Studies on the physiological role of brain-derived neurotrophic factor and neurotrophin-3 in knockout mice

PATRIK ERNFORS[1,2], JAN KUCERA[3]*, KUO-FEN LEE[1], JANET LORING[1] and RUDOLF JAENISCH[1]

[1]Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA, [2]Department of Biophysics and Biochemistry, Karolinska Institute, Stockholm, Sweden and [3]Department of Neurology, Boston University School of Medicine, Boston, Massachusetts, USA

ABSTRACT Brain-derived neurotrophic factor and neurotrophin-3 deficient mice were generated by gene targeting. The analysis of these mice has led to the characterization of their role in the survival of neurons in the peripheral nervous system. NT-3 deficient mice displayed severe movement defects and most died shortly after birth. The mutation causes loss of substantial portions of cranial and spinal peripheral sensory and sympathetic neurons. Significantly, spinal proprioceptive afferents and their peripheral sense organs (muscle spindles and Golgi tendon organs) were completely absent in homozygous mutant mice. BDNF deficient mice displayed deficiencies in coordination of movements and balance. Excessive loss of neurons was detected in most of the peripheral sensory ganglia examined, but the survival of sympathetic neurons was not affected. The most marked reduction of neurons was observed in the vestibular ganglion, leading to a loss of innervation of the sensory epithelia of the vestibular compartments of the inner ear.

KEYWORDS: *neurotrophins, peripheral nervous system, programmed cell death, neurotrophic factor*

Introduction

During development of the mammalian nervous system neurons are produced in excess, and during a restricted time period a majority degenerate in a process termed programmed cell death. The neurotrophic nerve growth factor (NGF) was identified more than three decades ago. Pioneering work assessing its biological functions led to the hypothesis that NGF regulates the survival of neurons during the period of programmed cell death. NGF is synthesized by the peripheral cells that receive neuronal innervation and is retrogradely transported to the cell body. Because it is synthesized in limited amounts, only the neurons that successfully compete, survive, and the rest undergo programmed cell death. A retrograde fashion of neurotrophic interactions in the peripheral nervous system (PNS) has been supported by several lines of evidence. Enlargement of the target field reduces the loss of neurons during naturally occurring cell death (Hollyday and Hamburger, 1976; Narayan and Narayan, 1978; Boydston and Sohal, 1979). Conversely, extirpation of the target field results in excessive loss of innervating neurons (Hamburger, 1958; Prestige, 1967; Carr and Simpson, 1978; Chu-Wang and Oppenheim, 1978). Although not yet proven, it is assumed that the regulation of cell death by target-derived NGF ensures that target cells are innervated by the correct number and type of nerve fibers.

The protein purification and DNA cloning of brain-derived neurotrophic factor (BDNF) (Barde *et al.*, 1982; Leibrock *et al.*, 1989) led to the discovery that NGF is the prototype of a family of neurotrophic factors including four structurally related proteins known as neurotrophins. In addition to NGF, BDNF, neurotrophin-3 (NT-3) (Ernfors *et al.*, 1990a; Hohn *et al.*, 1990; Jones and Reichardt, 1990; Kaisho *et al.*, 1990; Maisonpierre *et al.*, 1990; Rosenthal *et al.*, 1990) and neurotrophin-4/5 (NT-4) (Berkemeier *et al.*, 1991; Hallböök *et al.*, 1991; Ip *et al.*, 1992) are members of this gene family. Neurotrophin mRNAs are expressed in peripheral target tissues of sensory and sympathetic innervation during development in a cell-specific, partially overlapping pattern (Ernfors *et al.*, 1992; Pirvola *et al.*, 1992; Arumae *et al.*, 1993; Ibañez *et al.*, 1993). The spatial and temporal specific expression of neurotrophins during development suggests that the expression of these factors is precisely regulated to ensure the correct development of the nervous system.

Neurotrophins exert their physiological effects by binding to receptors with different affinities (Banerjee *et al.*, 1973; Herrup and Shooter, 1973; Richardson *et al.*, 1986; Sutter *et al.*, 1979). All neurotrophins bind a 75,000 dalton (P75) receptor with similar low-affinities (Ernfors *et al.*, 1990a; Rodriguez-Tebar *et al.*, 1990, 1992; Hallböök *et al.*, 1991; Squinto *et al.*, 1991). P75 is expressed in the populations of neurons known to respond to the neurotrophins (Ernfors *et al.*, 1988, 1989, 1991; Koh *et al.*, 1989; Large *et al.*, 1989; Hallböök *et al.*, 1990; Pioro and Cuello, 1990). The biological role of the P75 has not been resolved yet,

***Address for reprints:** Department of Neurology, Boston University School of Medicine, Boston, Massachusetts 02118, USA. FAX: 1-617.2586505.

0214-6282/95/$03.00

since signal transduction is mediated only by binding of the ligands to the high-affinity receptors. However, the phenotype of mice carrying a mutation of the P75 gene suggests that the low-affinity receptor is also important for neurotrophin interactions *in vivo* (Lee *et al.,* 1992) and it modulates ligand interactions with the high-affinity receptor (Davies *et al.,* 1993; Lee *et al.,* 1994). The high-affinity receptors are restricted to a sub-population of neurons expressing P75 (Banerjee *et al.,* 1973; Herrup and Shooter, 1973; Sutter *et al.,* 1979; Richardson *et al.,* 1986). The identification and cDNA cloning of the members of the trk family of tyrosine kinase receptors lead to the discovery that these are signal transducing receptors for the neurotrophins. NGF binds and activates the trkA receptor (Cordon-Cardo *et al.,* 1991; Kaplan *et al.,* 1991; Klein *et al.,* 1991), BDNF and NT-4 share the signal-transducing receptor trkB (Berkemeier *et al.,* 1991; Glass *et al.,* 1991; Soppet *et al.,* 1991; Squinto *et al.,* 1991) and NT-3 binds and activates trkC (Lamballe *et al.,* 1991). There are conflicting results on whether NT-3 also interacts with the trkA and trkB receptors. However, in a more neuronal-like context of PC12 cells, NT-3 insufficiently activates the trkA and trkB receptors (Ip *et al.,* 1993). Until recently there has been little information on the function of neurotrophins *in vivo.* The establishment of mice carrying inactivated genes for the neurotrophins (Crowley *et al.,* 1994; Ernfors *et al.,* 1994a,b; Jones *et al.,* 1994) has provided a unique opportunity to study their physiological functions *in vivo.*

Results and Discussion

BDNF knockout mice develop with severe deficits in the peripheral sensory nervous system and lack vestibular functions

Mice carrying a deletion of the BDNF gene were generated by homologous recombination in embryonic stem (ES) cells. These mice provide a powerful tool in studies of the physiological functions of BDNF during development (Ernfors *et al.,* 1994a). Mice carrying a deleted BDNF gene (BDNF mutant mice) developed with a marked reduction of neurons in all peripheral sensory ganglia examined (Table 1). Homozygous mutant mice were reduced in size and most died during the second postnatal week. A few mutant mice survived for three months. The mice displayed a striking phenotype with deficiencies in coordination of movements and balance, head bobbing and tilting, and spinning in periods of hyperactivity followed by immobility. This behavior is suggestive of an abnormal labyrinth innervation of the vestibular compartments of the inner ear (see below).

