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

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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; Hausmann 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-Tebay 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.
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 Shocfer, 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 neurotrophists 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 neurones 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 neurones in all the peripheral sensory ganglia examined, showing for the first time that BDNF is required at physiological concentrations for the survival of neurones in vivo (Ernfors et al., 1994a). However, the survival of α-motor neurones and sympathetic superior cervical ganglion neurones 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 neurones nor γ-motor neurones were severely affected by the lack of BDNF (Ernfors et al., 1994a and unpublished results). The finding that α-motor neurones develop in BDNF mutant mice was unexpected. Lesioned neonatal motor neurones as well as embryonic spinal cord motor neurones 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 neurones (Koliatsos et al., 1994), it is possible that NT-4, but not BDNF, is physiologically required for the survival of motor neurones. However, motor neurones 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 neurones in the BDNF mutant mice is consistent with previous culture studies, where these neurones 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 neurones do not contain any high-affinity receptors for BDNF (Rodriguez-Tebar and Barde, 1988).

BDNF mutant mice display a 45% loss of trigeminal ganglion neurones and a 50% loss of mesencephalic trigeminal neurones (Ernfors et al., 1994a), counted at postnatal day 14. The severe loss of trigeminal ganglion neurones 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 neurones is consistent with previous mRNA studies. All neurotrophins are expressed in a peripheral target for trigeminal neurones, 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 neurones express the BDNF receptor, trkB, (Klein et al., 1990; Ernfors et al., 1992) and BDNF elicits a survival effect on cultured trigeminal neurones (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 fibres are recruited until E15 (Davies and Lumsden, 1984). More than 50% of the trigeminal ganglion neurones 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 fibres to the whisker pad (Ernfors et al., 1994a). It is clear, however, that BDNF is physiologically important for the survival of these neurones at later stages because of the loss of almost half of the trigeminal ganglion neurones in the neonatal BDNF mutant mice. Central organization of trigeminal innervation is represented by barrels in the brain stem and barreis in the somatosensory cortex. The presence of both barreis (unpublished results) and barreis (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 appear normal in the BDNF mutant mouse.
TABLE 1

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

<table>
<thead>
<tr>
<th>Neuron Type</th>
<th>Wild Type</th>
<th>NT3-/-</th>
<th>BDNF-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeV</td>
<td>100±8</td>
<td>36±6</td>
<td>56±2</td>
</tr>
<tr>
<td>Vestibular ganglion</td>
<td>100±4</td>
<td>48±5</td>
<td>52±2</td>
</tr>
<tr>
<td>Nodose ganglion</td>
<td>100±3</td>
<td>95±5</td>
<td>18±4</td>
</tr>
<tr>
<td>L4 DRG</td>
<td>100±5</td>
<td>45±12</td>
<td>70±3</td>
</tr>
<tr>
<td>SCG</td>
<td>100±9</td>
<td>47±14</td>
<td>114±6</td>
</tr>
<tr>
<td>Facial mn</td>
<td>100±6</td>
<td>98±9</td>
<td>97±6</td>
</tr>
</tbody>
</table>

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)

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 utriclar 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 joker, 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
The NT-3 mutant mice displayed loss of neurons in all the ferented animals (Ranson, 1931; Wiesendanger, 1964), and 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). 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.

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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 afferents induces the formation of muscle spindles, and innervation by Ib neurons induces the formation of Golgi tendon organs (Zeleny, 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 isofrom, which is specifically expressed in the intramuscular 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 la 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 myotendinous 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 haplo-insufficiency 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 microscopic 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., 1982) 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 αδ 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. (1986) and digested with restriction endonucleases, fractionated by electrophoresis through 0.8% agarose gels, blotted with 20xSSC onto nylon membranes (Zetabind) and hybridized to a 32P 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 modamine conjugated secondary antiserm, 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 ultramicromtome and stained with toluidine blue. Tissue for electron microscopy was processed as described for the semi-thin sections.

Dil 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% Dil (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 Dil was applied to the 7th nerve; the nerve was cut distal to the applied Dil. 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 vibratome and viewed with rhodamine filter on a Zeiss microscope.
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