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Cranial sensory neuron development in the absence of brain-derived neurotrophic factor in BDNF/Bax double null mice

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Abstract

To investigate the role of brain-derived neurotrophic factor (BDNF) in differentiation of cranial sensory neurons in vivo, we analyzed development of nodose (NG), petrosal (PG), and vestibular (VG) ganglion cells in genetically engineered mice carrying null mutations in the genes encoding BDNF and the proapoptotic Bcl-2 homolog Bax. In *bax* $^{-/-}$ mutants, ganglion cell numbers were increased significantly compared to wild-type animals, indicating that naturally occurring cell death in these ganglia is regulated by Bax signaling. Analysis of *bdnf* $^{-/-}$ *bax* $^{-/-}$ mutants revealed that, although the Bax null mutation completely rescued cell loss in the absence of BDNF, it did not rescue the lethality of the BDNF null phenotype. Moreover, despite rescue of BDNF-dependent neurons by the *bax* null mutation, sensory target innervation was abnormal in double null mutants. Vagal sensory innervation to baroreceptor regions of the cardiac outflow tract was completely absent, and the density of vestibular sensory innervation to the cristae organs was markedly decreased, compared to wild-type controls. Moreover, vestibular afferents failed to selectively innervate their hair cell targets within the cristae organs in the double mutants. These innervation failures occurred despite successful navigation of sensory fibers to the peripheral field, demonstrating that BDNF is required locally for afferent ingrowth into target tissues. In addition, the *bax* null mutation failed to rescue expression of the dopaminergic phenotype in a subset of NG and PG neurons. These data demonstrate that BDNF signaling is required not only to support survival of cranial sensory neurons, but also to regulate local growth of afferent fibers into target tissues and, in some cells, transmitter phenotypic expression is required.

Keywords: Neurotrophin; Cell death; Differentiation; Transmitter expression; Target innervation; Vestibular; Nodose; Petrosal

Introduction

Brain-derived neurotrophic factor (BDNF) is required for the survival of large subsets of neurons in cranial sensory ganglia. Thus, genetically engineered loss of BDNF in mice results in the death of approximately 85% of vestibular ganglion (VG) cells, 50% of geniculate ganglion cells, 40–50% of visceral sensory neurons in the nodose (NG) and petrosal (PG) ganglia, and 20–30% of trigeminal sensory neurons before birth (Conover et al., 1995; Erickson et al., 1996; Fritzsch et al., 2004; Huang and Reichardt, 2001; Jones et al., 1994; Liu et al., 1995). Despite the importance of BDNF in survival of cranial sensory ganglion cells, the mechanisms by which BDNF regulates neuronal cell death are unknown. Moreover, the absolute survival requirement for BDNF has precluded analysis of other aspects of cranial sensory development, such as transmitter expression and target innervation, in BDNF null mutants in vivo.

Deletion of the proapoptotic gene *bax* eliminates developmental cell death resulting from genetic loss of nerve growth factor (NGF) or neurotrophin-3 (NT-3) in dorsal root sensory ganglia (DRG), indicating that NGF and NT-3

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normally suppress a Bax-dependent cell death pathway in sensory neurons (Patel et al., 2000, 2003). In the absence of NGF, target innervation and peptide expression by DRG neurons are deficient, indicating that NGF regulates phenotypic development of DRG neurons as well as survival. Moreover, *bax* null mutation dramatically reduces neuronal apoptosis in the developing trigeminal and cochleovestibular ganglia, both of which contain NGF- and NT-3-independent neurons, suggesting that Bax is also important for signaling by other neurotrophins (White et al., 1998). However, the role of Bax in developmental cell death of BDNF-dependent neurons has not been directly examined in vivo. Therefore, to understand how BDNF regulates cranial sensory neuron development, we generated mice carrying null mutations in both the *bdnf* and *bax* loci. We focused our analyses on neurons in the nodose (NG), petrosal (PG), and vestibular (VG) sensory ganglia, the three major sites of sensory neuron loss in BDNF null mutants (Conover et al., 1995; Fritzsch et al., 2004). Our data demonstrate that BDNF-mediated signaling is important not only for suppressing Bax-mediated cell death, but also for regulating initial sensory target innervation and transmitter phenotypic expression in vivo.

Methods

Transgenic animals

To generate BDNF/Bax double null mutants, mice heterozygous for a null mutation in the *bdnf* gene (Conover et al., 1995; Regeneron Pharmaceuticals) were crossed with mice heterozygous for the *bax* null mutation (Knudson et al., 1995; Jackson Laboratories) to generate mice that were heterozygous for both genes. These double heterozygotes were subsequently crossed to generate $bdnf^{-/-}$ $bax^{-/-}$ double null mutants. PCR genotyping was performed separately for the *bdnf* and *bax* mutations as previously described (Deckwerth et al., 1996; Erickson et al., 1996). We observed no differences in viability among wild-type and $bax^{-/-}$ mice. However, $bdnf^{-/-}$ and $bdnf^{-/-}$ $bax^{-/-}$ mice died within 5 days after birth.

Immunohistochemistry and cell counts

Newborn mice were deeply anesthetized with sodium pentobarbitol and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. Following perfusion, tissue blocks containing the NPG were postfixed overnight, infiltrated with 30% sucrose, and subsequently embedded and frozen in Tissue-Tek embedding compound (Sakura Finetek). Parasagittal sections (10 μm) were cut and thaw-mounted onto glass microscope slides. TH immunostaining was performed as previously described (Erickson et al., 1996) using a rabbit antityrosine hydroxylase (TH) antibody (Pelfreez) and goat antirabbit FITC (Cappel). For total cell counts, sections were stained with 0.1% cresyl violet acetate. TH immunostaining, cross-sectional area, and total cell counts were performed as previously described (Erickson et al., 1996). Whole mount preparations of aortic arches were collected and stained for PGP9.5 as previously described (Brady et al., 1999).