The BDNF mutant mice displayed a loss of neurons in all the peripheral sensory ganglia examined, showing for the first time that BDNF is required at physiological concentrations for the survival of neurons *in vivo* (Ernfors *et al.,* 1994a). However, the survival of α-motor neurons and sympathetic superior cervical ganglion neurons was not affected in BDNF mutant mice (Ernfors *et al.,* 1994a; Jones *et al.,* 1994). Recently we have counted ventral root axons in semi-thin sections and found that neither α-motor neurons nor γ-motor neurons were severely affected by the lack of BDNF (Ernfors *et al.,* 1994a and unpublished results). The finding that α-motor neurons develop in BDNF mutant mice was unexpected. Lesioned neonatal motor neurons as well as embryonic spinal cord motor neurons can be rescued from cell death by injecting BDNF (Oppenheim *et al.,* 1992; Sendtner *et al.,* 1992; Yan *et al.,* 1992; Koliatsos *et al.,* 1993). Because BDNF and NT-4 share signal transduction receptors, and NT-4 can also rescue lesioned neonatal motor neurons (Koliatsos *et al.,* 1994), it is possible that NT-4, but not BDNF, is physiologically required for the survival of motor neurons. However, motor neurons respond to a large number of trophic factors in culture (Hughes *et al.,* 1993). It is therefore also possible that they respond to several neurotrophic factors *in vivo,* and that the lack of a single factor does not critically compromise motor neuron survival. The normal development of superior cervical ganglion neurons in the BDNF mutant mice is consistent with previous culture studies, where these neurons have been shown to be unresponsive to BDNF in terms of survival and neurite outgrowth (Barde *et al.,* 1982; Lindsay *et al.,* 1985). Furthermore, chick sympathetic neurons do not contain any high-affinity receptors for BDNF (Rodriguez-Tebar and Barde, 1988).

BDNF mutant mice display a 45% loss of trigeminal ganglion neurons and a 50% loss of mesencephalic trigeminal neurons (Ernfors *et al.,* 1994a), counted at postnatal day 14. The severe loss of trigeminal ganglion neurons is represented by only a 16% reduction of volume of the trigeminal ganglion at P0 (Jones *et al.,* 1994). A physiological role for BDNF on trigeminal neurons is consistent with previous mRNA studies. All neurotrophins are expressed in a peripheral target for trigeminal neurons, the whisker pad (Bandtlow *et al.,* 1987; Ernfors *et al.,* 1992; Ibañez *et al.,* 1993). At E13 NGF, NT-3 and NT-4 mRNAs are expressed in the surface epithelium and proximal mesenchyme of the rat whisker pad. At later stages of embryonic development the levels are markedly decreased in these structures (Ernfors *et al.,* 1992; Ibañez *et al.,* 1993) and at E16 and E18, NT-3 and NT-4 mRNAs are detected in whisker follicles (Ernfors *et al.,* 1992; Ibañez *et al.,* 1993). NT-4 mRNA is expressed in both the external and internal root sheath (ERS and IRS, respectively), while NT-3 mRNA is detected only in the ERS. BDNF mRNA is expressed throughout the deep mesenchyme in the maxillary process at E13, after which it decreases several fold (Ernfors *et al.,* 1992; Ibañez *et al.,* 1993). In agreement with this, trigeminal ganglion neurons express the BDNF receptor, trkB, (Klein *et al.,* 1990; Ernfors *et al.,* 1992) and BDNF elicits a survival effect on cultured trigeminal neurons (Lindsay *et al.,* 1985; Leibrock *et al.,* 1989; Ibañez *et al.,* 1993). The initial target encounter in the mouse occurs at E10.5 and peaks at E12, but new fibers are recruited until E15 (Davies and Lumsden, 1984). More than 50% of the trigeminal ganglion neurons degenerate during the period of naturally occurring cell death, which peaks between E13 and E15 (Davies and Lumsden, 1984). From whole-embryo immunostaining for neurofilament, mice lacking BDNF do not appear to have any deficiencies of the initial axon extension and growth of nerve fibers to the whisker pad (Ernfors *et al.,* 1994a). It is clear, however, that BDNF is physiologically important for the survival of these neurons at later stages because of the loss of almost half of the trigeminal ganglion neurons in the neonatal BDNF mutant mice. Central organization of trigeminal innervation is represented by barellets in the brain stem and barrells in the somatosensory cortex. The presence of both barellets (unpublished results) and barells (Jones *et al.,* 1994; unpublished results) in the BDNF mutant mice is an important finding. These results are consistent with the lack of BDNF mRNA

