masticatory muscles at birth. Thus, muscle-derived BDNF may play an early survival role in a subpopulation of TMN neurons, but it is unlikely to be involved in their long-term maintenance. Masticatory muscles may behave similar to limb muscles in terms of NT3 and BDNF expression. Both NT3 and BDNF mRNA was detected in embryonic limb muscles, but the level of BDNF expression is 1/20 of that of NT3 expression at birth (Henderson et al., 1993; Funakoshi et al., 1995). The probable retrograde support of TMN

neurons by muscle-derived NT3 and BDNF does not preclude a contributory role of autocrine or paracrine mechanisms in the neuron survival.

We observed that BDNF, but not NT3, is expressed in a subset of E17.5 TMN neurons, as revealed by the $lacZ$ reporter gene in NT3^{lacZneo} and BDNF^{lacZ} mice (data not shown).

Changing Responsiveness of TMN Neuron to NT3 and BDNF

Both TrkC and TrkB receptors are present on the cell surface of TMN neurons of wild-type newborns. Whether developing TMN neurons also express both kinds of Trk receptors before birth could not be ascertained because TMN neurons could not be reliably identified in the embryonic brain stem. However, our study suggests that coactivation of TrkC and TrkB receptors is not a requirement for the survival of TMN neurons because a number of neurons survived when either NT3 or BDNF were deleted. In terms of the survival outcome, two main populations of TMN neurons may exist during development, one critically dependent on TrkC/NT3 functioning and the other utilizing TrkB/BDNF signaling. Early TMN neurons may co-express TrkB and TrkC receptors, but might differ in their ability to access target neurotrophins, especially if the tissue distribution of BDNF, NT3, and NT4 was dynamically regulated both spatially and temporally. Alternatively, co-expression of TrkC and TrkB receptors might be a late-developing characteristic of TMN neurons rather than being present throughout the embryonic development.

The potential role of target neurotrophins in determining the neurotrophin responsiveness of proprioceptive neurons is unclear. Presumably, the critical dependence of TMN neurons on neurotrophins manifests itself at E11.5-E13.5 when their axon projections leave the brain stem (Widmer et al., 1998) and innervate target muscles. Encountering limiting amounts of muscle-expressed NT3 or BDNF may determine a neuron dependence on either NT3 or BDNF for survival. The apparent broadening of neurotrophin responsiveness of TMN neuron late in development might be an adaptive neuron characteristic in response to changing patterns of neurotrophin expression in target tissues. In masticatory muscles, these changes include downregulation of BDNF expression and progressive restriction of NT3 expression to spindles.

Both NT3 and BDNF may have postnatal functions in TMN neurons that are separate from their early survival roles. Expression of NT3, as indicated by the $lacZ$ reporter, in spindles innervated by BDNF-dependent TMN neurons attests that the complexity of roles of neurotrophins in neuronal development and function. NT3 expressed in postnatal spindles may have nonsurvival functions, such as controlling the conduction velocity and hence the caliber of proprioceptive afferents (Munson et

al., 1997), independent of whether NT3, BDNF, or NT4 supports the survival of proprioceptive neurons during development.

CONCLUSIONS

Multiple trophic factors including NT3, BDNF, and NT4 must be present for the development of a full complement of cranial proprioceptive neurons. Although TMN neurons may have the capacity to bind NT3, BDNF, or NT4, these neurotrophins cannot compensate for each other in the absence of one of the neurotrophins during embryogenesis. Sensory neurons subserving proprioception differ in neurotrophin requirements depending on whether they reside in the brain stem or dorsal root ganglia, presumably reflective of the types of neurotrophins expressed in relevant peripheral and/or central targets during neuron development.

MATERIALS AND METHODS
Mutant Animals

Mice carrying the mutated BDNF, NT3, or NT4 allele were generated in the BALB/c 129 strain of mouse as described previously (Ernfors et al., 1994a,b; Liu et al., 1995). Lines of mice lacking TrkC or TrkB receptors were those described previously by Tessarollo et al. (1997) and Klein et al. (1993). Polymerase chain reactions (PCR) and Southern blot analysis of DNA extracted from the tail were used to genotype mice as null mutant (-/-), heterozygous mutant (+/-), or wild-type (+/+). Single BDNF, NT3, or NT4 null mutants lacked both copies of the BDNF, NT3, or NT4 gene. Double (NT3;BDNF or NT4;BDNF) or triple (NT3;BDNF;NT4) null mutants were generated by mating mice heterozygous for single gene deletions. Wild-type littermates served as controls.

Expression of NT3 and BDNF in target muscles was examined in mutant mice in which one or both copies of the NT3 or BDNF genes were replaced by the *lacZ* reporter gene. The mutants used, NT-3*lacZneo* (Fariñas et al., 1996) and BDNF*lacZ* (creation to be described in detail elsewhere), were generated by gene targeting in embryonic stem (ES) cells. In both cases, the second exon coding for the neurotrophin is replaced by the *E. coli* lacZ gene beginning at the predicted start codon, and both are strongly predicted to be null alleles. NT-3*lacZneo* mice were genotyped using PCR. To genotype BDNF*lacZ* mice, one PCR primer (MBDSA10 : GTGGAGTTCTGCTAATGAGA) was located in the mouse genome outside the BDNF coding exon and the other primer was located in the *lacZ* gene (lacZN5 : GTGCTGCAAGGCGATTAAGT) (Vigers et al., in press).