Analysis of vestibular innervation

The lipophilic carbocyanine dyes, DiI (Molecular Probes) and PTIR 271 (PTI Research) were dissolved in xylene to generate a saturated solution. Filter paper (Schleicher and Schuell, 8-μm pores, nylon filter) was sectioned into thin strips that were soaked in the dye solutions. The dye was allowed to evaporate, and the dried filter strips were cut into small triangles with microscissors.

Perfusion-fixed heads were cut along the midline, and dye-impregnated filter fragments were inserted near the midline across the facial genu to label efferent fibers to the ear (Karis et al., 2001). Afferents were labeled by inserting a filter fragment with a different colored dye transversally into the anteroventral cochlear and adjacent vestibular nuclei immediately rostral to the seventh nerve root. Diffusion of the dyes required 52–72 h at 37°C in an incubator oven. During diffusion, brains were continuously kept in 4% paraformaldehyde in 0.1 M phosphate buffer.

After appropriate diffusion times as detailed previously (Maklad and Fritzsch, 2003), ears and vestibular ganglia were dissected and mounted flat in glycerol. The coverslipped dissected pieces of the ear were viewed without delay in a confocal microscope. All confocal imaging was performed with a BioRad Radiance 2000 confocal system mounted on a Nikon Eclipse 800 upright microscope. For excitation, we used the 568- and 635-nm band of the Kr/Ar laser. For maximal excitation and segregation of the dyes, we used the following settings: PMT for 568 with dichroic mirror set at 650 and emission filter set at 600-40, PMT for 635 with 100% power to PMT III and 650-lp emission filter. Image stacks were taken using the *Z*-axis control software, and *Z*-axis stacks were collapsed into a single image using the BioRad software. Images were organized into plates using Corel Draw software and printed on a Tektronix color laser printer.

Results

Cranial sensory neuron survival

To determine whether sensory neurons lacking Bax could survive in the absence of BDNF, we compared total neuron survival in the NG, PG, and VG of wild-type, $bax^{-/-}$, $bdnf^{-/-}$, and $bdnf^{-/-}$ $bax^{-/-}$ newborn mice. As described previously (see Introduction), each of these ganglia contains a large subset of neurons that require BDNF for survival and are therefore absent in $bdnf^{-/-}$ mutants (Figs. 1 and 2). In contrast, sensory neuron survival in $bax^{-/-}$ mutants was

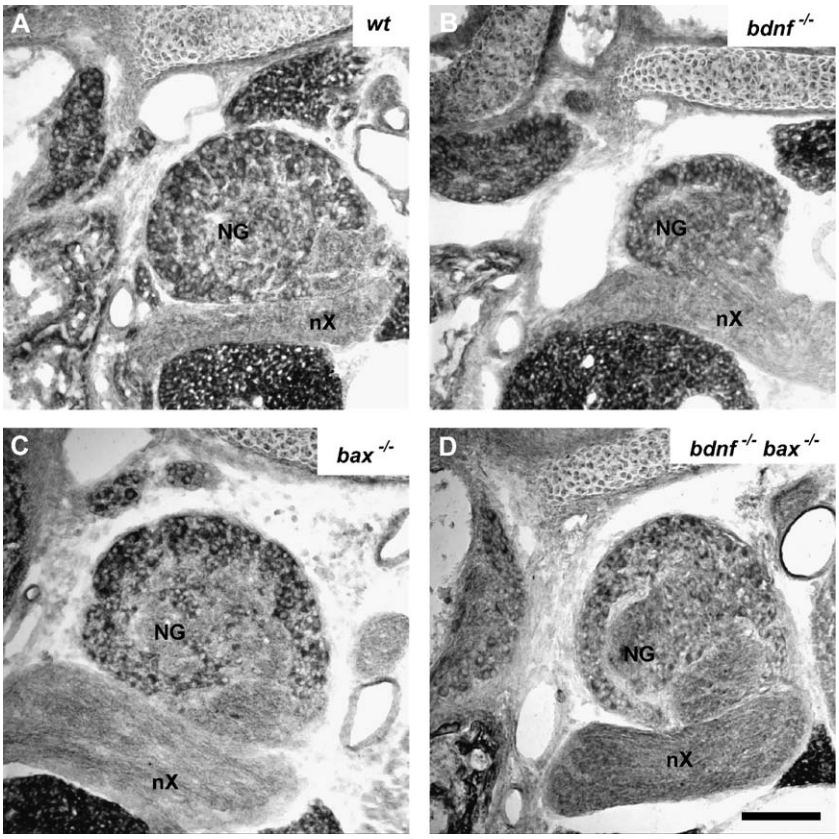

Fig. 1. *bax* null mutation rescues neuron survival in the absence of BDNF. Cresyl violet-stained sagittal sections through the nodose ganglion (NG) from (A) wild-type, (B) *bdnf*$^{-/-}$, (C) *bax*$^{-/-}$, and (D) *bdnf*$^{-/-}$ *bax*$^{-/-}$ newborn mice. nX indicates vagus nerve. Scale bar equals 100 μm.

significantly increased compared to wild-type controls in all three ganglia, indicating that Bax is required for naturally occurring developmental cell death in these populations. The mean diameter of neurons in *bax*$^{-/-}$ mice was significantly decreased compared to wild-type animals, suggesting either that the *bax* null mutation selectively rescued a population of small neurons or that the size of *bax*$^{-/-}$ neurons was reduced overall (Fig. 3). Frequency histogram analysis revealed a reduction in the diameters of the *bax*$^{-/-}$ population as a whole, rather than the appearance of a new population of small neurons (data not shown).