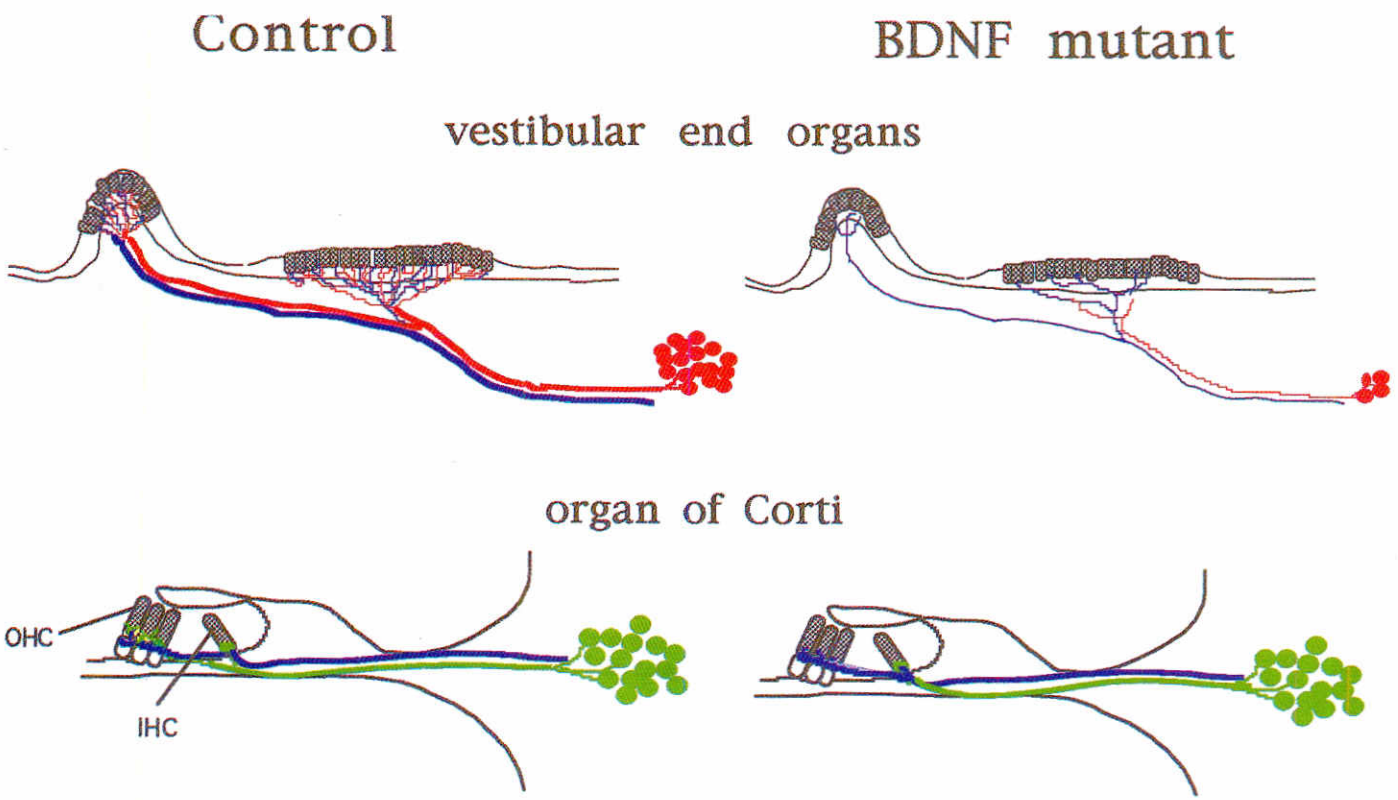

Fig. 1. Defects of inner ear innervation in BDNF knockout mice. (A) *Vestibular afferents (red) fail developmentally to innervate the sensory epithelia of the saccular and utricular macula and terminate in the adjacent connective tissue. Afferent fibers never reach the ampullary crista. More than 80% of the vestibular ganglion neurons are lost in the BDNF mutant mice. Efferent fibers (blue) innervate the hair cell normally, but most are lost between the first and fourth week after birth. The hair cells of the utricular macula and ampullary crista are shaded.* **(B)** *Spiral ganglion afferents (green) innervate the inner hair cells (IHC) of the organ of Corti, but fail developmentally to reach the outer hair cells (OHC). Efferents (blue) innervating the organ of Corti appears normal in the BDNF mutant mice.*

expression in these brain stem nuclei (Ernfors *et al.*, 1990b; Phillips *et al.*, 1990), and suggests that the lack of peripheral BDNF may not affect its central topographic innervation.

BDNF mutant mice display a 65% loss of nodose ganglion neurons and a 30% loss of lumbar dorsal root ganglion (DRG) neurons (Ernfors *et al.*, 1994a) showing that BDNF is required for the survival of these neurons *in vivo*. Consistent with these results, BDNF injected into the embryo prevents naturally occurring cell death of dorsal root and nodose ganglion neurons in the chick (Kalcheim *et al.*, 1987; Hofer and Barde, 1988), and most nodose and some embryonic dorsal root ganglion neurons express trkB mRNA (Klein *et al.*, 1990; Ernfors *et al.*, 1992). Furthermore, dorsal root ganglion neurons have been shown to contain high-affinity BDNF receptors (Rodriguez-Tebar and Barde, 1988). Because trkB mRNA expressing dorsal root ganglion neurons are mostly of medium size, it is believed that the BDNF responsive neurons include those mediating low-threshold mechanoreception (Mu *et al.*, 1993). In support of this, Jones *et al.* (1994) found a 37% loss of myelinated L5 dorsal root axons in mice lacking BDNF, which presumably convey mechanoreception. The affected modalities have, however, not been identified yet. Preliminary results show that the lack of BDNF does not cause deficiencies in the development of Paccinian corpuscles, muscle spindles or CGRP positive central fibers in spinal cord lamina III and IV (Ernfors *et al.*, 1994a; Jones *et al.*, 1994 and unpublished results).

The circling and spinning and the defects in coordination of movements of the BDNF mutant mice suggested an abnormal labyrinth innervation, consistent with previous mRNA and culture studies on the vestibuloacoustic system. The peripheral targets of the vestibuloacoustic ganglion contain trophic activity which supports the survival of these neurons in culture (Hauger *et al.*, 1989; Hemond and Morest, 1992). Recently, vestibular and spiral ganglion neurons have been shown to express trkB and trkC mRNAs (Ernfors *et al.*, 1992; Ylikoski, *et al.*, 1993), and the sensory epithelia of the inner ear contain mRNAs for BDNF and NT-3 but not NGF or NT-4 (Ernfors *et al.*, 1992; Pirvola *et al.*, 1992; Ylikoski, *et al.*, 1993). In the embryonic and neonatal rat, BDNF and NT-3 mRNAs are expressed in a partially overlapping pattern. NT-3 mRNA is localized to both the differentiating hair cells and surrounding supporting cells of the cochlear and vestibular sensory epithelia, whereas BDNF mRNA is localized exclusively to the differentiating hair cells (Pirvola *et al.*, 1992). In addition, BDNF, but not NT-3 mRNA, is expressed in the sensory epithelia of the crista ampullary (Ernfors *et al.*, 1992; Pirvola *et al.*, 1992). The expression of BDNF and NT-3 in the adult is more restricted. NT-3 mRNA is present in inner hair cells of the cochlea and BDNF mRNA in the

TABLE 1

LOSS OF SENSORY AND SYMPATHETIC NEURONS IN BDNF AND NT-3 MUTANT MICE

		percent of control (± SEM)
Trigeminal ganglion		
	wild type	100±8
	NT3-/-	36±6
	BDNF-/-	56±2
MeV		
	wild type	100±4
	NT3-/-	48±5
	BDNF-/-	52±2
Vestibular ganglion		
	wild type	100±3
	NT3-/-	96±5
	BDNF-/-	18±4
Nodose ganglion		
	wild type	100±5
	NT3-/-	53±18
	BDNF-/-	34±3
L4 DRG		
	wild type	100±5
	NT3-/-	45±12
	BDNF-/-	70±3
SCG		
	wild type	100±9
	NT3-/-	47±14
	BDNF-/-	114±6
Facial mn		
	wild type	100±6
	NT3-/-	98±9
	BDNF-/-	97±6

The values were not corrected for split nucleoli. Abbreviations: L4 DRG, lumbar level 4 dorsal root ganglion; MeV, mesencephalic trigeminal nucleus; mn, motor nucleus; SCG, superior cervical ganglion. (Data from Ernfors *et al.*, 1994a,b)

hair cells of the vestibular compartments (Ylikoski *et al.*, 1993). In culture, BDNF and NT-3 have mitogenic effects on vestibuloacoustic neurons (Represa *et al.*, 1993) and at later developmental stages act sequentially to promote the survival of neurons. Both spiral and vestibular ganglion neurons respond to BDNF and NT-3 at the time of target encounter; however, at later stages, neuronal survival is virtually only promoted by BDNF (Avila *et al.*, 1993). In agreement with these studies, more than 80% of the vestibular ganglion neurons were lost in the BDNF mutant mice, and innervation of the sensory epithelia of the saccular and utricular macula and the crista of the semicircular ducts was absent (Ernfors *et al.*, 1994a). The behavioral abnormalities of the BDNF mutant mice, including circling and spinning, was an expected consequence of defective vestibular innervation and is typically seen in mutant mice such as waltzer, shaker or jerker, which have innervation defects or malformation of the inner ear (Lyon and Searle, 1989). Preliminary results from studies of the BDNF mutant mice at embryonic and postnatal stages indicate no deficiencies of the initial target encounter of the saccular and utricular afferents. Shortly before birth, however, when afferents normally invade the sensory epithelia, the afferents of the BDNF mutant mice fail to contact the vestibular sensory epithelia and remain in the adjacent connective tissue (deficiencies of BDNF mutant mice are depicted in Fig. 1). These results suggest that BDNF acts at the cellular level and is a requirement for establishing afferent innervation of the saccular and utricular macula. Afferents innervating the sensory epithelia of the crista of the lateral semicircular duct never reach the ampulla, and there is an absence of afferents in both the epithelia and adjacent connective tissue. In contrast to the other vestibular compartments where both BDNF and NT-3 mRNAs are expressed, only BDNF mRNA is present in the crista of the semicircular ducts. Thus, depletion of BDNF in mice leads to a failure of both target encounter and innervation of the crista of the semicircular ducts. Initial efferent innervation of the vestibular inner ear compartments was not affected by the lack of BDNF. However, between postnatal days 6 and 25, when efferents make synaptic contacts and mature, the majority are lost. It is possible that synaptogenesis and maturation of the efferent system depend on afferent innervation, in which case, a lack of afferents may lead to the deficiencies of efferents. Within the cochlea, lack of BDNF did not affect the survival of spiral ganglion neurons, or the innervation of inner hair cells in the organ of corti. However, spiral ganglion afferents failed to innervate cochlear outer hair cells. Efferent innervation of the cochlea was not affected and both acetylcholine esterase-positive fibers and neurofilament 200 kDa-positive fibers were present at both

