

CORRIGENDUM

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Dissection of NT3 functions in vivo by gene replacement strategy

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SUMMARY

The development of the peripheral nervous system is governed in part by a family of neurotrophic factors that signal through Trk tyrosine kinase receptors. Neurotrophin 3 (NT3) ablation in mice causes a more severe neuronal phenotype than deletion of its receptor TrkC, suggesting that NT3 acts also through other non-preferred Trk receptors. To study the role of low-affinity ligand receptor interactions in vivo, we have replaced the *Nt3* gene with the gene for brain-derived neurotrophic factor (BDNF), a TrkB ligand. As in NT3 and TrkC null mice, the proprioception system of these mutants failed to assemble. However, sensory fiber projections in the embryonic spinal cord suggest chemotropic effects of BDNF in vivo. In the dorsal root ganglia, the developmental dynamic of neuron numbers demonstrates that NT3 is required for activation of TrkB during neurogenesis and that TrkA is required

during target tissue innervation. In the inner ear, the ectopic BDNF rescued the severe neuronal deficits caused by NT3 absence, indicating that TrkB and TrkC activate equivalent pathways to promote survival of cochlear neurons. However, specific increased innervation densities suggest unique functions for BDNF and NT3 beyond promoting neuronal survival. This mouse model has allowed the dissection of specific spatiotemporal Trk receptor activation by NT3. Our analysis provides examples of how development can be orchestrated by complex high- and low-affinity interactions between ligand and receptor families.

Key words: NT3, BDNF, TrkA, TrkB, TrkC, DRG, Cochlea, Sensory neurons, Mouse

INTRODUCTION

The neurotrophin family of secreted growth factors plays pivotal roles in the development of the peripheral nervous system (PNS) (Snider, 1994; Tessarollo, 1998). In mammals, this family comprises four known members: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4/5 (NT4/5) (Bothwell, 1995). Two types of receptors mediate their actions. The Trk family of tyrosine kinase receptors, to which neurotrophins bind with high affinity, and the so-called p75 low-affinity NGF receptor (LANR), a member of the tumor necrosis factor receptor family (Bothwell, 1995; Kaplan and Miller 1997). Binding experiments with cell lines have been used to determine the ligand-receptor relationship between neurotrophins and their receptors (Bothwell, 1995; Tessarollo, 1998). NGF binds only to TrkA, whereas BDNF and NT4/5, growth factors with dissimilar patterns of expression, bind to TrkB. NT3 signals mainly through TrkC but can also bind with lower affinity to TrkA and TrkB, and can signal, in some cellular environments, through these receptors (Snider, 1994; Bothwell, 1995).

The availability of genetically engineered mouse models obtained by targeted deletion of neurotrophins or their

receptors has led to important information about the neurotrophin requirement of specific cell populations of the PNS and the specificity of ligand/receptors interactions in vivo (Snider, 1994; Tessarollo, 1998). For example, both *Ngf*^{-/-} and *TrkA*^{-/-} (*Ntrk1*^{-/-} – Mouse Genome Informatics) mice have similar phenotypes. Ablation of either gene results in the virtual absence of the superior cervical ganglia (SCG) neurons and insensitivity to pain, owing to the loss of small-diameter nociceptive sensory neurons. By contrast, the phenotype of BDNF and TrkB knockout mice is not identical because of the presence of another physiologically relevant ligand, NT4/5. Interestingly, BDNF and NT4/5 double mutations cause PNS losses comparable with the ones caused by the *TrkB* null mutation, confirming the relevance of the same receptor to both neurotrophins in vivo. For example, the geniculate ganglion is lost completely in TrkB null mutants or in BDNF-NT4/5 double mutants but shows only 50% reduction in either BDNF- or NT4/5-deficient mice (Tessarollo, 1998). However, the abnormal rescue of the BDNF null mice phenotype by NT4/5 knock-in in the BDNF locus suggests that these neurotrophins may differentially activate TrkB and its downstream signals in vivo (Fan et al., 2000).

NT3 and TrkC knockout mice display remarkable differences. Even though both exhibit the same deficiencies in

movement and posture due to proprioception defects, spinal and cephalic sensory ganglia display greater loss of neurons in *Nt3*^{-/-} than in *TrkC*^{-/-} (*Ntrk3*^{-/-} – Mouse Genome Informatics) mice (Tessarollo, 1998). Moreover, while the *TrkC* null mutant mice have no deficiencies in the SCG, *Nt3*^{-/-} mice exhibit severe neuronal losses, indicating that also in the sympathetic system NT3 activates other receptors (Tessarollo et al., 1997).

Thus, these mouse models have provided essential information on the neurotrophin requirements of peripheral neurons *in vivo*. One major obstacle in understanding neurotrophin actions other than survival has been the early demise of PNS neurons before target interactions (ElShamy and Ernfors, 1996; Fariñas et al., 1996; Liebl et al., 1997; Tessarollo et al., 1994). An interesting approach has recently been used to circumvent this problem. Snider and colleagues (Patel et al., 2000) have generated mice that lack not only NGF or *TrkA* but also the proapoptotic BAX protein. This strategy blocks cell death in the PNS and suggested that *TrkA*/NGF signaling is not required for innervation of central targets, as dorsal root ganglia (DRG) axons extended centrally into the dorsal roots and established collaterals in the superficial laminae of the spinal cord. This study also showed that NGF is still necessary for biochemical differentiation of DRG neurons (Patel et al., 2000).

Analysis of NT3 signaling is complicated by its potential interactions with *TrkA* and *TrkB*, and suggests that a similar rescue strategy would not differentiate between the role of different receptors. For example, NT3 mutant mice lose about 70% of DRG sensory neurons, whereas only 20% (Klein et al., 1994) to 35% are lost in the *TrkC*-deficient mice (Tessarollo et al., 1997). Two hypotheses have been put forward to explain the severe sensory losses of NT3 null mice. Initial studies suggested that *TrkC*-expressing proliferating precursors in the DRG undergo apoptosis when NT3 is absent, resulting in a depletion of neuron precursors (ElShamy and Ernfors, 1996). However, a subsequent study of *TrkC*- and NT3-deficient DRG suggested that failure of NT3 to activate *TrkB* during the proliferation stage of neurogenesis is responsible for these neuron losses (Fariñas et al., 1998; Fariñas et al., 1996). Equally conflicting results have been obtained from the analysis of the inner ear of NT3 and *TrkC* null mice. NT3 mutant mice lose 85% of the cochlear sensory neurons (Fariñas et al., 1994; Tessarollo et al., 1997), whereas 55% (Schimmang et al., 1995) to 70% losses (Tessarollo et al., 1997) have been reported for *TrkC*-deficient mice. In addition, initial studies have proposed differential functions for BDNF and NT3 suggesting that BDNF supports outer hair cell innervation whereas NT3 supports inner hair cell innervation (Ernfors et al., 1995). By contrast, subsequent analysis has shown that NT3 null mutants lose spiral neurons in a topologically restricted fashion and that the outer hair cell innervation is more reduced than the inner hair cell innervation in either BDNF or NT3 null mutants, suggesting that spatial-temporal gradients of neurotrophin expression control inner ear innervation (Fritzsch et al., 1997). Such gradients were recently visualized using NT3 and BDNF *lacZ* fusion reporter systems (Fariñas et al., 2001).

To study the temporal activation of *Trk* receptors and to dissect the dynamics of NT3 requirement by sensory neurons in development, we have replaced the *Nt3* gene with *Bdnf* (B/N allele). This strategy was aimed at eliminating activation of

TrkA and *TrkC*, while preserving only *TrkB*-specific signaling by 'NT3'. For the DRG, we found that BDNF in place of NT3 can rescue a substantial number of neurons during neurogenesis suggesting a major role for *TrkB* activation at this stage. However, during the period of target tissue innervation when the majority of DRG sensory neurons express *TrkA*, we observed a substantial loss of neurons in the B/N DRG, indicating that at this stage *TrkA* activation by NT3 becomes crucial for promoting sensory neuron survival. In the inner ear, we found that the B/N allele could rescue almost completely the sensory losses caused by NT3 deficiency in agreement with the reported expression of both *TrkB* and *TrkC* by spiral neurons (Fariñas et al., 2001; Pirvola et al., 1994). This result indicates that either NT3 or BDNF can support spiral neurons and that gradients of neurotrophin expression control inner ear innervation (Fariñas et al., 2001). In the spinal cord of the B/N mice, we have detected central sensory fibers, which suggests a chemoattractant function of BDNF on subpopulations of *TrkB*-expressing neurons (Song and Poo, 1999; Wang and Tessier-Lavigne, 1999). By contrast, thermo- and nociceptive neurons do not require NGF for sending collaterals into the dorsal laminae (Patel et al., 2000). Thus, our data indicate that not all spinal sensory neurons behave in a similar fashion and some subpopulations that express *TrkB* may require neurotrophin for central projections of collaterals to appropriate regions of the spinal cord (Song and Poo, 1999; Wang and Tessier-Lavigne, 1999).