In *bdnf*$^{-/-}$ *bax*$^{-/-}$ mice, ganglion cell numbers were indistinguishable from those in *bax*$^{-/-}$ mutants, indicating that the *bax* null mutation prevented cell death that would have resulted from the loss of BDNF. In all three ganglia, *bax*$^{-/-}$ and *bdnf*$^{-/-}$ *bax*$^{-/-}$ neurons were smaller than wild-type cells (Figs. 2 and 3). In addition, however, *bdnf*$^{-/-}$ *bax*$^{-/-}$ NG and VG neurons were also significantly smaller compared to *bax*$^{-/-}$, indicating that BDNF availability can influence cell size independent of survival.

Sensory target innervation in bdnf$^{-/-}$ *bax*$^{-/-}$ *mutants*

Given that the *bax* null mutation rescued survival of BDNF-dependent cells in *bdnf*$^{-/-}$ *bax*$^{-/-}$ mutants, we used these animals to examine whether the loss of BDNF resulted in phenotypic deficits in cranial sensory neurons, including target innervation and transmitter expression. To analyze potential effects of BDNF depletion on sensory target innervation, we examined the density of sensory axons in the vestibular apparatus and cardiac outflow tract, peripheral targets of the VG and NG, respectively.

Vestibular innervation

Previous work has shown that *bdnf*$^{-/-}$ mutants lose almost all fibers to the canal cristae sensory organs soon after the onset of innervation (Bianchi et al., 1996; Fritzsch et al., 1997; Tessarollo et al., 2004). Indeed, we observed only an occasional fiber extending toward the anterior and horizontal cristae and no fibers extending to the posterior canal cristae in newborn *bdnf*$^{-/-}$ mutants (Fig. 4). The few fibers that did extend to the cristae sensory organs showed no branching within the cristae organs and no structures resembling terminals on hair cells. In contrast to this paucity of canal cristae innervation in *bdnf*$^{-/-}$ mutants, many more fibers extended to the anterior and horizontal cristae in *bdnf*$^{-/-}$ *bax*$^{-/-}$ double null mutants. Moreover, these fibers showed terminal swellings reminiscent of afferent terminals. However, as with *bdnf*$^{-/-}$ mutants, no fibers were found to extend to the posterior crista in *bdnf*$^{-/-}$ *bax*$^{-/-}$ mutants. Fibers to the anterior and horizontal cristae were disorgan-

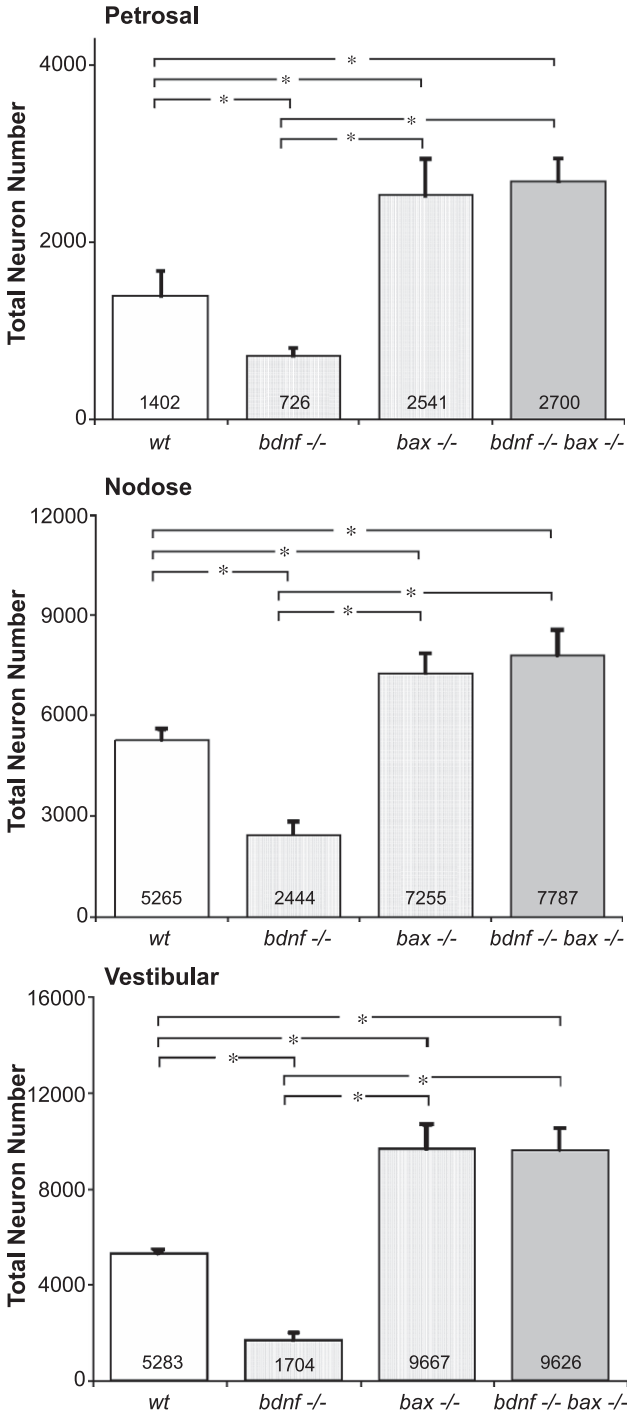

Fig. 2. Morphometric analysis of total neuron survival in the petrosal, nodose, and vestibular ganglia in wild-type (wt), *bdnf* null (*bdnf*$^{-/-}$), *bax* null (*bax*$^{-/-}$), and double *bdnf/bax* null (*bdnf*$^{-/-}$ *bax*$^{-/-}$) newborn mice. Each bar represents the mean ± SEM of cell counts from six different animals (see Methods for details). *$P < 0.05$, ANOVA.

ized and extended both inside and outside the sensory epithelium with limited preference for the epithelium. Thus, despite complete rescue of VG neuron survival in *bdnf*$^{-/-}$ *bax*$^{-/-}$ animals, the *bax* null mutation failed to rescue the wild-type pattern of specific vestibular innervation directed exclusively to the cristae sensory organs. Examination of single *bax* null mutants showed no qualitative changes in the pattern of vestibular innervation (Fig. 4).