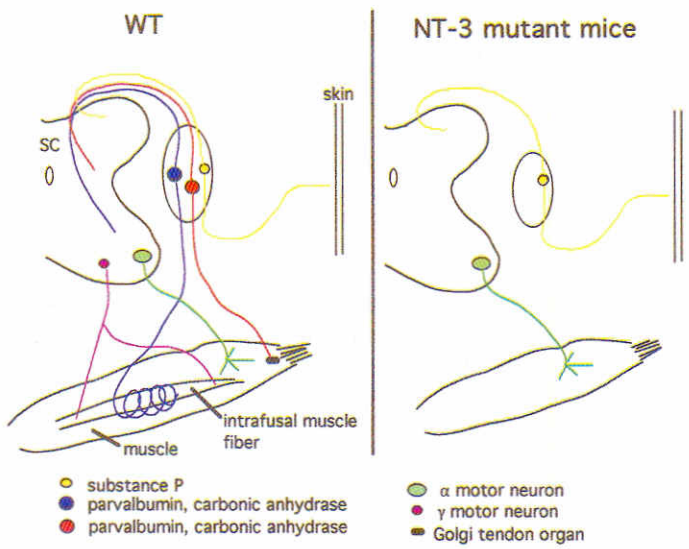

Fig. 2. Loss of limb proprioceptive afferents and fusimotor efferents in NT-3 mutant mice. *The NT-3 mutant mice lack the two main proprioceptive sensory end organs, muscle spindles (innervated by Ia and II afferents) and Golgi tendon organs (Innervated by Ib afferents). The proprioceptive Ia afferents are absent in the spinal cord and no afferents are present in the muscle. There is a loss of more than 50% of the lumbar dorsal root ganglion neurons. Immunocytochemistry revealed an absence of carbonic anhydrase and parvalbumin positive dorsal root ganglion neurons, which are markers associated with proprioceptive neurons. Furthermore, the lack of NT-3 also leads to a loss of the motor efferents innervating muscle spindles. In addition to a loss of the components associated with the muscle spindle, Golgi tendon organs are absent in the NT-3 mutant mice. Whereas all major components mediating limb proprioceptive functions are absent in the NT-3 mutant mice, cutaneous substance P-positive fibers, Paccininan corpuscles and deep nerve fibers in the joint and tendon appear normal.*

the inner and outer hair cells. Thus, these results suggest novel roles for the neurotrophins. BDNF appears to be required at physiological concentrations, not only for the survival of particular neurons, but also for collateral branching and innervation of some targets.

NT-3 knockout mice develop with severe deficits in the peripheral sensory and sympathetic nervous system and lack limb proprioception

Mice carrying a deletion of the NT-3 gene were generated by homologous recombination in ES cells. Mice carrying a deleted BDNF gene (NT-3 mutant mice) develop with a marked reduction of neurons in most peripheral sensory ganglia examined (Table 1) (Ernfors *et al.,* 1994b). Homozygous mutant mice were reduced in size and the majority died shortly after birth. Those surviving postnatal day 0 often lived to the third postnatal week. Mutant mice displayed limb ataxia and an apparent inability to position the extremities properly when attempting to move. There was a tendency for all four limbs to intermittently stiffen in an extensor posture. This abnormality resembled the rigidity and cross-extensor reflex observed in limbs of experimentally deafferented animals (Ranson, 1931; Wiesendanger, 1964), and suggested a loss of proprioceptive sensation in the NT-3 mutant mice. The phenotype was apparent shortly after birth.

At autopsy, most peripheral ganglia were markedly smaller in the homozygous mutant mice as compared with control mice. Loss of neurons in cranial and spinal ganglia was quantitated. The NT-3 mutant mice displayed loss of neurons in all the peripheral sensory and sympathetic ganglia examined (Table 1) (Ernfors *et al.,* 1994b). However, the survival of α-motor neurons was not affected in the NT-3 mutant mice. These results are consistent with previous results from culture studies and manipulations of the embryo, and confirm a physiological role for NT-3 during development of the nervous system.

The loss of neurons in the DRG and movement defects suggested a deficiency of limb proprioception. Limb proprioceptive sense organs include muscle spindles (innervated by type Ia and II afferents and fusimotor efferents), Golgi tendon organs (innervated by type Ib afferents), and joint receptors. During development, afferent innervation by Ia neurons induces the formation of muscle spindles, and innervation by Ib neurons induces the formation of Golgi tendon organs (Zelena, 1957; Kucera and Walro, 1992). Muscle spindles and Golgi tendon organs were counted in the soleus muscle in semi-thin plastic sections. Control mice contained an average of 11.4 muscle spindles, whereas mutant mice were completely devoid of spindles in all hindlimb muscles, including the soleus (Ernfors *et al.,* 1994b). The loss of spindles was revealed by the absence of encapsulated bundles of small caliber muscle fibers innervated by afferents. The muscles of mutant mice were also devoid of any expression of the slow-tonic myosin heavy chain isoform, which is specifically expressed in the intrafusal muscle spindles (Kucera and Walro, 1992) and electron microscopic examination of mutant muscles revealed no evidence of any afferent nerve fiber-muscle contacts. Mice heterozygous for the NT-3 mutation displayed half the normal complement of muscle spindles, indicating that NT-3 is produced at limiting amounts in the embryo. The loss of the peripheral Ia afferents was represented also by a loss of the central Ia fibers in the spinal cord. The proprioceptive

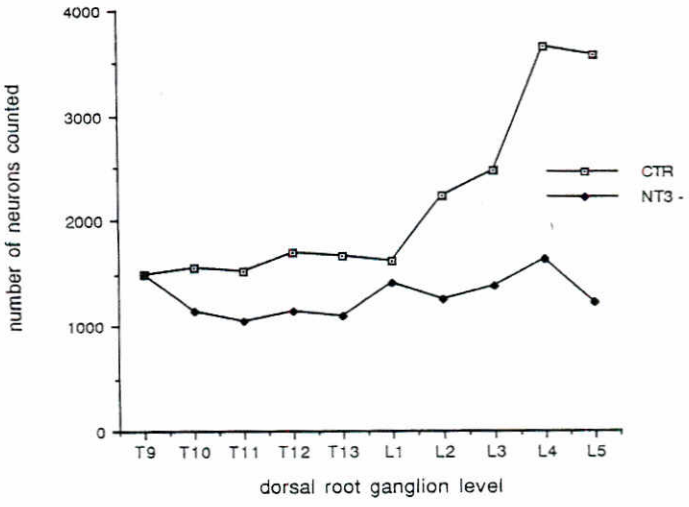

Fig. 3. Loss of neurons in the dorsal root ganglia of some thoracic and lumbar levels. *The most pronounced loss of neurons can be seen in ganglia supplying the limbs.*