MATERIALS AND METHODS

Generation of B/N mice

Two and 5 kb respectively of the 5' and 3' genomic DNA that flanks the *Nt3*-coding sequence were fused to the *Bdnf*-coding region with chimeric NT3/BDNF oligonucleotides by fusion PCR. The entire region was sequenced to verify that no errors were introduced during the cloning process. The selectable pGK*neobpA* marker, flanked by *loxP* sites, was placed downstream of the NT3 polyadenylation signal (Fig. 1A). Electroporation and selection were performed using the CJ7 ES cell line as described elsewhere (Tessarollo et al., 1994). DNA derived from G418/FIAU resistant ES clones was screened using a diagnostic *ScaI* restriction enzyme digestion and the 5' probe external to the targeting vector sequence indicated in Fig. 1A. Recombinant clones containing the predicted rearranged bands were obtained at a frequency of 1/18. Targeted clones were then transfected with a Cre-expressing vector (Life Technologies, Gaithersburg, MD) for neo removal. Two independent targeted ES cell recombinant clones injected into C57Bl/6 blastocysts generated chimeras that transmitted the mutated B/N (BDNF over NT3) allele to the progeny. Mating of heterozygous B/N mice gave rise to homozygous mutant mice at the expected frequency of about 25%.

Northern blot analysis

Total RNA was isolated using Trizol (Life Technologies, Gaithersburg, MD). RNAs (10 µg/lane) were separated on agarose-formaldehyde gels, transferred to nylon membranes, and hybridized at 65°C (at 68°C for BDNF) O/N to ³²P-CTP-labeled cDNA antisense probes in 1% BSA, 1mM EDTA, 7% SDS, 0.5M phosphate buffer pH 7.2. Following hybridization, blots were washed for 30 minutes at 65°C twice with 1 mM EDTA, 0.5 M phosphate buffer, 5% SDS and twice with 1 mM EDTA, 0.5 M phosphate buffer, 1% SDS and then exposed to X-ray film. Equal loading of the lanes was assessed by hybridizing the membranes with a cDNA cyclophilin probe (not shown). Full-length BDNF and NT3 rat coding sequences were used

Fig. 1. Replacement of the endogenous NT3-coding region with BDNF. (A) Diagram of the replacement vector and strategy used to 'knock-in' BDNF into the NT3 locus. Probes external to the targeting vector were the same as previously described (Tessarollo et al., 1994). Successful targeting of the locus with the replacement vector is revealed by a change of the wild-type-specific 6.5 kb *ScaI* DNA restriction fragment (wt) to an 8.5 kb *ScaI* fragment (mt). Removal of the neo selectable marker cassette by CRE-mediated recombination of the targeted allele is indicated by the switch of the 8.5 kb *ScaI* fragment (mt) to a 7.0 kb fragment (mt (cre)). X, *XbaI*; S, *ScaI*; B, *BamHI*; E, *EcoRI*; Sm, *SmaI*. (B) Southern blot analysis of DNA from wild-type embryonic stem (ES) cells (+/+), a heterozygous B/N ES cell clone (+/mt), and a heterozygous B/N ES cell clone following Cre-mediated recombination (+/mt (cre)). The *ScaI* restriction enzyme digestion and the (NT3) 5' probe indicated in A were used to detect the rearrangements in the murine NT3 locus. The BDNF-coding region (BDNF) and the neo (neo) probes were hybridized to parallel blots. Bacteriophage lambda DNA cut with *HindIII* was used as the size markers. (C) To ensure that the recombinant vector could produce biologically active BDNF, we introduced the NT3/BDNF chimeric genomic sequence into the pMEX vector which drives expression from an MSV-LTR promoter (Martin-Zanca et al., 1989). The supernatant of NIH 3T3 cells transfected with the pMEXneo-BDNF expression vector was harvested and tested on *TrkB*-expressing PC12 cells for the presence of BDNF activity. The presence of BDNF in the media was revealed by neurite outgrowth (bottom right). Unconditioned media (top left) and conditioned media by NIH 3T3 cells transfected with the pMEXneo (top right) did not induce neurite extension. Treatment of the PC12 cells with 1ng/ml recombinant BDNF was used as positive control (bottom left).

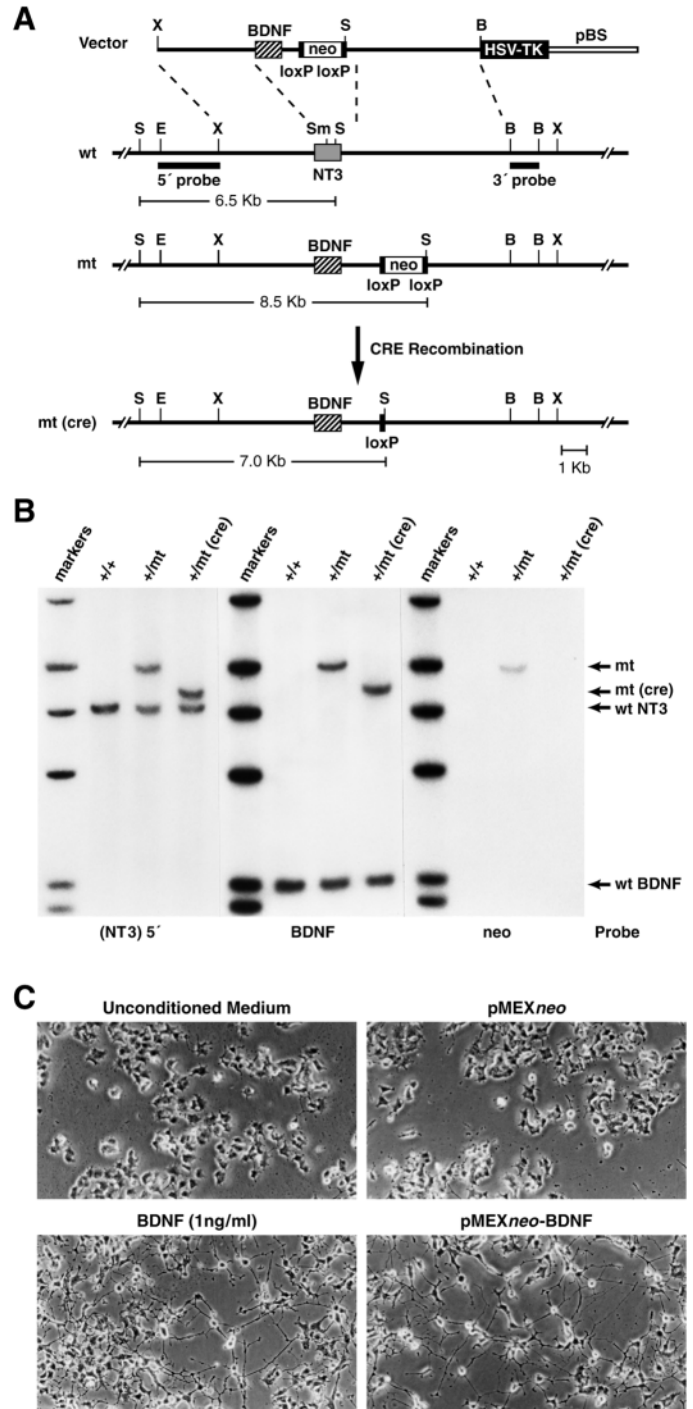
as template in an asymmetric PCR reaction to synthesize ^{32}P -CTP-labeled cDNA antisense probes.

Animals, histological techniques and in situ hybridization

Timed embryos were obtained by overnight mating of heterozygotes. The morning when the vaginal plug was observed was considered embryonic (E) day 0.5. Gravid uteri were removed from timed pregnant females at different stages of gestation (E11.5, 12.5, 13.5, 14.5, 15.5), and embryos dissected and fixed overnight in either 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.2) for in situ hybridization or Carnoy's fixative (60% ethanol, 30% chloroform, 10% acetic acid) for immunostaining and cell counts. Anesthetized neonate animals were perfused with 4% PFA for retrograde labeling experiments or decapitated and fixed in Bouin's fixative for neuronal counts, as described elsewhere (Tessarollo et al., 1997). Tissues from one mutant and one wild-type littermate were embedded in the same block, sectioned at 5 μm thickness and mounted on sialinated slides. In situ hybridization protocols employing the full-length antisense coding sequence of the NT3 and BDNF genes, and the previously described *TrkB*- and *TrkC*-specific probes (Tessarollo et al., 1993) were performed as previously reported (Tessarollo and Parada, 1995). All analyses were carried out on age-matched mice processed in parallel. All animals employed in this study were genotyped by DNA blot analysis as described (Tessarollo et al., 1997; Tessarollo et al., 1994).