Vascular innervation

NG neurons provide baroreceptor innervation to specialized regions of the cardiac outflow tract that include the aortic arch, the origin of the right subclavian artery, and the carotid sinus. Baroreceptor afferents are BDNF-dependent and completely absent in newborn *bdnf*$^{-/-}$ mutants (Brady et al., 1999). We therefore used the baroreceptor innervation of the cardiac outflow tract as a model to examine the

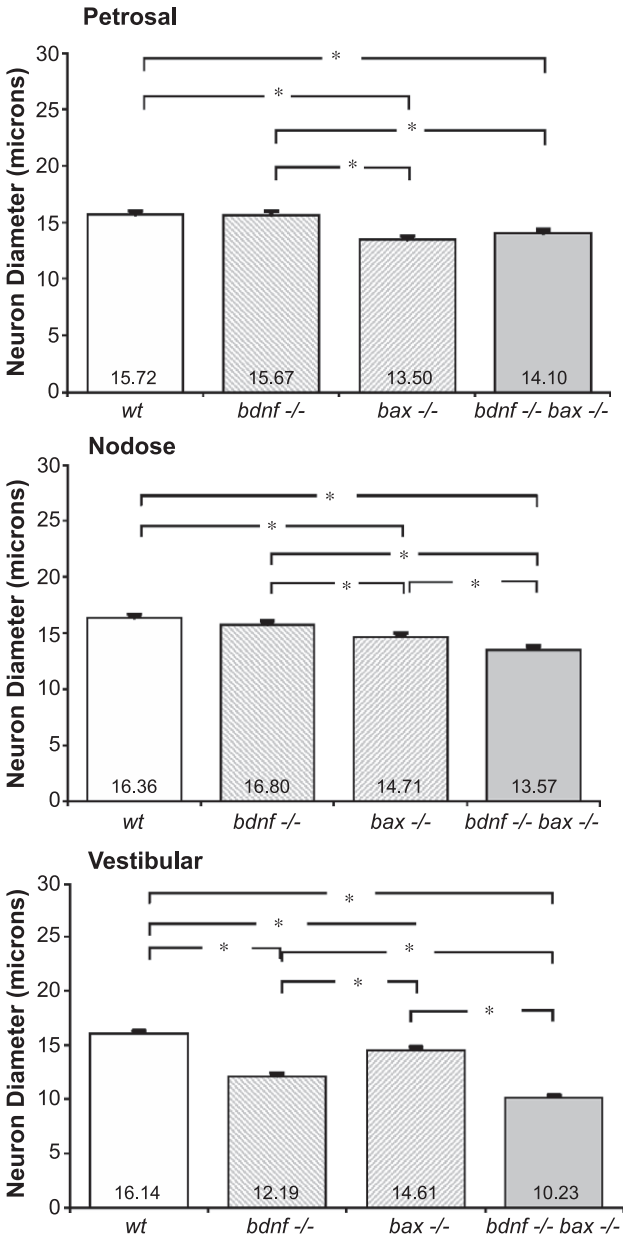

Fig. 3. Morphometric analysis of neuronal cell diameters in the petrosal, nodose, and vestibular ganglia in wild-type (wt), *bdnf* null (*bdnf*$^{-/-}$), *bax* null (*bax*$^{-/-}$), and double *bdnf/bax* null (*bdnf*$^{-/-}$ *bax*$^{-/-}$) newborn mice. Each bar represents the mean ± SEM of measurements taken from 100 neurons from six different animals (see Methods for details). *$P < 0.05$, ANOVA.