Ia afferents are part of the muscle-motor neurons-muscle circuit that mediates the stretch reflex and make monosynaptic contacts with spinal cord motor neurons. The deficiency was revealed by the absence of DiI traced central DRG afferents innervating spinal cord layer IX (Ernfors *et al.,* 1994b). In addition to the loss of Ia afferents and their sensory organs, NT-3 mutant mice lacked Golgi tendon organs. Whereas control mice contained 3-4 Golgi tendon organs in the myotendonous junction, Golgi tendon organs were completely absent in NT-3 mutant mice (Ernfors *et al.,* 1994b). Thus, the mutation affected both type Ia and Ib afferents. In addition to a complete absence of muscle spindles and Golgi tendon organs, the mutant mice lacked the γ-peak of motor axons in the ventral root. This deficiency was revealed by the absence of the small-caliber myelinated nerve fibers in a frequency histogram of fiber sizes of the L4 ventral root (unpublished results). Mice heterozygous for the mutation contained approximately half the normal complement of small-myelinated fibers in the ventral root, indicating a haploinsufficiency of NT-3 also for the development of γ-motor neurons. Thus, NT-3 appears physiologically required for the development of both muscle spindle afferents and efferents (Fig. 2).

We have addressed the role of neurotrophins in the differentiation of the proprioceptive neurons and endorgans by electron microscopical examination of spindle development in NT-3 mutant mice. In E15-17 control mice, undifferentiated nerve-muscle contacts were observed. Definitive motor nerve-muscle contacts were observed at E17-18 by the presence of axon terminals with synaptic vesicles and a distinct basal lamina with a relatively wide synaptic cleft. Probable and definite sensory-muscle contacts were identified in control mice at E17 and E18, respectively, present only on muscle fibers enclosed by spindle capsule. NT-3 mutant mice contained motor nerve-muscle contacts, but no afferents or encapsulated muscle fibers at E17 and E18 (unpublished results). Thus, because a degeneration of newly-formed spindles was not detected, NT-3 appears to be required for the induction of muscle spindles. This suggests that

NT-3 could be required for the collateral sprouting and target innervation, as has been shown for BDNF in the inner ear (see above). Together, these findings show that NT-3 is required for the development of the afferent and efferent components of the limb proprioceptive system and that the induction of the spindles may depend on NT-3.

Proprioceptive DRG neurons are believed to stain for parvalbumin and carbonic anhydrase. Parvalbumin positive neurons in the L4 DRG are completely missing in NT-3 mutant mice and only a few carbonic anhydrase positive neurons are present (Ernfors *et al.,* 1994b). An absence of proprioceptive DRG neurons is correlated with a marked reduction in the number of neurons in the lumbar DRGs, but a more subtle loss in the thoracic ganglia (Fig. 3). This agrees with the notion that proprioceptive neurons are more abundant in the cervical and lumbar DRGs, which project to the limbs, than at the thoracic levels. Unexpectedly, the examination of plastic sections of the DRG did not reveal the loss of any single ultrastructural class of neurons in NT-3 mutant mice. Both small, medium and large neurons were present in NT-3 mutant mice, indicating that proprioceptive DRG neurons may not be of a single class of DRG neurons. In a size-frequency histogram, however, the neuronal loss appeared to occur predominantly in the population of large neurons (unpublished results). These results suggest that NT-3 is required for either the survival of committed proprioceptive neurons or the differentiation of the proprioceptive phenotype.

The source(s) of NT-3 that support the DRG neurons which require NT-3 for survival is not known. Principally, proprioceptive DRG neurons could be supported by NT-3 from the central or peripheral target, from Schwann cells in the nerve, or from the dependent DRG neuron itself. Spinal cord motor neurons express NT-3 mRNA prior and during muscle spindle induction and this expression is down-regulated shortly after birth (Ernfors and Persson, 1991), when the muscle spindles are formed. NT-3 mRNA is also known to be expressed in the E15 rat muscle (Henderson *et al.,* 1993), although the exact temporal and spatial expression during development are not known. In addition, NT-3 mRNA is expressed by developing DRG neurons (Ernfors *et al.,* 1992) and in the adult sciatic nerve (Funakoshi *et al.,* 1993). Thus, NT-3 produced in any, or all, of these tissues could be the source of the factor required for the development of muscle spindles. A more detailed mRNA expression study in fetal tissues should shed some light on this issue.

Dorsal root ganglion neurons convey many different modalities of sensory information from the outside world to the brain. This requires a large number of different types of neurons, characterized by their peripheral and central projections, ultrastructure, size, membrane properties, neurotransmitter and other phenotypic markers. Expression of mRNAs for the trkA, trkB and trkC has been associated with small, medium and large diameter DRG neurons (Mu *et al.,* 1993), respectively, and proprioceptive and other low-threshold neurons are believed to be included in the populations of medium and large diameter neurons. Antibody deprivation *in utero* or inactivation of NGF or the NGF receptor (trkA) gene in mice leads to a loss of up to 80% of the DRG neurons belonging to the population of small diameter DRG neurons (Johnson *et al.,* 1980; Crowley *et al.,* 1994; Klein *et al.,* 1994). These neurons project to superficial layers of the spinal cord dorsal horn (Ruit *et al.,* 1992) and presumably subserve nociception and thermoreception. The selective role of NGF for nociception has functionally been characterized and NGF supports the Aδ cutaneous pain receptors *in vivo* (Ritter *et al.,* 1991). The loss of primarily the medium and large diameter DRG neurons and loss of the proprioceptive system in NT-3 mutant mice agrees with previous culture studies where NT-3 supported survival of DRG neurons that innervate muscle (Hory-Lee *et al.,* 1993), and suggests that the survival of DRG neurons of different sensory modalities may depend on different neurotrophins *in vivo.*

Materials and Methods

Gene targeting, generation of mutant mice and Southern blot analysis

BDNF and NT-3 mutant mice were established as described in Ernfors *et al.* (1994a,b). For embryonic studies, the day of vaginal plug was considered as day 0. For genotyping of mice, DNA was purified according to Laird *et al.* (1991) and digested with restriction endonucleases, fractionated by electrophoresis through 0.8% agarose gels, blotted with 20xSSC onto nylon membranes (Zetabind) and hybridized to a ^{32}P labeled fragment external to the targeting construct.

Histological methods

For immunocytochemistry, mice were perfused with 4% paraformaldehyde, dissected, and postfixed for 2 h. Sections were cut at 7 μm on a cryostat and preincubated in dilution buffer (0.5 M NaCl, 0.01 M phosphate buffer pH 7.3, 3% bovine serum albumin and 0.3% triton X100) for 1 h followed by overnight incubation with the indicated concentration of antisera in dilution buffer. After 4 washes in phosphate buffered saline, sections were incubated for 2 h with the appropriate rhodamine conjugated secondary antiserum, washed 3 times for 10 min, covered with glycerol/PBS (9:1) for viewing. For immunocytochemical detection of slow tonic MHC, the hindlimbs were quenched in isopentane cooled to -160°C with liquid nitrogen, and cut transversely into serial sections of 8 μm thickness from the ankle to the knee in a cryostat. The sections were immunoreacted with ALD 19 antibody using the avidin-biotin complex method, as previously described (Kucera and Walro, 1992).