Neuronal cell counts and immunocytochemistry

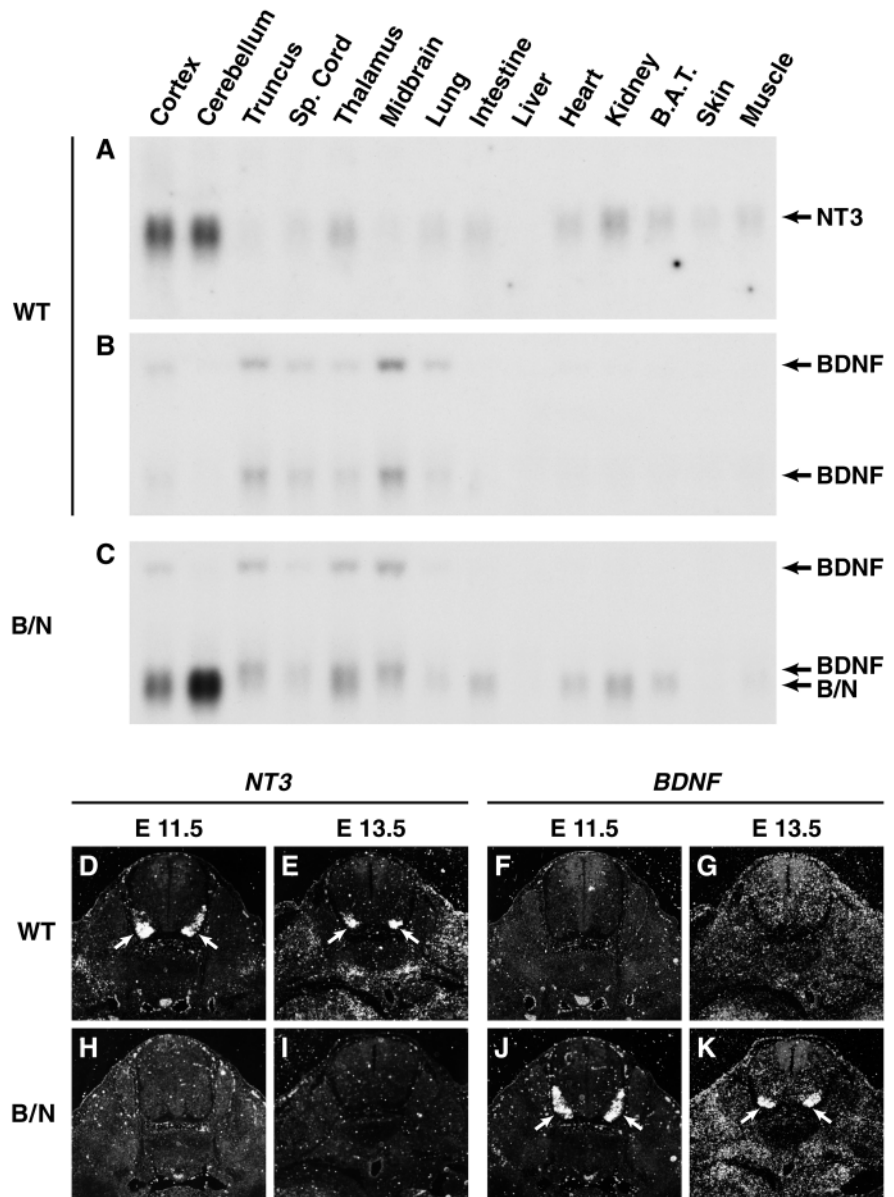
One mutant and one wild-type embryo fixed in Carnoy's solution were embedded in the same paraffin block and serially sectioned at 5 μm . For neuronal counts, the sections were immunostained with antibodies to the 150 kDa neurofilament subunit as described (Fariñas et al., 1998). Individual ganglia were identified using the ribs as landmarks. Cells were counted in every fourth section to calculate the total number in the ganglion, and numbers were multiplied by four and



corrected. For neonatal neuronal counts, the heads (for C1 and T1 counts) or spinal cords (for L1 counts) of one mutant and one wild-type littermate were embedded in the same block, sectioned at 5 μm sagittally, for C1 and T1 analysis, or transversally, for L1 analysis, and Nissl-stained with 0.1% Cresyl Violet. Neurons with a clear nucleus and nucleolus were counted in every sixth section, and the sum of counts multiplied by 6. To assure unbiased analysis, blind neuronal counts were made.

Primary antibodies included rabbit polyclonal antibodies to the neurofilament subunit of relative molecular mass 150,000 (Chemicon, Temecula, CA; dilution 1:2,000) and the monoclonal antibody against parvalbumin (Swant Swiss antibodies; Bellinzona, Switzerland;

Fig. 2. BDNF expression in B/N mutant mice. (A-C) Northern blot analysis of BDNF transcripts in tissues dissected from newborn wild-type (WT) and B/N mice. For each sample, 10 µg of total RNA were analyzed. The NT3-coding sequence used to probe the wild-type samples identified a unique ~1.4 Kb band (A). Hybridization of the B/N tissues with the NT3 probe resulted in the total absence of signal, as expected (not shown). Hybridization of the WT samples with the BDNF cDNA probe identified the expected 1.6 and 4.0 kb bands corresponding to the endogenous products of the *Bdnf* gene (B; Maisonpierre et al., 1990). Hybridization of the B/N tissues with the BDNF probe revealed, in addition to the endogenous BDNF-specific pattern of expression, an additional band (B/N) of approximately 1.4 kb (C). Note that the pattern of expression of the recombinant BDNF (B/N) parallels that of NT3. Blots were hybridized with a cyclophilin-specific probe to assure equal RNA loading (not shown). B.A.T., brown adipose tissue. (D-K) BDNF replaces NT3 expression in the mutant B/N mouse at the cellular level. In situ hybridization analysis of NT3 and BDNF expression in wild-type and B/N animals during embryonic development. Transverse dark-field views of wild-type (D-G) and B/N (H-K) embryos at embryonic stage E11.5 (D,F,H,J) and E13.5 (E,G,I,K) hybridized with an NT3- (D,E,H,I) or BDNF-specific (F,G,J,K) probe. Note the precise replacement of NT3 specific signal in the lateral motor column (arrows) of the wild-type spinal cord (D,E) by BDNF hybridization in the B/N mutant. (J,K).



dilution 1:5000). For bright field immunocytochemistry, sections were incubated with the appropriate biotinylated secondary antibody followed by avidin-biotin-peroxidase complex (Vector Labs, Burlingame, CA), according to the instructions of the manufacturer. Sections were developed in 0.05% 3-3'-diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide in 0.1 M Tris-HCl (pH 7.5), dehydrated and mounted with DPX.

Using one-way analysis of variance (ANOVA), mean neuronal counts from each ganglion (C1, T1, L1) for *TrkC*^{-/-}, *B/N*^{-/-} and *Nt3*^{-/-} mice were compared at E12.5, E13.5, E14.5 and at P0. The significance of any differences between strains at each timepoint was determined by Tukey post-hoc testing.

Dil tracing, spindle and nerve fiber counts

E15.5 and newborn mutant and normal littermates were decapitated and perfused with phosphate-buffered saline and 4% PFA, eviscerated, and incubated overnight at 4°C in 4% PFA. The skin was removed and crystals of DiI (Molecular Probes) were inserted into axial muscles or DRG. After 5-11 days of incubation in 10% formalin (37°C), the spinal cords were removed, embedded in 3.5%

agar/sucrose and sectioned transversely at 100 µm on a vibratome. Sections were viewed on a Zeiss Axiophot microscope using the rhodamine filter set to visualize DiI-labeled afferents. For muscle spindle counts, tissues from newborn mutant and wild-type littermates were prepared as previously described (Kucera et al., 1995). Muscle spindles were identified and counted in the soleus (SOL), medial gastrocnemius (MG) and plantaris (PL). Myelinated axons of the nerve to the SOL muscles were also counted in neonate, as described (Kucera et al., 1995). The total number of nerve fibers (both myelinated and unmyelinated) of mutant and wild-type newborns was determined from electron micrographs taken at 5000× magnification. Differences between mutant and wild-type values were analyzed using Student's *t*-test.

Analysis of inner ear innervation

We have analyzed a total of 15 mutant ears (three ears from P6, four ears each from P3, P1 and E 14.5 null mutants) and compared them with P1 NT3 null mutants and P6 and P1 wild-type control animals. Analysis consisted of labeling the afferent and efferent fibers to the ear from the brainstem using DiI. After insertion of the DiI-soaked

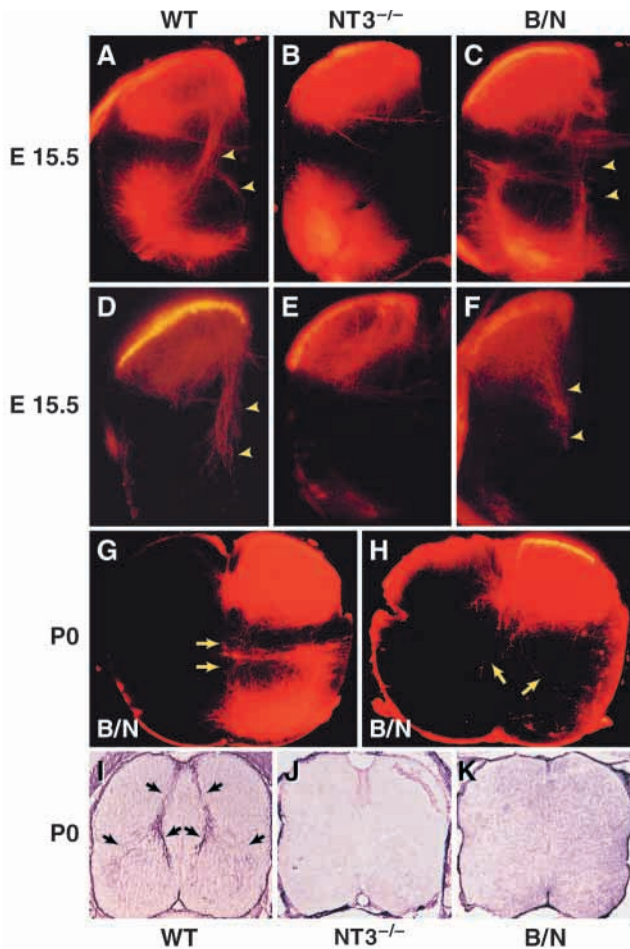


Fig. 3. Development of central fibers in B/N mutant mice. Retrograde labeling of sensory neurons and motoneurons was performed using the lipid-soluble fluorescent tracer DiI in axial muscles (A-C,G) or dorsal root ganglia (D-F,H) at the lumbar level (see Materials and Methods). Micrographs of sections, through the lumbar spinal cord of E 15.5 embryos after DiI tracing show a normal complement of Ia fibers in control mice (arrowheads, A,D), which is absent in *Nt3*^{-/-} mutant mice (B,E). Sections through the spinal cord of a B/N mouse reveals the presence of some fibers projecting ventrally in the spinal cord (arrowheads, C,F). Labeling performed in the axial muscles (G) or in the DRG (H) of B/N mice at stage P0 shows the presence of only residual central fibers (arrows). Immunohistochemical analysis of P0 spinal cords from wild-type (I), *Nt3*^{-/-} (J) or B/N (K) animals using an antibody against parvalbumin (PV), a marker specific for proprioception fibers, shows presence of PV-specific staining in only the wild-type spinal cord (arrows, I).