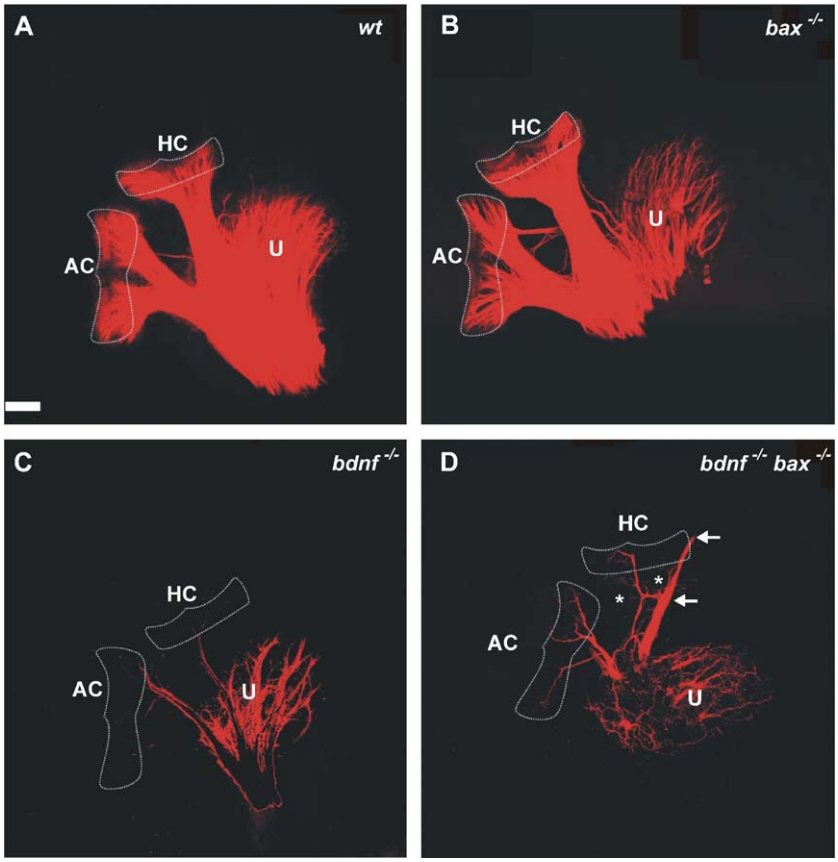

Fig. 4. Sensory innervation of the vestibular apparatus in wild-type (wt), *bax*$^{-/-}$, *bdnf*$^{-/-}$, and *bdnf*$^{-/-}$ *bax*$^{-/-}$ newborn mice. Whole mount preparations showing carbocyanine dye labeling of vestibular nerve afferent fibers. The utricle (U) and anterior (AC) and horizontal (HC) canal cristae are densely and selectively innervated in wild-type (A) and *bax*$^{-/-}$ (B) mice. In contrast, only occasional afferent fibers are found in the cristae organs in *bdnf*$^{-/-}$ mice (C). In *bdnf*$^{-/-}$ *bax*$^{-/-}$ mice (D), many more afferent fibers extend to the canal cristae than in *bdnf*$^{-/-}$ mice (C), however, the fibers are not specifically targeted to innervate only the cristae sensory epithelia. As shown in (D), many fibers either project beyond (arrows) or terminate proximal to (asterisks) the HC epithelium. Scale bar equals 100 μm.

role of BDNF in regulating target innervation by NG sensory neurons. In wild-type animals, baroreceptor afferents form a dense circumferential fiber plexus in the vascular adventitia with terminals extending into the tunica media. Fig. 5A shows the normal pattern of innervation of the right subclavian artery, which arises from sensory branches of the recurrent laryngeal nerve. Fig. 5B shows the normal pattern of innervation of the aortic arch, which arises from the aortic depressor nerve, a sensory branch of the vagus. Baroreceptor fibers were absent in *bdnf*$^{-/-}$ mutants (Figs. 5C and D), whereas *bax*$^{-/-}$ animals exhibited the wild-type pattern of innervation (Figs. 5E and F). In contrast, no baroreceptor fibers were present in the aortic arch in *bdnf*$^{-/-}$ *bax*$^{-/-}$ animals (Figs. 5G and H), indicating that the *bax* null mutation failed to rescue the *bdnf* null phenotype.

Our analysis of vestibular and vascular targets in *bdnf*$^{-/-}$ *bax*$^{-/-}$ animals demonstrates that BDNF is required not only for survival of cranial sensory neurons, but also for regulating peripheral innervation as well. The deficits we observed in double null mutants could reflect a requirement for BDNF in sensory fiber pathfinding, invasion of the target field, and/or maintenance of target innervation. To distinguish among these possibilities, we compared the pattern of baroreceptor fibers in wild-type, *bdnf*$^{-/-}$, *bax*$^{-/-}$, and *bdnf*$^{-/-}$ *bax*$^{-/-}$ animals at the onset of initial target innervation, between E13.5 and E14.5. As shown in Fig. 6, the aortic depressor branch of the vagus is found in close apposition to the wall of the aortic arch in both wild-type and *bdnf*$^{-/-}$ *bax*$^{-/-}$ animals, indicating that baroreceptor fibers are able to navigate to the arch in the absence of BDNF. However, in contrast to wild-type animals, virtually no baroreceptor fibers are found within the vascular wall in the double null mutants.

Sensory transmitter phenotype

A subset of BDNF-dependent NG and PG neurons is dopaminergic (Erickson et al., 1996; Hertzberg et al., 1994), and they can be distinguished by their expression of the catecholamine synthesizing enzyme tyrosine hydroxylase (TH). Therefore, to examine the effect of BDNF loss on neurochemical differentiation of cranial sensory neurons, we compared the number of TH immunoreactive NG and PG