For histology of muscle spindles, Golgi tendon organs and Pacinian corpuscles, mice were perfused, the hindlimbs excised and embedded into plastic. Serial sections of 1 μm thickness were cut from the ankle to the knee on an ultramicrotome and stained with toluidine blue. Tissue for electron microscopy was processed as described for the semi-thin sections.

DiI tracing

P0 or P6 mice were fixed for 6 h to overnight in 4% paraformaldehyde. For tracing of DRG afferents, the cervical spinal cord with the dorsal root ganglia attached was dissected, the pia membrane was carefully removed and the ventral roots were cut. The spinal cords were pinned down on sylgard gel Petri dishes with insect pins and submerged in 4% paraformaldehyde. Individual C5-6 dorsal root ganglia were injected with 0.25% DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes) in dimethyl formamide using a 20 μm thick glass pipette, and incubated for 7 days at 37°C. For tracing of vestibular afferents, the brain was carefully lifted from the olfactory bulbs and a crystal of DiI was applied to the 7th nerve; the nerve was cut distal to the applied DiI. The brains were incubated for 2 weeks at 50°C. The spinal cords and brains were rinsed several times in phosphate buffered saline followed by embedding in 2% agar at 50°C. Cross sections (70 μm) were cut on a vibratom and viewed with rhodamine filter on a Zeiss microscope.

Acknowledgments

We greatly appreciated technical assistance from Jessica Dausman and Ruth Curry. This research was supported by the NIH grant # 5-R35-CA44339 to RJ, and NIH grant # 5-R01-NS25796 to JK. PE was supported by a fellowship from the Swedish Medical Research Council and KFL by Amgen Inc.

References

ARUMAE, U., PIRVOLA, U., PALGI, J., KIEMA, T.R., PALM, K., MOSHNYAKOV, M., YLIKOSKI, J. and SAARMA, M. (1993). Neurotrophins and their receptors in rat peripheral trigeminal system during maxillary nerve growth. *J. Cell Biol. 122:* 1053-1065.

AVILA, M.A., VARELA-NIETO, I., ROMERO, G., MATO, J.M., GIRALDEZ, F., VAN DE WATER, T.R. and REPRESA, J. (1993). Brain-derived neurotrophic factor and neurotrophin-3 support survival and neuritogenesis response of developing cochleovestibular ganglion neurons. *Dev. Biol. 159:* 266-275.

BANDTLOW, C.E., HEUMANN, R., SCHWAB, M.E. and THOENEN, H. (1987). Cellular localization of nerve growth factor synthesis by *in situ* hybridization. *EMBO J. 6:* 891-899.

BANERJEE, S.P., SNYDER, S.H., CUATRECASAS, P. and GREENE, L.A. (1973). Binding of nerve growth factor receptor in sympathetic ganglia. *Proc. Natl. Acad. Sci. USA 70:* 2519-23.

BARDE, Y-A., EDGAR, D. and THOENEN, H. (1982). Purification of a new neurotrophic factor from mammalian brain. *EMBO J. 1:* 549-553.

BERKEMEIER, L., WINSLOW, J., KAPLAN, D., NICOLICS, K., GOEDDEL, D. and ROSENTAL, A. (1991). Neurotropin-5: a novel neurotropic factor that activates trk and trkB. *Neuron 7:* 857-866.

BOYDSTON, W. and SOHAL, G. (1979). Grafting of additional periphery reduces embryonic loss of neurons. *Brain. Res. 178:* 403-410.

CARR, V. and SIMPSON, S. (1978). Proliferative and degenerative events in the early development of chick dorsal root ganglia. II. Response to altered peripheral fields. *J. Comp. Neurol. 182:* 741-756.

CHU-WANG, V. and OPPENHEIM, R. (1978). Cell death of motoneurones in the chick embryo spinal cord. I. A light and electron microscopy study of naturally occurring and induced cell loss during development. *J. Comp. Neurol. 177:* 33-58.

CORDON-CARDO, C., TAPLEY, P., JING, S., NANDURI, V., O'ROURKE, E., LAMBALLE, R., KOVARY, K., KLEIN, R., JONES, K., REICHARDT, L. and BARBACID, M. (1991). The trk tyrosine kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. *Cell 66:* 173-183.

CROWLEY, C., SPENCER, S.D., NISHIMURA, M.C., CHEN, K.S., PITTS-MEEK, S., ARMANINI, M.P., LING, L.H., McMAHON, S.B., SHELTON, D.L., LEVINSON, A.D. and PHILLIPS, H.S. (1994). Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. *Cell 76:* 1001-1012.

DAVIES, A. and LUMSDEN, A. (1984). Relation of target encounter and neuronal death to nerve growth factor responsiveness in the developing mouse trigeminal ganglion. *J. Comp. Neurol. 223:* 124-137.

DAVIES, A.M., LEE, K.F. and JAENISCH, R. (1993). p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. *Neuron 11:* 565-574.

ERNFORS, P. and PERSSON, H. (1991). Developmentally regulated expression of HDNF/NT-3 mRNA in rat spinal cord motoneurons and detection of BDNF mRNA in embryonic dorsal root ganglion. *Eur. J. Neurosci. 3:* 953-961.

ERNFORS, P., HALLBÖÖK, F., EBENDAL, T., SHOOTER, E., RADEKE, M. J., MISKO, T. P. and PERSSON, H. (1988). Developmental and regional expression of β-Nerve Growth Factor receptor mRNA in the chick and rat. *Neuron 1:* 983-996.

ERNFORS, P., HENSCHEN, A., OLSON, L. and PERSSON, H. (1989). Expression of nerve growth factor receptor mRNA is developmentally regulated and increased after axotomy in rat spinal cord motoneurons. *Neuron 2:* 1605-1613.

ERNFORS, P., IBAÑEZ, C.F., EBENDAL, T., OLSON, L. and PERSSON, H. (1990a). Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain. *Proc. Natl. Acad. Sci. USA 87:* 5454-5458.

ERNFORS, P., LEE, K-F. and JAENISCH, R. (1994a). Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature 368:* 147-150.

ERNFORS, P., LEE, K-F., KUCERA, J. and JAENISCH, R. (1994b). Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell 77:* 503-512.

ERNFORS, P., MERLIO, J-P. and PERSSON, H. (1992). Cells expressing mRNA for neurotrophins and their receptors during embryonic rat development. *Eur. J. Neurosci. 4:* 1140-1158.

ERNFORS, P., WETMORE, C., ERIKSDOTTER-NILSSON, M., BYGDEMAN, M., STRÖMBERG, I., OLSON, L. and PERSSON, H. (1991). The nerve growth factor receptor gene is expressed in both neuronal and non-neuronal tissues in the human fetus. *Int. J. Dev. Neurosci. 9:* 57-66.

ERNFORS, P., WETMORE, C., OLSON, L. and PERSSON, H. (1990b). Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. *Neuron 5:* 511-526.

FUNAKOSHI, H., FRISEN, J., BARBANY, G., TIMMUSK, T., ZACRISSON, O., VERGE, V.M. and PERSSON, H. (1993). Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. *J. Cell Biol. 123:* 455-465.