filter strips, the fixed heads were incubated for four days at 36°C until the dye had diffused into the fine axonal terminals. The inner ears were then dissected and mounted flat in glycerol. Images were captured using a cooled CCD camera and were photographed. The P6 ears were analyzed by confocal microscopy (BioRad Radiance 200). Image stacks of flat mounted cochlea were taken and collapsed in the z-axis to reveal the entire pattern of innervation in the control mice. In the B/N mice, two sets of fibers were collapsed: one set below and another one on top of the basilar membrane. Cochlea were subsequently osmicated, embedded in epoxy resin and thick and ultra-thin sections were cut to reveal the pattern of innervation and synapses on various cells. One mutant and one wild-type cochlea were reacted for acetylated tubulin using an Alexa 568-conjugated secondary

antibody, frozen and sectioned at 20 μm thickness, and viewed in a confocal microscope using the residual autofluorescence to visualize cellular details.

RESULTS

Replacement of the murine NT3 coding sequence with BDNF

Using fusion-PCR, we have generated a replacement-type targeting vector with the BDNF-coding region substituting the NT3 coding sequence (Fig. 1). To ensure that the vector could produce biologically active BDNF, we introduced the NT3/BDNF chimeric genomic sequence into the pMEX expression vector (Martin-Zanca et al., 1989). The supernatant from NIH 3T3 cells transfected with this vector was tested for the presence of BDNF activity on *TrkB*-expressing PC12 cells (Fig. 1C). The induction of neuron-like differentiation of PC12 cells by the supernatant indicated that this vector could produce biologically active BDNF (Fig. 1C). The targeting vector was then introduced into ES cells. Subsequent to selection of correctly targeted clones we transiently expressed the *cre* recombinase gene in the targeted ES cells to remove the neo gene (Fig. 1B). Mice bearing the new allele with BDNF under the control of the NT3 locus were generated as previously described and the new allele was termed *B/N* (BDNF over NT3) (Tessarollo et al., 1994).

Expression of the B/N allele parallels that of the wild-type *Nt3* gene

The objective of our approach depended on the ability to reproduce the precise developmental expression pattern of NT3 with the exogenous *Bdnf* gene under the control of the NT3 promoter. Therefore, we analyzed the expression of the B/N allele in comparison with NT3 and BDNF. The B/N allele produced an mRNA that was similar in size to the endogenous NT3 transcripts (Fig. 2C). Northern blot analysis of tissues obtained from B/N mutant mice and wild-type control littermates revealed that indeed the exogenous *Bdnf* gene was expressed in a pattern overlapping that of the wild-type *Nt3* gene (Fig. 2). For example, in the B/N mutant mouse a BDNF-specific band (B/N band) is present in cortex, intestine, heart and kidney at levels that are comparable with NT3 in the control. In the mutant mouse, we did not observe significant changes in expression pattern of the endogenous *Bdnf* gene, compared with the wild type animal, indicating that the exogenous BDNF allele did not alter the expression level of the endogenous BDNF locus (Fig. 2). Occasionally, in the B/N mouse we have observed small differences in the levels of the endogenous BDNF and the B/N transcripts compared with BDNF and NT3 levels in wild-type controls. However, these differences were not consistent between different animals and we attributed them to variations in tissue dissections (data not shown). One notable exception is the cerebellum, where the relative level of the B/N transcripts was consistently significantly higher than that of NT3 in the wild-type animal (see Discussion).

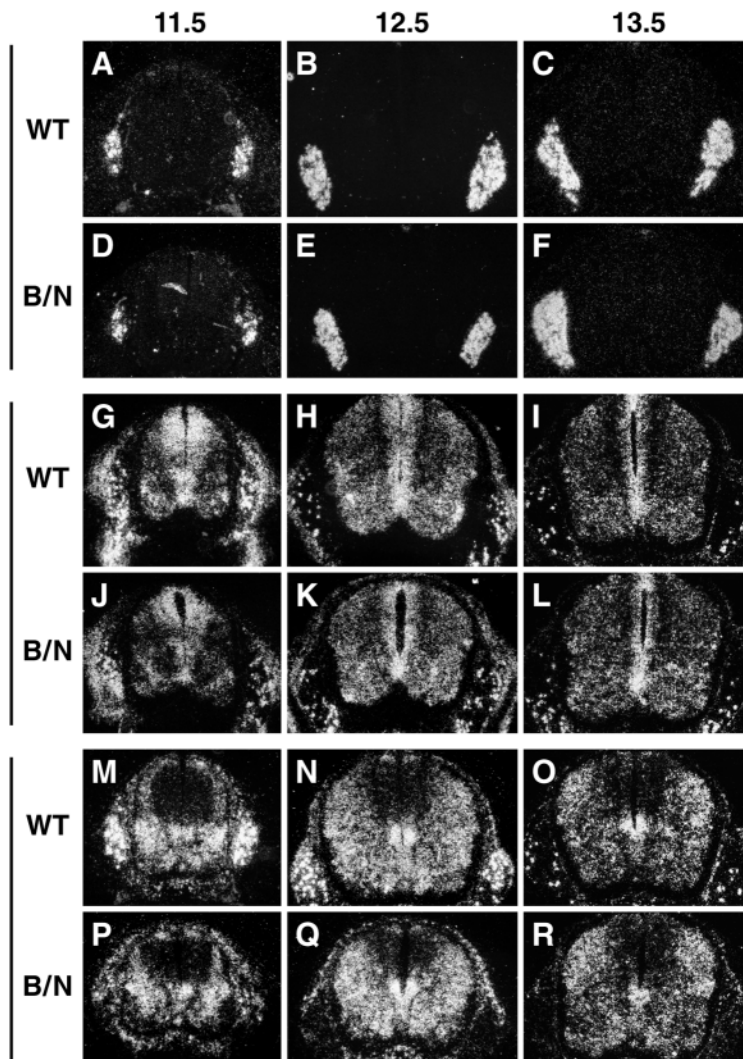
The specificity of the spatial-temporal replacement of NT3 expression with the B/N allele was also confirmed at the cellular level by in situ hybridization analysis. Mutant embryos at stage E11.5 and E13.5 of development show BDNF

Fig. 4. Expression of Trk receptors in DRG neurons of B/N mutant embryos. In situ hybridization analysis of TrkA (A-F), TrkB (G-L) and TrkC (M-R) expression in transverse sections of wild-type (WT) and mutant (B/N) animals between day 11.5 and day 13.5 of embryonic development. In the spinal cord, the expression patterns of Trk genes are similar in both wild-type and B/N embryos suggesting that neurotrophins do not influence the expression of their receptor in these neurons. In the DRG of B/N mutant mice, there is a reduction in TrkC expression already at E11.5 (P) and by E 12.5 (Q) there are no TrkC-expressing neurons. TrkB-positive neurons, however, are present in the B/N embryos at all stages (J-L) in a similar fashion as observed in the wild-type DRG (G-I).

expression in the lateral motor column of the spinal cord instead of the normal NT3 expression (Fig. 2D-K). Collectively, these data demonstrate NT3-specific spatial-temporal control of expression of the exogenous *Bdnf* gene by the regulatory elements of the *Nt3* locus in both neuronal as well as non neuronal tissues.

Chemotropic effects of BDNF on central afferent projections

The B/N mutant mice display the same behavioral phenotype, including abnormal movements and postures attributed to defects in proprioception that are characteristic of *Nt3*- and *TrkC*-deficient mice (not shown; Ernfors et al., 1994; Fariñas et al., 1994; Tessarollo et al., 1994; Klein et al., 1994; Tessarollo et al., 1997). Proprioception deficit in NT3 null mice is characterized by loss of Ia peripheral afferent projections and of muscle spindles in the limbs (Kucera et al., 1995). In addition, spinal cord collateral branches of Ia proprioceptive neurons are completely missing at any developmental stage (Ernfors et al., 1994; Tessarollo et al., 1994; Fig. 3B). In the B/N mutant mouse, by contrast, by retrograde labeling of B/N sensory and motor neurons innervating lumbar and thoracic axial muscles we found central neuronal fibers projecting ventrally in the spinal cord at day 15.5 of embryonic development (Fig. 3C). As TrkC-expressing neurons are missing in the DRG of B/N mice at E12.5 and E13.5, while TrkB expression is not altered (Fig. 4), these data suggest that expression of BDNF instead of NT3 in the motoneuron region of the spinal cord has chemotropic effects on central projections that express TrkB. To assure that the observed fibers were central sensory projections, we selectively retrograde labeled dorsal root at E15.5. As shown in Fig. 3F, a substantial number of fibers projected ventrally in the B/N spinal cord, in contrast to NT3 deficient spinal cord where these fibers are completely absent (Fig. 3E). However, these projections appear abnormal when compared with those in control mice, indicating that other factors in addition to neurotrophins are involved in the precise routing of central sensory fibers. Despite this apparent embryonic rescue, by P0 only few residual projections towards motoneurons persisted, consistent with the mouse behavior and indicating



proprioception deficits (Fig. 3G,H). To determine whether these residual fibers belong to proprioceptive neurons, we analyzed mutant spinal cord for expression of parvalbumin (PV), a proprioceptive neuronal marker (Ernfors et al., 1994). Similar to what was observed in the NT3 (Fig. 3J) or *TrkC*^{-/-} (data not shown) mice, no PV-positive fibers were detected in the thoracic or lumbar B/N mutant spinal cord, suggesting that most projecting fibers observed during embryogenesis fail to differentiate and degenerate by birth (Fig. 3K).