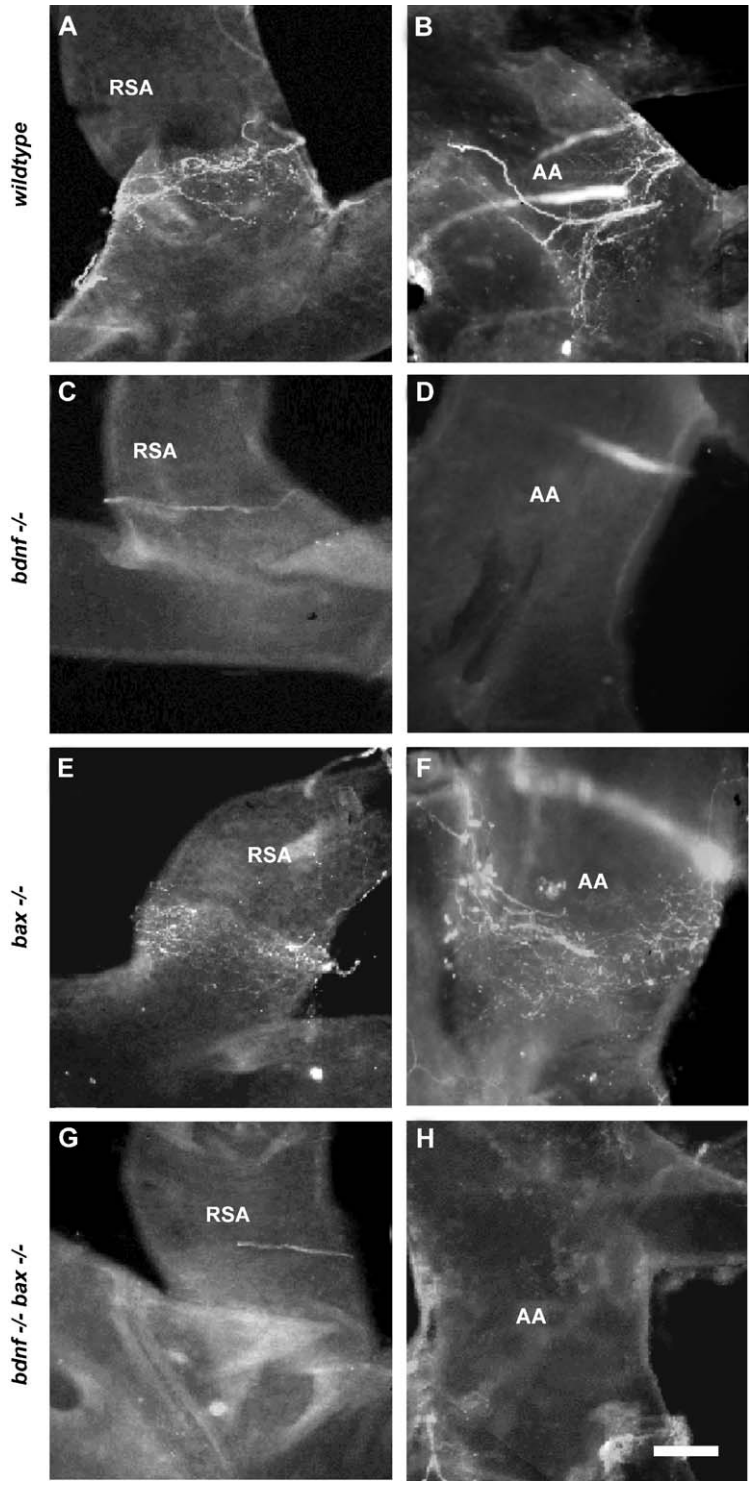

Fig. 5. Baroreceptor innervation of the right subclavian artery (A, C, E, G) and aortic arch (B, D, F, H) in wild-type (A and B), *bdnf*$^{-/-}$ (C and D), *bax*$^{-/-}$ (E and F), and *bdnf*$^{-/-}$ *bax*$^{-/-}$ (G and H) newborn mice. Whole mount preparations stained with antibodies against PGP 9.5 to reveal nerve fibers and terminal swellings. AA indicates aortic arch; RSA, right subclavian artery. Scale bar equals 250 μm.

neurons in wild-type, *bax*$^{-/-}$, *bdnf*$^{-/-}$, and *bdnf*$^{-/-}$ *bax*$^{-/-}$ newborn mice. As previously described (Erickson et al., 1996), TH cell numbers are severely reduced in *bdnf*$^{-/-}$ mice (Fig. 7). In contrast, the number of TH cells was increased in *bax*$^{-/-}$ animals compared to wild-type. This increase was disproportionately greater than the increase in total neuron number, resulting in a higher percentage of TH cells in *bax*$^{-/-}$ ganglia compared to wild-type (*bax*$^{-/-}$, 10% ± 0.44% vs. wild-type, 5% ± 0.54%). In *bdnf*$^{-/-}$ *bax*$^{-/-}$ animals, on the other hand, the number of TH cells

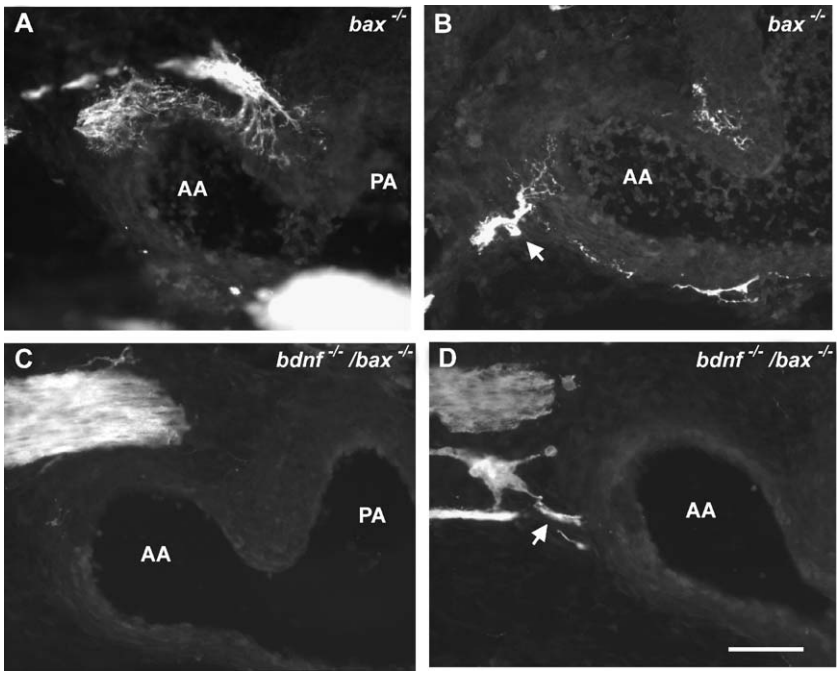

Fig. 6. Baroreceptor innervation of the aortic arch in E14.5 $bax^{-/-}$ (A and B) and $bdnf^{-/-}$ $bax^{-/-}$ (C and D) mouse embryos (PGP 9.5 immunostaining of 12-μm sections). Despite the presence of baroreceptor nerves in close apposition to the aortic arch in $bdnf^{-/-}$ $bax^{-/-}$ animals (arrow in D), no fibers enter the vascular adventitia as in wild-type (not shown) or $bax^{-/-}$ animals (A and B). AA indicates aortic arch; PA, pulmonary artery. Scale bar equals 75 μm.

was significantly decreased compared to $bax^{-/-}$ animals and was intermediate between the wild-type and $bdnf^{-/-}$ values.