GLASS, D.J., NYE, S.H., HANTZOPOULOS, P., MACCHI, M.J., SQUINTO, S. P., GOLDFARB, M. and YANCOPOULOS, G.D. (1991). TrkB mediates BDNF/NT-3-dependent survival and proliferation in fibroblasts lacking the low affinity NGF receptor. *Cell 66:* 405-131.

HALLBÖÖK, F., AYER-LELIEVRE, C., EBENDAL, T. and PERSSON, H. (1990). Expression of nerve growth factor receptor mRNA during early development of the chicken embryo: emphasis on cranial ganglia. *Development 108:* 693-704.

HALLBÖÖK, F., IBAÑEZ, C.F. and PERSSON, H. (1991). Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in *Xenopus* ovary. *Neuron 6:* 1-20.

HAMBURGER, V. (1958). Repression versus peripheral control of differentiation in motor hypoplasia. *Am. J. Anat. 102:* 365-410.

HAUGER, S., BOOK, K. and MOREST, D. (1989). Trophic support of the developing cochleovestibular ganglion by its peripheral target *in vitro* does not depend on neuronal cell division. *Neuroscience 33:* 241-251.

HEMOND, S.G. and MOREST, D.K. (1992). Trophic effects of otic epithelium on cochleo-vestibular ganglion fiber growth *in vitro*. *Anat. Rec. 232:* 273-284.

HENDERSON, C.E., CAMU, W., METTLING, C., GOVIN, A., POULSEN, K., KARIHALOO, M., RULLAMAS, J., EVANS, T., McMAHON, S.B. and ARMANINI, M.P. (1993). Neurotrophins promote motor neuron survival and are present in embryonic limb bud. *Nature 363:* 213-214.

HERRUP, K. and SHOOTER, E.M. (1973). Properties of the B-nerve growth factor receptor of avian dorsal root ganglia. *Proc. Natl. Acad. Sci. USA 70:* 3884-3888.

HOFER, M.M. and BARDE, Y-A. (1988). Brain-derived neurotrophic factor prevents neuronal death *in vivo*. *Nature 331:* 261-262.

HOHN, A., LEIBROCK, J., BAILEY, K. and BARDE, Y.A. (1990). Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature 344:* 339-341.

HOLLYDAY, M. and HAMBURGER, V. (1976). Reduction of naturally occurring motor neuron loss by enlargement of the periphery. *J. Comp. Neurol. 170:* 311-320.

HORY-LEE, F., RUSSEL, M., LINDSAY, R.M. and FRANK, E. (1993). Neurotrophin-3 supports the survival of developing muscle sensory neurons in culture. *Proc. Natl. Acad. Sci. USA 90:* 2613-2617.

HUGHES, R.A., SENDTNER, M. and THOENEN, H. (1993) Members of several gene families influence survival of rat motoneurons *in vitro* and *in vivo*. *J. Neurosci. Res. 36:* 663-671.

IBAÑEZ, C.F., ERNFORS, P., TIMMUSK, T., IP, N.Y., ARENAS, E., YANCOPOLOUS, G.D. and PERSSON, H. (1993). Neurotrophin-4 is a target-derived neurotrophic factor for neurons of the trigeminal ganglion. *Development 117:* 1345-1353.

IP, N., IBAÑEZ, C., NYE, S., McCLAIN, J., JONES, P., GIES, D., BELLUSCIO, L., LE BEU, M., ESPINOSA III, M., SQUINTO, R., PERSSON, H. and YANCOPOLOUS, G. (1992). Mammalian neurotrophin-4: Structure, distribution and receptor specificity. *Proc. Natl. Acad. Sci. USA 89:* 3060-3064.

IP, N., STITT, T.N., TAPLEY, P., KLEIN, R., GLASS, D., FANDL, J., GREENE, L., BARBACID, M. and YANCOPOLOUS, G. (1993) Similarities and differences in

the way neurotrophins interact with the trk receptors in neuronal and nonneuronal cells. *Neuron 10:* 137-149.

JOHNSON, E., GORIN, P., BRANDEIS, L. and PEARSON, J. (1980). Dorsal root ganglion neurons are destroyed by exposure *in utero* to maternal antibodies to nerve growth factor. *Science 210:* 916-918.

JONES, K. and REICHARDT, L. (1990). Molecular cloning of a human gene that is a member of the nerve growth factor family. *Proc. Natl. Acad. Sci. USA 87:* 8060-8064.

JONES, K.R., FARINAS, I., BACKUS, C. and REICHARDT, L.F. (1994). Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. *Cell 76:* 989-999.

KAISHO, Y., YOSHIMURA, K. and NAKAHAMA, K. (1990). Cloning and expression of a cDNA encoding a novel human neurotrophic factor. *FEBS Lett. 266:* 187-191.

KALCHEIM, C., BARDE, Y-A., THOENEN, H. and LEDOUARIN, N.M. (1987). *In vivo* effect of brain-derived neurotrophic factor on the survival of developing dorsal root ganglion cells. *EMBO J. 6:* 2871-2873.

KAPLAN, M., MARTIN-ZANCA, D. and PARADA, L. (1991). Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene produced by NGF. *Nature 350:* 158-160.

KLEIN, R., JING, S.Q., NANDURI, V., O'ROURKE, E. and BARBACID, M. (1991). The trk proto-oncogene encodes a receptor for nerve growth factor. *Cell 65:* 189-971.

KLEIN, R., MARTIN, Z.D., BARBACID, M. and PARADA, L.F. (1990). Expression of the tyrosine kinase receptor gene trkB is confined to the murine embryonic and adult nervous system.. *Development 109:* 845-50.

KLEIN, R., SILOS, S.I., SMEYNE, R.J., LIRA, S.A., BRAMBILLA, R., BRYANT, S., ZHANG, L., SNIDER, W.D. and BARBACID, M. (1994). Disruption of the neurotrophin-3 receptor gene trkC eliminates la muscle afferents and results in abnormal movements. *Nature 368:* 249-51.

KOH, S., OYLER, G.A. and HIGGINS, G.A. (1989). Localization of nerve growth factor receptor messenger RNA and protein in the adult rat brain. *Exp. Neurol. 106:* 209-226.

KOLIATSOS, V.E., CAYOUETTE, M.H., BERKEMEYER, L.R., CLATTERBUCK, R.E., PRICE, D.L. and ROSENTHAL, A. (1994). Neurotrophin-4/5 is a trophic factor for mammalian facial motor neurons. *Proc. Natl. Acad. Sci. USA 91:* 3304-3308.

KOLIATSOS, V.E., CLATTERBUCK, R.E., WINSLOW, J.W., CAYOUETTE, M.H. and PRICE, D.L. (1993). Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons *in vivo*. *Neuron 10:* 359-367.

KUCERA, J. and WALRO, J.M. (1992). Superfluousness of motor innervation for the formation of muscle spindles in neonatal rat. *Anat. Embryol. 186:* 301-309.

LAIRD, P.W., ZILDERVELD, A., LINDERS, K., RUDNICKI, M.A., JAENISCH, R. and BERNS, A. (1991). Simplified mammalian DNA isolation procedure. *Nucleic Acids Res. 19:* 4293.

LAMBALLE, F., KLEIN, R. and BARBACID, M. (1991). trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. *Cell 66:* 967-791.