As some fibers are present in the B/N spinal cord, we next examined whether DRG neurons also project into limbs of B/N mice. The soleus muscle which is innervated by the tibial nerve whose proprioceptive afferent component resides principally in L4 and L5 DRGs, had 39±2 (*n*=3) and 80±3 (*n*=3) entering axons in B/N and wild-type newborn mice, respectively. A similar 50% deficit was observed in *Nt3*^{-/-} newborns, and is a result of the loss of muscle afferents and gamma motoneuron efferents (Kucera et al., 1995). In addition, no muscle spindles, the end organs of proprioception, were observed in muscles of the axial paraspinal muscles, hindlimb or forelimb in B/N neonates (data not shown). The absence of spindles is most likely due to an absence of Ia afferent projections to the muscle in B/N mice, because spindles can form only at sites where

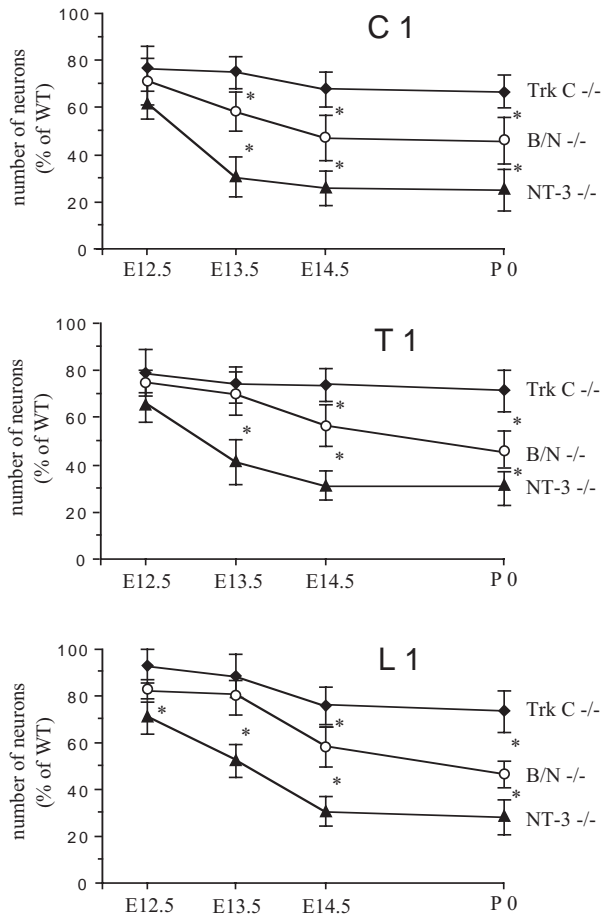


Fig. 5. DRG neuron numbers in NT3- or TrkC-deficient and B/N mutant mice. Graphic representation of the number of neurons in cervical 1 (C1), thoracic 1 (T1) and lumbar 1 (L1) DRG at E12.5, E13.5, E14.5 and P0 as shown in Table 1. Data from mutant mice are expressed as a percentage of the neuron numbers of wild-type littermates. * indicates significant differences ($P < 0.05$) between strains.

group Ia proprioceptive neurons contact primary myotubes (Kucera and Walro, 1990; Zelena, 1957). Thus, BDNF can

support a small number of afferent projections to the ventral regions of the spinal cord in the absence of NT3 but can not substitute for NT3 as a differentiation and/or survival factor for limb proprioceptive afferents, or mediate the formation of muscle spindles.

Differential rescue of DRG sensory neurons at neurogenesis and target tissue innervation by B/N

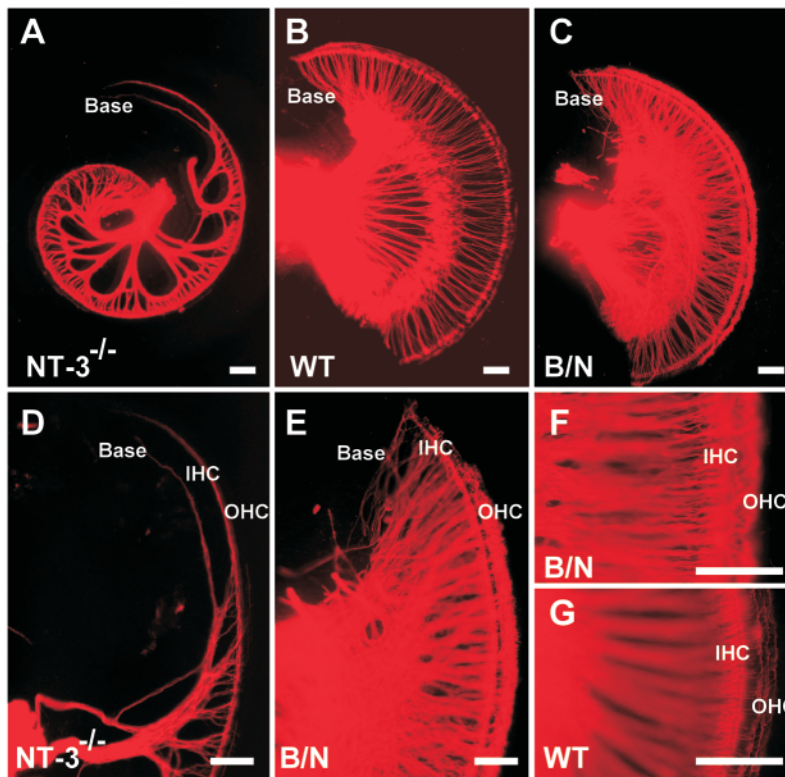
Comparison of neonatal mice deficient in *TrkC* or *Nt3* has shown that there is a much greater deficiency in the number of spinal sensory neurons in the ligand mutant. This suggests that NT3 acts through other receptors in addition to TrkC presumably supporting the survival of non-proprioceptive neurons. If so, replacement of NT3 with BDNF, a preferred TrkB ligand, should rescue at least in part the neuronal losses caused by NT3 deficiency. During embryogenesis, sensory neurons undergo dynamic changes in neurotrophin receptor expression, raising the possibility that sequential activation of different Trk receptors by NT3 may be required to support neuronal survival (Davies 1994; Fariñas et al., 1998). As the effect of NT3 on sensory neurons may vary between ganglia at different sites of the spinal cord, we investigated the dynamics of neuronal losses in cervical 1 (C1), thoracic 1 (T1) and lumbar 1 (L1) DRG during embryonic development in B/N mice compared with NT3 and TrkC null mutant animals (Table 1, Fig. 5). At E13.5, immediately after most DRG neurons are born (Lawson and Biscoe, 1979) all three ganglia of B/N mice contained significantly more neurons than the NT3 nulls. In T1 and L1 DRG, neuronal losses in B/N mice were similar to that observed in *TrkC*^{-/-} mice. A similar trend was also observed in C1. Collectively, these results suggest that BDNF expressed in the NT3 locus can rescue neurons during the major period of neurogenesis, and support the notion that NT3 can function through TrkB during neurogenesis (Fariñas et al., 1998). We next investigated the nature of this rescue by studying the dynamics of Trk-expressing neurons in wild-type and B/N DRG between days 11.5 and 13.5 of development (Fig. 4). In situ hybridization analysis showed that, as observed in the NT3 knockout mouse (Tessarollo et al., 1994; Fariñas et al., 1998), neurons that express TrkC are already reduced at E11.5 and are completely absent by E12.5 (Fig. 4P-R). In NT3 null mice, the DRG TrkB neuronal population is 40-50% reduced at E13.5

Table 1. Total numbers of neurons in DRG of normal and mutant mice

Mice	E 12.5			E 13.5			E 14.5			P 0		
	Wild type	Mutant	%	Wild type	Mutant	%	Wild type	Mutant	%	Wild type	Mutant	%
Trk C												
C1	5734±287 (3)	4370±415 (3)	-24	7260±620 (6)	5432±383 (6)	-25	8188±145 (2)	5540±403 (2)	-32	8070±736 (3)	5332±293 (3)	-34
T1	5229±223 (3)	4130±410 (3)	-21	5811±420 (4)	4302±325 (5)	-26	6345±424 (2)	4673±329 (2)	-26	7389±547 (2)	5259±471 (2)	-28
L1	5016±337 (3)	4635±368 (3)	-8	5808±504 (6)	5141±481 (6)	-12	5970±375 (2)	4498±378 (2)	-25	7535±542 (2)	5504±491 (2)	-27
B/N												
C1	5053±361 (5)	3593±354 (5)	-29	7991±646 (12)	4666±394 (10)	-42	8116±436 (4)	3841±365 (4)	-53	7442±314 (8)	3411±336 (8)	-54
T1	4260±334 (4)	3184±166 (4)	-25	5495±479 (11)	3851±358 (11)	-30	5940±277 (3)	3357±300 (3)	-44	7333±637 (6)	3267±321 (6)	-55
L1	4394±233 (4)	3655±129 (4)	-17	5932±535 (10)	4785±411 (10)	-19	6996±314 (4)	4078±352 (4)	-42	7182±652 (4)	3323±103 (4)	-54
NT3												
C1	5187±425 (4)	3208±226 (4)	-38	7698±748 (4)	2323±196 (3)	-70	8888±484 (2)	2310±170 (2)	-74	7286±550 (2)	1857±148 (2)	-75
T1	4368±315 (4)	2886±238 (4)	-34	6268±604 (4)	2569±246 (4)	-59	7155±290 (2)	2335±141 (2)	-67	6555±267 (2)	2004±161 (2)	-69
L1	4382±340 (4)	3103±223 (4)	-29	6873±359 (4)	3470±239 (4)	-50	7790±304 (2)	2395±156 (2)	-69	7071±683 (2)	1977±148 (2)	-72

Tissue sections of wild-type and mutant littermate embryos at E12.5, E13.5, E14.5 or P0 were prepared as described in Materials and Methods. The numbers of ganglia (DRG) analyzed are indicated in parenthesis. Values obtained from the B/N DRG were analyzed in comparison with those from NT3- and TrkC-deficient mice as described in Materials and Methods.