Discussion

The present findings demonstrate that the *bdnf* null mutation results in cranial sensory neuron loss only in the presence of functional *bax* alleles. When *bax* is deleted, loss of BDNF has no effect on the number of NG, PG, or VG neurons, indicating that BDNF supports survival in these ganglia by suppressing Bax-dependent cell death. These results are consistent with recent demonstrations that the *bax* null mutation prevents death of spinal sensory neurons resulting from deletion of NGF (Patel et al., 2000) and neurotrophin-3 (Patel et al., 2003). Thus, inhibition of Bax-dependent cell death appears to be a common mechanism underlying support of sensory neuron survival by multiple neurotrophins (Patel et al., 2000).

Despite rescue of sensory neuron survival, the *bax* null mutation did not rescue the lethality of the *bdnf* null phenotype, as $bdnf^{-/-}$ $bax^{-/-}$ animals died soon after birth with the same latency and frequency as $bdnf^{-/-}$ animals. Previous studies in our laboratory demonstrated that BDNF is required for development of normal respiratory activity and that newborn $bdnf^{-/-}$ mutants exhibit severely depressed and irregular breathing (Erickson et al., 1996) that likely contributes to death during early postnatal life. This abnormal respiratory phenotype is related in part to the loss of BDNF-dependent chemoafferent neurons in the PG that provide excitatory drive to the respiratory central pattern generator (Erickson et al., 1996). Many of these neurons are dopaminergic (Katz and Black, 1986). Thus, the loss of peripheral target innervation and TH expression by PG neurons in $bdnf^{-/-}$ $bax^{-/-}$ mice may result in a functional chemoafferent deficit that reproduces the effect of PG cell loss in $bdnf^{-/-}$ animals. In addition, we recently found that BDNF and its receptor, TrkB, are expressed by neurons within the respiratory central pattern generator itself and that BDNF can modulate the central respiratory rhythm (Thoby-Brisson et al., 2003). Therefore, the lethality associated with the $bdnf^{-/-}$ and $bdnf^{-/-}$ $bax^{-/-}$ phenotypes may result from deficits in central respiratory output (Balkowiec and Katz, 1998), as well as excitatory chemoafferent drive. Moreover, the absence of baroreceptor innervation in $bdnf^{-/-}$ $bax^{-/-}$ mice suggests that cardiovascular homeostasis is likely to be disrupted in these animals as well.

Developmental expression of BDNF in peripheral tissues is tightly associated with the onset of innervation by sensory fibers (Brady et al., 1999; Farinas et al., 2001). Thus, initial expression of BDNF mRNA and protein in the cardiac outflow tract coincides with the arrival of baroreceptor afferents from the NG and is localized to sites at which the sensory axons enter the vascular wall (Brady et al., 1999). No baroreceptor fibers were observed within the aortic arch in either fetal or newborn $bdnf^{-/-}$ $bax^{-/-}$ mice, despite the presence of the aortic depressor nerve in close apposition to the vessel wall. This finding indicates that, in the absence of BDNF, baroreceptor fibers are still able to navigate to the periphery but are unable to enter their target field. Thus, the absence of peripheral baroreceptor fibers in newborn

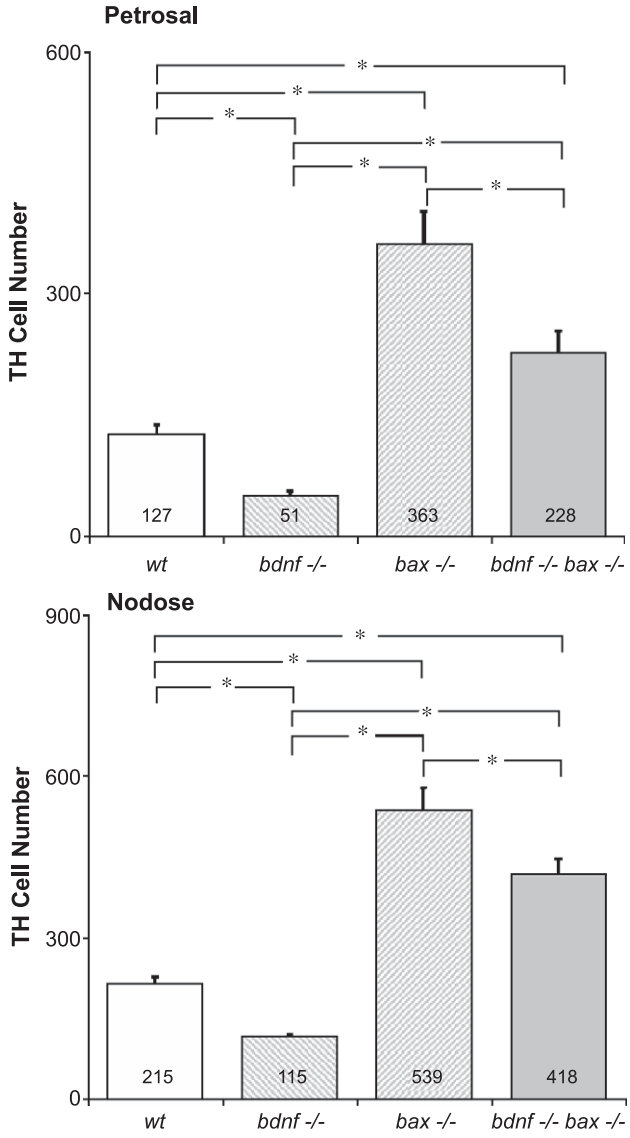

Fig. 7. *bax* null mutation does not completely rescue tyrosine hydroxylase (TH) expression in petrosal and nodose primary sensory neurons. Morphometric analysis of TH-positive cell numbers in the petrosal and nodose ganglia in wild-type (wt), *bdnf* null ($bdnf^{-/-}$), *bax* null ($bax^{-/-}$), and double *bdnf*/*bax* null ($bdnf^{-/-}$ $bax^{-/-}$) newborn mice. Each bar represents the mean ± SEM of cell counts from six different animals (see Methods for details). *$P < 0.05$, ANOVA.