Numbers of Spindles as Indicators of Proprioceptive Afferents

Muscle spindles and proprioceptive neurons are developmentally linked. No spindles develop in the absence of proprioceptive neurons. Each spindle is innervated by one group Ia proprioceptive afferent and 0–3 group II afferents; thus spindles and Ia proprioceptive neurons exist in a 1:1 relationship, and numbers of group II proprioceptive afferents also correlate with numbers of spindles (Zelená, 1957; Kucera and Walro, 1990). A full complement of spindles is present in mouse muscles at birth (Kozeka and Ontell, 1981), thus sections of the craniums of postnatal (P) day 0–4 mice were surveyed for the presence of spindles. Mice anesthetized with sodium pentobarbital (50 mg/kg b.wt., i.p.) were perfused with glutaraldehyde-paraformaldehyde fixative. Epoxy-embedded heads were serially cross-sectioned in the coronal plane at 1 μm thickness. Every 20th section was stained with toluidine blue. Four jaw muscles— the anterior deep masseter (AM), zygomaticomandibularis (ZM), superficial masseter (SM), and internal pterygoideus (PI)—were selected for counts of spindles. Spindle equators were counted so that no spindle was counted twice. Spindle counts were compared between mutant and wild-type muscles using analysis of variance and a Student-Newman-Keuls test.

Immunonocytochemistry of Neurons and Muscle

Types of Trk receptor proteins expressed by TMN neurons and types of myosins present in intrafusal fibers were determined by immunocytochemistry. TrkB or TrkC proteins were detected using a previously characterized polyclonal rabbit trkB$_{23-36}$ antibody against the extracellular regions of rat TrkB receptor (Yan et al., 1997) and a goat antibody against extracellular domains of rat TrkC receptor (Fariñas et al., 1998; Huang et al., 1999). These antibodies are specific and do not cross-react with other forms of Trk. Brain stems of neonatal and adult wild-type mice were fixed with 4% paraformaldehyde, cryoprotected with 30% sucrose, and serially sectioned at 10 or 20 μm. Sections were reacted with primary anti-Trk antibodies as described previously (Yan et al., 1997; Farinas et al., 1998). Binding of anti-Trk antibodies was detected using biotinylated secondary antibodies and Vectastain detection kits (Vector Laboratories, Burlingame, CA). Co-expression of TrkB and TrkC antigens was detected by fluorescent microscopy using a goat anti-rabbit secondary antibody coupled to FITC and a biotinylated rabbit anti-goat secondary antibody, followed by streptavidin-conjugated Texas Red (Huang et al., 1999). The proportion of TMN neurons expressing TrkB was determined from serial brain stem sections stained alternately with the Nissl stain or processed for TrkB immunocytochemistry. The incidence of neurons co-expressing TrkB and TrkC was determined by counting all neurons expressing the two antigens in a random sample of TMN neurons in serial brain stem sections.

We used the S46 antibody to determine whether intrafusal muscle fibers of spindles in neurotrophin-

deficient mice express slow-developmental myosin heavy chain, a spindle marker whose expression is dependent on Ia afferent innervation (Kucera et al., 1998). Heads of mutant and wild-type littermates were excised at P0–P4, fresh frozen, cut transversely into serial 8 μm thick sections and reacted with the S46 antibody reactive to the slow-developmental MyHC using the ABC (avidin-biotin complex) peroxidase method (Kucera et al., 1998).

We used β-galactosidase histochemistry to visualize expression of the *lacZ* reporter gene in NT3^{lacZneo} and BDNF^{lacZ} mutants, using methods described elsewhere (Fariñas et al., 1994; Vigers et al., in press). For staining of cranial whole-mounts, heads fixed with 4% paraformaldehyde were stained overnight at 37°C in X-gal solution at pH 7.3. For staining of sections, heads were stained with X-gal, cryoprotected, frozen, and sectioned at 8 μm (E14.5 BDNF^{lacZ}) or 20 μm (E14.5 and E17.5 NT3^{lacZneo}) thickness. Sections were counterstained with neutral red (BDNF^{lacZ}) or eosin (NT3^{lacZneo}). Blue reaction product marked sites of *lacZ* expression.

Counts of TMN Neurons

The cell bodies of cranial proprioceptive afferents were counted in the trigeminal mesencephalic nucleus (TMN) in brain stem (Copray and Liem, 1993). Paraformaldehyde-fixed brain stems of wild-type and mutant neonates were cryoprotected with 30% sucrose, frozen, serially cut at a thickness of 10 μm and stained with cresyl violet or the Nissl stain (Ernfors et al., 1994b). Counts of TMN neurons containing a nucleolus were not corrected for double nucleoli. A Student's t-test was used to compare neuron counts in mutant and wild-type mice.

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