Fig. 6. Rescue of cochlear innervation by the B/N allele at P1. Whole mount of transganglionically retrograde labeled ears showing the distribution of afferent fibers to the basal turn in a *Nt3*^{-/-} mutant (A,D), a wild-type control (B,G) and a B/N mutant mouse at P1 (C,E,F). The B/N mouse shows a dense complement of spiral ganglion neurons and radial fibers throughout the basal turn up to the basal end of the cochlea (Base) similar to the wild-type mouse. In contrast to an almost complete absence of basal turn radial fibers and reduced density of the outer hair cell innervation in the *Nt3*^{-/-} mice (D), the B/N mutant ear shows dense radial fibers (E) and innervation of both outer hair (OHC) and inner hair cells (IHC). Note that the density of the outer hair cell innervation appears to be even higher in the B/N mice than in wild-type control animals when similar areas of the cochlea are compared (B,C,F,G). Scale bars: 100 μ m.

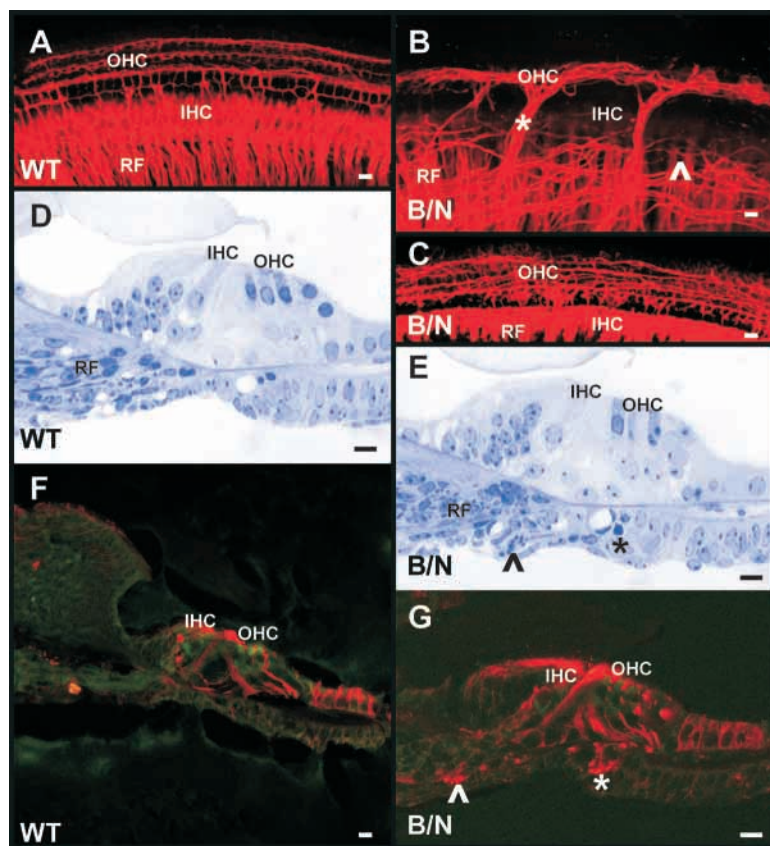


(Fariñas et al., 1998). However, quantitative analysis of TrkB expression demonstrated a normal sized population in the B/N DRG at E13.5 (Fig. 4G,L; Table 2). This result is important because it demonstrates that the TrkB neuronal populations in the B/N mouse are restored to a physiological level and are not abnormally expanded by the ectopic expression of BDNF. Thus, expression of BDNF activity from the NT3 locus rescues TrkB-expressing neurons which would otherwise be lost in the NT3 deficient mouse. These data demonstrate that activation of TrkB by NT3 is a crucial function during neurogenesis.

At E13.5, most DRG neurons express TrkA, marking the beginning of the target tissue innervation period (Fig. 4C; Fariñas et al., 1998). By this stage, total neuron numbers in *TrkC*^{-/-} and *Nt3*^{-/-} DRG have reached about the levels found at P0 (Fariñas et al., 1998; Liebl et al., 1997; Table 1) with the exception of L1. In this ganglion, the severe losses observed at birth are reached at E14.5, most probably because of the rostrocaudal temporal gradient of DRG maturation (Fariñas et al., 1998; Lawson and Biscoe, 1979; Ma et al., 1999). However, in B/N mice, neuron numbers of all DRG decline significantly between E13.5 and E14.5. This result

shows that the bulk of neuronal losses (~25%) detected at P0 in B/N mice compared with TrkC null mutants occurs in the earliest phase of the target tissue innervation period. As the TrkB population is normal (see above), this loss must occur

Fig. 7. Basal turn cochlear innervation at P7 in wild-type and B/N mice. A collapsed z-series of confocal images shows the radial fibers (RF) extending to the inner hair cells (IHC) and outer hair cells (OHC) in the control animal (A). The innervation of the outer hair cells is less well organized in the organ of Corti in mutant animals (C) and additional fibers can be seen running underneath the organ of Corti in a separate bundle (* in B) and between the radial fibers (^ in B). None of these fibers exists in control animals. Radial sections reveal the presence of longitudinally cut radial fibers in the control animals (D) and additional transversally cut spiraling fiber bundles (^,*) below the organ of Corti among the tympanic border cells in the mutant mice (E). These fibers may be myelinated (^ in E). Immunohistochemistry (red) for acetylated tubulin (F,G) can visualize these fibers only in the mutant (^,* in G). Scale bar: 10 μ m.



within the TrkA neuronal population (Table 1; Fig. 5). Thus, our data demonstrate a physiological role for NT3 as a ligand of TrkA, the most abundant Trk receptor in DRG during axon outgrowth.

The B/N allele supports the development of inner ear spiral neurons

Spiral ganglion sensory neurons innervate the cochlea from the apex to the basal turn. These cells give rise to innervation of both the inner hair cells and outer hair cells via numerous radial fibers (Fig. 6B). In NT3 null mutants, both the basal turn spiral neurons and the radial fibers emanating from them are absent (Fig. 6A,D; Fritsch et al., 1997). By contrast, at P0 the B/N mouse shows the presence of spiral neurons that extend as far to the basal turn as in wild-type animals (Fig. 6B,C). As in wild type animals, numerous fibers extend radially from these sensory neurons to the basal turn to innervate densely the inner and outer hair cells. Contrary to the NT3 null mutants, where the density of outer hair cell innervation in the basal turn is particularly reduced or even absent, the B/N mice show dense innervation, in particular, of outer hair cells (Fig. 6D,E). This apparently complete innervation rescue is paralleled by an almost complete rescue of neurons in the spiral ganglion of B/N mice (85% of wild type; 8646 ± 936 in B/N ($n=4$) versus 10740 ± 894 in wild type ($n=4$)) compared with NT3- (15% of wild type) and TrkC- (30% of wild type) deficient mice (Tessarollo et al., 1997). These data are in agreement with the overlapping expression of TrkB and TrkC in spiral neurons, and suggest that if both receptors are expressed in the same neuron, substituting BDNF for NT3 will not affect neuronal survival (Fariñas et al., 2001; Pirvola et al., 1994).

To further investigate the extent of the innervation supported by the neurotrophin replacement, we analyzed the cochlea at P6. As seen at P0, B/N mice showed a complete restoration of radial fibers to the basal turn, but again the innervation of the three rows of outer hair cells was somewhat disorganized (compare Fig. 7A with 7C). However, transmission electron microscopy showed that these fibers formed normal synapses with the hair cells and an occasional contact with Deiter's cells, as reported for wild-type mice (data not shown) (Slepecky, 1996). Thus, NT3 replacement with BDNF promotes the development of inner and outer hair cell innervation.