$bdnf^{-/-}$ $bax^{-/-}$ mice results from an early failure of target innervation rather than a failure to maintain established projections. These data are the first demonstration that BDNF is required for establishment of visceral sensory innervation.

In the ear, $bdnf^{-/-}$ $bax^{-/-}$ mice exhibit decreased innervation density to the canal cristae despite complete rescue of vestibular ganglion cells. The innervation pattern in these animals is very similar to that in mice expressing BDNF under control of the NT-3 promoter ($NT\text{-}3^{tgBDNF}$) in the absence of endogenous BDNF (Tessarollo et al., 2004). However, in contrast to $NT\text{-}3^{tgBDNF}$ mice, the limited fiber growth to the canal cristae in $bdnf^{-/-}$ $bax^{-/-}$ mutants is disorganized, and the fibers exhibit little overt targeting to the cristae organs. These data indicate that NT-3, which is normally present in the vestibular sensory epithelium, has only a limited ability to promote directed growth of vestibular fibers in *bdnf*/*bax* double null mutants. In the ear, as in the cardiac outflow tract, BDNF appears not to be required for sensory axons to reach their target organs but is required to organize innervation within the target field (Tessarollo et al., 2004).

Our observations are consistent with previous reports that initial outgrowth of sensory fibers occurs independently of neurotrophins (Lumsden and Davies, 1983; Tessarollo et al., 2004; Vogel and Davies, 1991). On the other hand, cutaneous sensory axons fail to extend into hindlimb nerves in $ngf^{-/-}$ $bax^{-/-}$ and $trkA^{-/-}$ $bax^{-/-}$ mice, indicating that NGF, unlike BDNF, may be required for development of long projecting sensory nerves (Patel et al., 2000). Whether sensory fibers are unable to grow into the vascular wall or the sensory epithelia of the ear in the absence of BDNF or are simply not attracted to do so remains to be defined. A chemoattractive role for BDNF in sensory innervation would be consistent with observations that neuronal processes are attracted by artificial gradients of BDNF in vitro (Song et al., 1997) and in vivo. For example, developing sensory axons are sequestered at sites of BDNF overexpression (Krimm et al., 2001; Ringstedt et al., 1999), and focal application of antibodies against BDNF can arrest axonal outgrowth in vivo (Tucker et al., 2001). Similarly, the lack of targeted vestibular sensory innervation resulting from misexpression of BDNF under control of the NT-3 promoter further supports a role of BDNF in directed fiber growth (Tessarollo et al., 2004).

We previously demonstrated that baroreceptor innervation is also abnormal in mice in which the BDNF receptor, TrkB, is mutated at the Shc adaptor protein binding site (Postigo et al., 2002). In Shc mutants, sensory fibers are present within the vascular wall; however, the density of innervation is reduced compared to wild-type animals. The number of BDNF-dependent NG neurons appears unaffected by the Shc mutation (Minichiello et al., 1998), indicating that TrkB/Shc signaling regulates target innervation density independent of survival. These observations contrast with the present finding that, in the absence of BDNF in $bdnf^{-/-}$ $bax^{-/-}$ mice, no baroreceptor fibers enter the target field at all, despite complete rescue of NG neuron survival. Therefore, these data raise the possibility that the ability of sensory axons to invade the target field and innervation density, respectively, may be independently regulated by distinct BDNF-TrkB signaling pathways. Further work is required, for example, to determine whether the trkB/PLC-γ adaptor pathway (Minichiello et al., 1998) is involved in regulating target invasion by BDNF-dependent sensory afferents.

Our data also demonstrate that BDNF is required in vivo for expression of tyrosine hydroxylase (TH) by a subset of NG and PG sensory neurons. TH is a selective marker of

dopaminergic neurons in these ganglia (Katz et al., 1983) and is expressed subsequent to peripheral target innervation (Katz and Erb, 1990). The failure of the *bax* null mutation to completely rescue TH expression in *bdnf*$^{-/-}$ *bax*$^{-/-}$ mice may indicate a direct role for BDNF in regulating expression of the dopaminergic phenotype in some cells. Alternatively, deficits in target innervation in *bdnf*$^{-/-}$ *bax*$^{-/-}$ mice may result in the loss of other target derived cues that are required for dopaminergic expression. However, although target tissues support survival of NG and PG neurons, there is no evidence that target innervation per se promotes expression of the dopaminergic phenotype (Hertzberg et al., 1994). On the other hand, TH expression in cranial sensory neurons is strongly regulated by depolarizing stimuli in vitro (Brosenitsch and Katz, 2001; Brosenitsch et al., 1998; Hertzberg et al., 1995) and by physiologic stimulation in vivo (Brosenitsch and Katz, 2002). One possibility, therefore, is that BDNF upregulates expression and/or activation of molecules that are required for activity-dependent TH expression in these cells, including voltage-gated calcium channels, mitogen-activated protein kinase (MAPK), and the phosphorylated form of cyclic AMP response element binding protein (CREB) (Brosenitsch and Katz, 2001; Brosenitsch et al., 1998). BDNF has also been shown to increase expression of Phox2a (Holm et al., 2003), a homeodomain transcription factor expressed by dopaminergic PG neurons (Brosenitsch and Katz, 2002) that can drive TH expression (Lo et al., 1999). Although further studies are required to define the mechanism of BDNF action, our data parallel observations that expression of the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) by DRG neurons is abolished in *trkA* $^{-/-}$ *bax*$^{-/-}$ mice in which the neurons survive in the absence of NGF signaling (Patel et al., 2000). However, unlike dopaminergic properties, developmental expression of SP in sensory neurons is largely independent of neuronal activity (Brosenitsch et al., 1998), indicating that neurotrophin regulation of dopaminergic and peptidergic expression, respectively, may involve distinct mechanisms.

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