LARGE, T.H., WESKAMP, G., HELDER, J.C., RADEKE, M.J., MISKO, T.P., SHOOTER, E.M. and REICHARDT, L.F. (1989). Structure and developmental expression of the nerve growth factor receptor in the chicken nervous system. *Neuron 2:* 1123-1134.

LEE, K-F., DAVIES, A. and JAENISCH, R. (1994). Embryonic dorsal root sensory and neonatal sympathetic neurons display decreased sensitivity to NGF. *Development 120:* 1027-1033.

LEE, K-F., LI, E., HUBER, L.J., LANDIS, S.C., SHARPE, A.H., CHAO, M.C. and JAENISCH, R. (1992). Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. *Cell 69:* 737-749.

LEIBROCK, J., LOTTSPEICH, F., HOHN, A., HOFER, M., HENGERER, B., MASIAKOWSKI, P., THOENEN, H. and BARDE, Y.A. (1989). Molecular cloning and expression of brain-derived neurotrophic factor. *Nature 341:* 149-151.

LINDSAY, R.M., THOENEN, H. and BARDE, Y-A. (1985). Placode and neural crest-derived sensory neurons are responsive at early developmental stages to brain- derived neurotrophic factor. *Dev. Biol. 112:* 319-328.

LYON, M.F. and SEARLE, A.G. (1989). *Genetic Variants and Strains of the Laboratory Mouse,* 2nd ed. Oxford Univ. Press, Oxford.

MAISONPIERRE, P.C., BELLUSCIO, L., SQUINTO, S., IP, N.Y., FURTH, M.E., LINDSAY, R.M. and YANCOPOLOUS, G.D. (1990). Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. *Science 247:* 1446-1451.

MU, X., SILOS-SANTIAGO, I., CARROLL, S.L. and SNIDER, W.D. (1993). Neurotrophin receptor genes are expressed in distinct patterns in developing dorsal root ganglia. *J. Neurosci. 13:* 4029-4041.

NARAYAN, C. and NARAYAN, Y. (1978). Neuronal adjustment in developing nuclear centres of the chick embryo following transplantation of an additional optic primordium. *J. Embryol. Exp. Morphol. 44:* 53-70.

OPPENHEIM, R.W., QIN-WEI, Y., PREVETTE, D. and YAN, Q. (1992). Brain-derived neurotrophic factor rescues developing avian motoneurons from cell death. *Nature 360:* 755-757.

PHILLIPS, H.S., HAINS, J.M., LARAMEE, G.R., ROSENTHAL, A. and WINSLOW, J.W. (1990). Widespread expression of BDNF but not NT-3 by target areas of basal forebrain cholinergic neurons. *Science 250:* 290-294.

PIORO, E.P. and CUELLO, A.C. (1990). Distribution of nerve growth factor receptor-like immunoreactivity in the adult rat central nervous system. Effect of colchicine and correlation with the cholinergic system-I. Forebrain. *Neuroscience 34:* 57-87.

PIRVOLA, U., YLIKOSKI, J., PALGI, J., LEHTONEN, E., ARUMÄE, U. and SAARMA, M. (1992). Brain-derived neurotrophic factor and neurotrophin 3 mRNAs in the peripheral target fields of developing inner ear ganglia. *Proc. Natl. Acad. Sci. USA 89:* 9915-9919.

PRESTIGE, M. (1967). The control of cell numbers in the spinal ganglia of *Xenopus laevis* tadpoles. *J. Embryol. Exp. Morphol. 17:* 453-471.

RANSON, S.W. (1931). Rigidity in deafferented limbs. *J. Comp. Neurol. 52:* 341-346.

REPRESA, J., AVILA, M.A., ROMERO, G., MATO, J.M., GIRALDEZ, F. and VARELA-NIETO, I. (1993). Brain-derived neurotrophic factor and neurotrophin-3 induce cell proliferation in the cochleovestibular ganglion through a glycosylphosphatidylinositol signaling system. *Dev. Biol. 159:* 257-265.

RICHARDSON, P.M., VERGE, V.M.K. and RIOPELLE, R.J. (1986). Distribution of neuronal receptors for nerve growth factor in the rat. *J. Neurosci. 6:* 2312-2321.

RITTER, A.M., LEWIN, G.R., KREMER, N.E. and MENDELL, L.M. (1991). Requirement for nerve growth factor in the development of myelinated nociceptors *in vivo*. *Nature 350:* 500-512.

RODRIGUEZ-TEBAR, A. and BARDE, Y-A. (1988). Binding characteristics of brain-derived neurotrophic factor to its receptors on neurons from the chick embryo. *J. Neurosci. 8:* 3337-42.

RODRIGUEZ-TEBAR, A., DECHANT, G. and BARDE, Y-A. (1990). Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. *Neuron 4:* 487-492.

RODRIGUEZ-TEBAR, A., DECHANT, G., GÖTZ, R. and BARDE, Y-A. (1992). Binding of neurotrophin 3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. *EMBO J. 11:* 917-922.

ROSENTHAL, A.,V., GOEDDEL, D.V., NGUYEN, T., LEWIS, M., SHIH, A., LARAMEE, G.R., NIKOLICS, K. and WINSLOW, J. W. (1990). Primary structure and biological activity of a novel human neurotrophic factor. *Neuron 4:* 767-773.

RUIT, K.G., ELLIOT, J.L., OSBORNE, P.A., JAN, Q. and SNIDER, W.D. (1992). Selective dependence of mammalian dorsal root ganglion neurons on nerve growth factor during embryonic development. *Neuron 8:* 573-587.

SENDTNER, M., HOLTMANN, B., KOLBECK, R., THOENEN, H. and BARDE, Y. (1992). Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. *Nature 360:* 757-759.

SOPPET, D., ESCANDON, E., MARAGOS, J., MIDDLEMAS, D.S., REID, S.W., BLAIR, J., BURTON, L.E., STANTON, B.R., KAPLAN, D.R., HUNTER, T. (1991). The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. *Cell 65:* 895-9032.

SQUINTO, S.P., STITT, T.N., ALDRICH, T.H., DAVIS, S., BIANCO, S.M., RADZIEJEWSKI, C., GLASS, D.J., MASIAKOWSKI, P., FURTH, M.E. and VALENZUELA, D.M. (1991). trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. *Cell 65:* 885-931.

SUTTER, A., RIOPELLE, R.J., HARRIS-WARRICK, R.M. and SHOOTER, E.M. (1979). Nerve growth factor receptors: characterization of two distinct classes of binding sites on chick embryo sensory ganglia cells. *J. Biol. Chem. 254:* 5972-82.

WIESENDANGER, M. (1964). Rigidity produced by deafferentation. *Acta Physiol. Scand. 62:* 160-168.

YAN, Q., ELLIOT, J. and SNIDER, W.D. (1992). Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death. *Nature 360:* 753-755.

YLIKOSKI, J., PIRVOLA, U., MOSHNYAKOV, M., PALGI, J., ARUMAE, U. and SAARMA, M. (1993). Expression patterns of neurotrophin and their receptor mRNAs in the rat inner ear. *Hear. Res. 65:* 69-78.

ZELENA, J. (1957). The morphogenetic influence of innervation on the ontogenetic development of muscle-spindles. *J. Embryol. Exp. Morphol. 5:* 283-292.