In addition to the radial fibers going to the organ of Corti, we detected bundles of fibers at irregular intervals (* in Fig. 7B) that passed underneath the organ of Corti and spiraled in a longitudinal bundle underneath the outer hair cells. Furthermore, fibers spiraling between radial fibers near the inner hair cells (^ in Fig. 7B) were also observed. Thick and ultra-thin sections showed that the additional fiber bundles visualized in the whole mounted cochlea did not enter the organ of Corti. Instead, these fibers, many of them myelinated, ran between the tympanic border cells near the inner hair cells and outer hair cells, forming conspicuous bundles that are cut transversally in the radial sections (Fig. 7D,E). These fiber bundles were never found in the control littermates, which showed only longitudinally sectioned fibers of radial bundles heading towards the inner hair cells (Fig. 7D). Acetylated

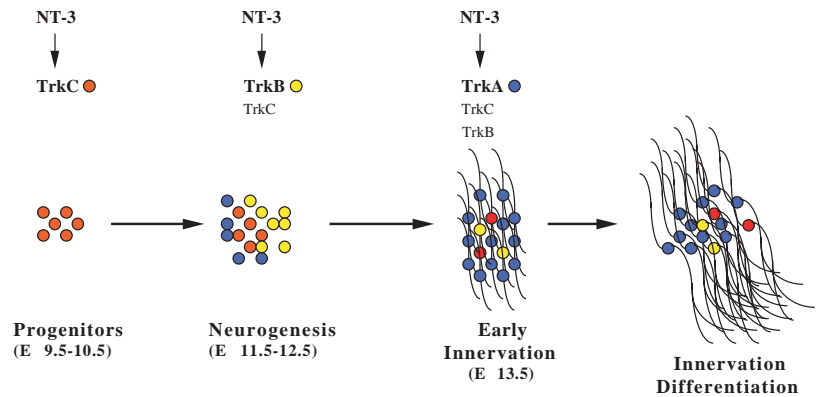


Fig. 8. Schematic of NT3 activation of Trk receptors during DRG development.

tubulin immunocytochemistry revealed again the presence of these longitudinal fiber bundles among the tympanic border cells below the organ of Corti. No synaptic contacts were detected between the tympanic border cells and the fibers ramifying between them (data not shown). These data show that the apparently increased innervation density observed in B/N mice is in part due to aberrant fiber development. Though the origin of these fibers is unclear, this result indicates that BDNF and NT3 have unique functions beyond cell survival (McAllister et al., 1997).

DISCUSSION

Using gene targeting technology, we have generated a mouse model in which NT3 has been replaced by BDNF to investigate the significance of NT3 interactions with its non-preferred receptor TrkB. This strategy was aimed at eliminating TrkC and TrkA activation by the product of the NT3 locus, while preserving only spatiotemporal activation of the TrkB receptor. Analysis of this mouse model has led to three major observations about neurotrophin function in vivo. First, the proprioception deficit caused by NT3 absence is not rescued by BDNF, indicating that this system is strictly dependent on NT3. Interestingly, however, expression of BDNF instead of NT3 in the lateral motor column leads to the generation of spinal cord central fibers, indicating chemotropic effects of BDNF on subpopulations of sensory neurons in vivo. Second, the dynamics of neuronal numbers in the DRG during neurogenesis and target tissue innervation demonstrates a physiological role of NT3 interactions with non-preferred Trk receptors. In particular, the quantitative and qualitative rescue

Table 2. Number of TrkB-expressing neurons in DRG of B/N and wild-type mice at E13.5

Ganglion	Wild type	B/N
C1	344±51 (4)	336±24 (3)
T1	360±56 (4)	312±54 (4)
L1	346±18 (3)	322±44 (3)

One mutant and one control embryo were embedded in the same paraffin block and serially sectioned at 5 μ m. In situ hybridization analysis was performed every sixth section using a TrkB-specific probe. For each ganglion, the sum of TrkB-positive neurons was multiplied by six and expressed as mean number \pm s.e.m. Number of ganglia analyzed are in parenthesis.

caused by the gene replacement in the number of sensory neurons during neurogenesis demonstrates activation of the TrkB receptor by NT3 at this developmental stage. Furthermore, the extensive decline in neuron numbers, starting at E13, when the majority of these neurons switch to TrkA receptor expression unveils a novel role for NT3 in supporting spinal sensory neuron survival through TrkA during the earliest phases of target tissue innervation (Fig. 8). Third, in the inner ear, the severe innervation deficits caused by NT3 deletion are almost completely rescued by BDNF. Spiral neurons express both TrkB and TrkC (Fariñas et al., 2001; Pirvola et al., 1994). Thus, our data shows that inner ear innervation can be supported by TrkB alone if the proper spatiotemporal expression of ligand is preserved.

Expression of exogenous and endogenous BDNF in the B/N mouse

Expression analysis in the B/N mouse showed that the distribution pattern of the exogenous BDNF transcripts is similar to that of the wild-type NT3 mRNA in neuronal as well as non neuronal tissues. Thus, our manipulations have preserved the regulatory function of the NT3 locus. It has been reported that neurotrophins may regulate their own level or that of other neurotrophins (Apfel et al., 1996; Leingärtner et al., 1994; Lindholm et al., 1994; Michael et al., 1997). We observed an overall conservation of the pattern of expression of the endogenous BDNF allele, suggesting that, at least at the tissue level, the exogenous BDNF does not affect its transcriptional activity. However, in the B/N mutant cerebellum, there is a significantly higher level of B/N mRNA compared with NT3 transcript levels in the wild-type mouse. This suggests that the exogenous BDNF stimulates NT3 promoter activity (Fig. 2). It has been reported that the addition of BDNF to granule cell or hippocampal neuron cultures increases NT3 mRNA levels (Leingärtner et al., 1994; Lindholm et al., 1994). Our data support this finding and suggest that BDNF may have a role in activating NT3 expression in specific regions of the brain. By contrast, the level of BDNF mRNA is not affected by loss of NT3 or NGF (Kolbeck et al., 1999). More detailed analyses at the cellular level, employing this mouse model in conjunction with other neurotrophin-deficient mice are required to address the physiological relevance of *Nt3* gene activation by BDNF.

In addition to their specific Trk tyrosine kinase receptors, neurotrophins also bind the so-called p75 low-affinity neurotrophin receptor (Kaplan and Miller, 1997; Bibel and Barde, 2000). As p75 is highly expressed in the sensory neuronal populations analyzed in this study, it is possible that a neurotrophin replacement may impact on neurotrophin effects mediated by this receptor. However, it has been shown that BDNF and NT3 elicit similar biological responses when binding to p75. For example, while all neurotrophins stimulate p75-mediated sphingomyelin hydrolysis with similar kinetics in mouse fibroblasts, BDNF and NT3 show a more potent effect than NGF (Dobrowsky et al., 1995). However, only NGF, but not BDNF or NT3, induces p75-mediated activation of NF- κ B (Kaplan and Miller, 1997; Bibel and Barde 2000). Similarly, only NGF causes association of p75 with the intracellular NADE protein (Mukai et al., 2000) or p75-mediated regulation of the subcellular localization of the zinc-finger protein SC-1 (Chittka and Chao, 1999). Thus, the

specific replacement of NT3 with BDNF is not expected to alter activation of p75 significantly as NT3 and BDNF have similar effects on p75.

BDNF expression in the lateral motor column of the spinal cord supports the development of central afferent projections

The B/N mutant mouse completely lacks proprioceptive functions, as shown by the behavioral deficit and absence of muscle spindles. As only muscle-specific expression of NT3 can rescue these deficits (Wright et al., 1997), our data confirm that the proprioceptive neurons are entirely dependent on TrkC activity. Nevertheless, we have detected the presence of central afferent projections at embryonic day E15.5 in the spinal cord of mice expressing the B/N allele (Fig. 3). By contrast, central fibers are completely missing in mice that lack NT3 or its receptor TrkC (Klein et al., 1994; Tessarollo et al., 1994). The B/N-BDNF is expressed by the lateral motor column in a region that normally expresses NT3 (Fig. 2; Schecterson and Bothwell, 1992). Thus, these fibers may be derived from misrouted TrkB-expressing neurons that are sensitive to BDNF gradients in the spinal cord. Despite mounting experimental evidence that neurotrophic factors may serve as axon guidance molecules during neuronal development in vitro (Ming et al., 1997; Song and Poo, 1999) such a notion is still controversial in vivo (Chen and Frank, 1999; Oakley et al., 1995; Patel et al., 2000). The recent finding that *TrkA*^{-/-} sensory neurons rescued by deletion of the pro-apoptotic *Bax* gene can extend collaterals into the superficial lamina of the spinal cord suggests that NGF/TrkA signaling does not have chemoattractive roles in vivo (Patel et al., 2000). This idea is also supported by the fact that NGF is not present at significant levels in the spinal cord (Shelton and Reichardt, 1986) and that it has a late onset of expression in the peripheral targets (Davies et al., 1987). However, both BDNF and NT3 are expressed much earlier during development (Maisonpierre et al., 1990), making them potential candidates for a role in axonal growth and guidance. Furthermore, BDNF and NT3 exert much stronger effects than NGF as in vitro growth cone guidance molecules and in modulating the response of growth cones to axon guidance molecules (Paves and Saarma, 1997; Song and Poo, 1999; Tuttle and O'Leary, 1998). However, it is not known which specific group(s), if any, of sensory neurons are sensitive to BDNF and/or NT3 as guidance cues. In chick, injections of a NT3 neutralizing antibody in the spinal cord does not reduce the number of Ia afferent projections or the extent of their ventral extension, suggesting that NT3 does not have chemotropic effects on Ia fibers (Oakley et al., 1995), which are known to belong to TrkC-expressing proprioceptive neurons (Klein et al., 1994). Thus, it appears that only TrkB-expressing fibers may be sensitive to BDNF or possibly even NT3 gradients. Interestingly, in a transgenic mouse expressing NT3 under the control of the nestin promoter a few central fibers develop but fail to contact the motoneurons in the lateral column. Instead, they project to the midline of the spinal cord, an area coinciding with highest levels of NT3 expression (Ringstedt et al., 1997). As the identity of those fibers is not known, including whether they express TrkB or TrkC, it is conceivable that TrkB-expressing central sensory fibers may be the ones that responded to the ectopic NT3. Therefore, while TrkA-expressing nociceptive and TrkC-expressing

proprioceptive axons may not require NGF and NT3, respectively, for reaching their central target, TrkB-expressing axons may sense and react to BDNF or even NT3 gradients.

Different neurotrophins can have opposite effects on growth cone behavior *in vitro*, depending on the context in which they function (Song and Poo, 1999). Thus, the lack of a unified view of their role as chemoattractant molecules on sensory neurons *in vivo* may be symptomatic of the heterogeneity among sensory neuron populations (Paves and Saarma, 1997; Scott, 1992; Tuttle and O'Leary, 1998).

Temporal activation of TrkB and TrkA receptors by NT3 during DRG development

Expression of Trk receptors in the DRG is dynamically regulated during development (Fig. 8; Fariñas et al., 1998; Ma et al., 1999; Tessarollo et al., 1993; Tessarollo et al., 1994). The transcription factors neurogenin 1 and 2 are required to generate all classes of Trk-expressing DRG neurons. However, the mechanisms that determine the expression of specific Trk receptors is still unknown (Anderson, 1999; Ma et al., 1999). TrkC is the main receptor expressed by progenitor cells (Tessarollo et al., 1993). During neurogenesis, cells expressing TrkA, TrkB and TrkC are all well represented in the DRG (Fariñas et al., 1998). However, after all neurons are born, at the beginning of the period of target tissue innervation, most neurons express TrkA. This dynamic pattern of neurotrophin receptor expression may reflect the changes in neurotrophin dependency of DRG neurons during development (Davies, 1994). However, it is still not clear what roles specific neurotrophins play in DRG neuronal survival during embryogenesis. For example, NT3 plays a major role in DRG development, as indicated by the severe losses in the sensory ganglia of *Nt3*^{-/-} mice (Ernfors et al., 1994; Fariñas et al., 1994; Tessarollo et al., 1997). However, mice that lack all isoforms of the high-affinity receptor TrkC do not lose as many neurons, suggesting that some TrkA- or TrkB-expressing neurons also depend on NT3 (Tessarollo et al., 1997). Two different mechanisms have been proposed to explain the severe neuronal losses caused by NT3 deficiency. As these losses appear early in development before the target tissue innervation period (ElShamy and Ernfors, 1996; Fariñas et al., 1996; Liebl et al., 1997; Tessarollo et al., 1994) and seem to coincide with an increased level of apoptosis of precursors, it has been first suggested that proliferating precursors undergo apoptosis when NT3 is absent, resulting in a depletion of pro-neuronal progenitors (ElShamy and Ernfors, 1996). However, analysis of the rate of proliferation and differentiation of precursor cells in NT3-deficient mice has led to the proposal that a decrease in neurogenesis and not apoptosis is responsible for the severe sensory losses (Fariñas et al., 1996). Furthermore, the pattern of expression of Trk receptors in proliferating neuroblasts suggests that NT3 is required to maintain neurons that express TrkB but not neurons expressing TrkA (Fariñas et al., 1998). Our data support a role for NT3 on TrkB-expressing neurons at neurogenesis, as this neuronal population was rescued at this stage. This specific effect was true for all DRG analyzed, although the rescue was less prominent in the C1 ganglion. This result may reflect unique characteristics of this particular DRG. For example, the role of NT3 activation of different Trk receptors may vary among DRG at this early developmental

stage or NT3 may act on the cell cycle control of sensory precursors cells (ElShamy et al., 1998).

While the B/N allele could rescue some neurons during neurogenesis, a large number is lost soon after most neurons switch to TrkA expression at the start of target tissue innervation (Table 1; Fig. 5). The dynamic of this loss is similar in the different ganglia, but in agreement with the reported rostrocaudal gradient of maturation of DRG neurons it occurs more rapidly at the C1 level (Lawson and Biscoe, 1979). The rapid loss of neurons immediately after neurogenesis was unexpected in light of the reports suggesting that only NGF is required at this stage (Davies, 1994; White et al., 1996). During the earliest stages of target tissue innervation, immediately after sensory neurons switch to TrkA expression they survive only briefly without neurotrophins *in culture* (Buchman and Davies, 1993). Because of this sensitivity to neurotrophin deprivation our data is consistent with a role for NT3 in supporting neurons during this delicate transition to a complete dependence on NGF (Fig. 8; Davies et al., 1987; White et al., 1996). Expression data have shown that the peripheral axons of sensory neurons that undergo a switch in neurotrophin dependence are exposed to NT3 en route to their peripheral tissues where NGF is produced. Thus, these neurons may see only NT3 during the earliest stage of target tissue innervation (Fariñas et al., 1998). Alternatively, NGF and NT3 may both be required for sensory neuron survival at this specific stage as demonstrated for neurons of the sympathetic lineage for which simultaneous requirement of both NGF and NT3 has been reported (Tafreshi et al., 1998).

The B/N allele supports sensory innervation of the inner ear

We found that replacement of NT3 with BDNF rescues almost completely the severe inner ear neuronal and innervation losses caused by NT3 deletion. The implication of this rescue is significant in light of the current controversy about the role of NT3 and BDNF in inner ear development. Initial analysis of NT3- and BDNF-deficient mice suggested that these neurotrophins act on separate subsets of spiral ganglion neurons, with BDNF and NT3 supporting outer and inner hair cell innervation, respectively (Ernfors et al., 1995). This hypothesis was supported by the apparently restricted loss of neurons that innervate outer hair cells of the cochlea in BDNF and TrkB mutants, and the complete loss of inner hair cell innervation in the NT3 and TrkC mutant mice (Ernfors et al., 1995; Schimmang et al., 1995). In this case, the B/N allele should have had almost no effect on restoring the innervation losses caused by NT3 ablation.

More recent studies of the expression of neurotrophins and their receptors (Fariñas et al., 2001) and of inner ear innervation losses in mutant mice (Fritzsch et al., 1999) have led to the hypothesis that region-specific expression of BDNF and NT3 may control the spatial shaping of cochlear innervation (Fariñas et al., 2001). Analysis of *Nt3* mutants indicates an almost complete loss of innervation in the basal turn and a more severe reduction of the outer hair cell than the inner hair cell innervation (Fritzsch et al., 1997). Similar spatial effects on cochlear innervation are also present in TrkC receptor mutants (Fritzsch et al., 1998). As *in situ* hybridization studies showed that spiral sensory neurons express both the TrkB and TrkC receptors (Fariñas et al., 2001; Pirvola et al.,

1994) it appeared that region-specific expression of the neurotrophins might account for the differential sparing of sensory neurons along the cochlea. The limited loss of spiral sensory neurons in the B/N mice indicates that only some signal through both TrkC and TrkB receptor is needed for survival of all neurons.

Indeed, recent work described a developmental countercurrent of neurotrophin expression in the cochlea, with *BDNF* expanding from the apex toward the base and *Nt3* expanding from the base toward the apex as development progresses (Fariñas et al., 2001). In agreement with this scenario, replacement of NT3 with BDNF causes an almost complete rescue of the topological loss of basal turn spiral neurons caused by lack of NT3 (Fig. 6). Most importantly the effect of the exogenous BDNF on the basal turn innervation cannot be explained by a simple additive effect of endogenous and exogenous BDNF. In fact, the innervation rescue in the B/N mouse occurs before expression of the endogenous BDNF in the basal turn (data not shown). Beyond the 'simple' rescue of spiral sensory neurons in the basal turn we have also observed an even increased density of outer hair cells innervation in the basal turn at birth. Closer examination at P7 showed that many of the fibers that appear to reach the outer hair cells are actually never entering the organ of Corti. Instead, they remain between the tympanic border cells where they run for various distances longitudinally underneath the organ of Corti (Fig. 7). These fibers, contrary to the rescued afferent and efferent fibers contacting the hair cells do not form recognizable synapses with border cells. The endogenous expression of NT3, which is predominately in supporting cells in the embryonic basal turn and the inner hair cells in the adult basal turn (Fariñas et al., 2001; Pirvola et al., 1994) does not correlate well with the innervation of hair cells in normal cochlea. Thus, it appears that BDNF, which is expressed exclusively in hair cells in the basal turn, may be more important in the cochlea for short range navigation of ingrowing afferents to reach preferentially the hair cells. Consistent with this assumption is our finding of misdirected growth of some fibers which spiral underneath the organ of Corti in the B/N mice. We assume that the synthesis of BDNF under NT3 promoter control in the cochlear supporting cells will lead to a diffusion away from those cells. Such a diffusion halo appears to allow afferent fibers to extend into the foreign territory of tympanic border cells and to survive there at least until P7. Later stages, which are known to display exclusive expression of NT3 in inner hair cells, need to be examined to reveal the ultimate fate of these fibers.

In summary, we have shown that replacement of NT3 with BDNF can support some neuronal systems that are impaired by NT3 deficiency. This rescue revealed for the first time physiological spatial-temporal interactions of NT3 with the non-preferred TrkA and TrkB receptors. Further analysis of this as well as of similar mouse models may allow the dissection of some of the most complex traits of neurotrophin functions in vivo.

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