Target-derived BMP signaling limits sensory neuron number and the extent of peripheral innervation in vivo

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Accepted 18 November 2003

Development 131, 1175-1186
Published by The Company of Biologists 2004
doi:10.1242/dev.01013

Summary

The role of target-derived BMP signaling in development of sensory ganglia and the sensory innervation of the skin was examined in transgenic animals that overexpress either the BMP inhibitor noggin or BMP4 under the control of a keratin 14 (K14) promoter. Overexpression of noggin resulted in a significant increase in the number of neurons in the trigeminal and dorsal root ganglia. Conversely, overexpression of BMP4 resulted in a significant decrease in the number of dorsal root ganglion neurons. There was no significant change in proliferation of trigeminal ganglion neurons in the noggin transgenic animals, and neuron numbers did not undergo the normal developmental decrease between E12.5 and the adult, suggesting that programmed cell death was decreased in these animals. The increase in neuron numbers in the K14-noggin animals was followed by an extraordinary increase in the density of innervation in the skin and a marked change in the pattern of innervation by different types of fibers. Conversely, the density of innervation of the skin was decreased in the BMP4 overexpressing animals. Further Merkel cells and their innervation were increased in the K14-noggin mice and decreased in the K14-BMP4 mice. The changes in neuron numbers and the density of innervation were not accompanied by a change in the levels of neurotrophins in the skin. These findings indicate that the normal developmental decrease in neuron numbers in sensory ganglia depends upon BMP signaling, and that BMPs may limit both the final neuron number in sensory ganglia as well as the extent of innervation of targets. Coupled with prior observations, this suggests that BMP signaling may regulate the acquisition of dependence of neurons on neurotrophins for survival, as well as their dependence on target-derived neurotrophins for determining the density of innervation of the target.

Key words: BMP, Neurotrophin, Dorsal root ganglion, Trigeminal ganglion, Innervation, Noggin

Introduction

Excess neurons are generated during development of the vertebrate peripheral nervous system (PNS), and numbers are reduced to the required complement during a phase of programmed cell death that occurs shortly after the neurons innervate their targets. For most neurons in the PNS, this process is regulated by members of the neurotrophin family of growth factors that includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4 (NT4) (Hohn et al., 1990; Huang and Reichardt, 2001; Ip et al., 1992; Kirstein and Farinas, 2002; Leibrock, 1989; Maisonpierre et al., 1990; Rosenthal et al., 1990). At about the time that neurons innervate their targets, they become dependent upon the neurotrophins for their survival through unknown mechanisms, and the availability of growth factor then determines both the number of neurons that will survive and the density of innervation of the target structure (for a review, see Davies, 2003). The requirement of specific subsets of sensory neurons on the various neurotrophins and their cognate receptors has been corroborated in vivo through several techniques, including the analysis of mice with homozygous null mutations of the corresponding genes (Conover et al., 1995; Crowley, 1994; Ernfors et al., 1994a; Ernfors et al., 1994b; Farinas et al., 1994; Jones et al., 1994; Klein et al., 1993; Smeyne et al., 1994; Tessarollo et al., 1997). For example in the trkA null mutant (*Ntrk* null – Mouse Genome Informatics), 90% of the neurons are lost in the trigeminal ganglion, the primary sensory ganglion innervating the skin of the face and oral cavity, and accordingly several types of sensory endings formed by myelinated and unmyelinated axons are reduced or absent (Fundin et al., 1997a; Rice et al., 1998). Conversely, transgenic overexpression of neurotrophins in the skin leads to increases both in neuron numbers in sensory ganglia and in the density of innervation of the target (Albers et al., 1996; Albers et al., 1994; Davis et al., 1997; LeMaster et al., 1999; Rice et al., 1998).

Although the mechanisms by which neurons become dependent upon neurotrophins are uncertain, several lines of evidence suggest that members of the bone morphogenetic protein (BMP) family of the TGFβ superfamily are involved. Embryonic ganglia throughout the PNS express BMP receptors

(Zhang et al., 1998), indicating their potential for BMP sensitivity, and members of the BMP family of ligands including BMP2, BMP4 and BMP7 are expressed in the developing skin during the time that this target organ is innervated (Jones et al., 1991; Lyons et al., 1989; Lyons et al., 1990; Winnier et al., 1995). BMPs limit population size of a number of embryonic cell types, including neocortical ventricular zone cells both by promoting exit from cell cycle and by inducing apoptosis (Furuta et al., 1997; Mabie et al., 1999). Moreover, BMP treatment of MAH cells, a sympathoadrenal cell line (Birren and Anderson, 1990) promotes exit from cell cycle and induces apoptosis and neurotrophin dependence (Song et al., 1998). BMPs also promote apoptotic cell death of postmigratory enteric and sympathetic neural precursors unless rescued by gut-derived factors (Pisano et al., 2000; Chalazonitis et al., 2003). Finally, treatment of cultured embryonic sympathetic neuroblasts with BMP4 induces premature dependence of the neurons on neurotrophins for survival (Gomes and Kessler, 2001), suggesting that the BMPs might be the crucial factors that induce neurotrophin dependence during development.

As homozygous deletion of *Bmp2* or *Bmp4* or their receptors causes lethality in early development (Winnier et al., 1995; Zhang and Bradley, 1996; Beppu et al., 2000; Mishina et al., 1995), we chose a strategy of overexpressing an inhibitor of BMP signaling, noggin (Zimmerman et al., 1996) in skin to define the role of BMP signaling on the development of peripheral sensory neurons in vivo. We also generated mice that overexpress BMP4 in skin under the same keratin14 (K14) promoter to look for complementary findings. Our observations indicate that BMPs may regulate both the final neuron number in sensory ganglia and the extent of innervation of targets. Coupled with prior observations, this suggests that BMP signaling may regulate the acquisition of neuronal dependence upon neurotrophins for survival, as well as their dependence on target-derived neurotrophins for determining the density of innervation of the target.

Materials and methods

Generation of K14-noggin and K14-BMP4 transgenic mice

The generation of the K14-noggin mice has been described elsewhere (Guha et al., 2002). Briefly, *noggin* or *Bmp4* cDNA was subcloned downstream of the keratin14 promoter in the K14-hGH cassette vector (Cheng et al., 1992), which contains 2.1 kilobase pairs (kbps) of the human keratin 14 gene and a 1.8 kbp intron containing sequence from the human growth hormone (hGH) gene. Two lines of similar phenotype were obtained for the K14-noggin transgenic mice and three lines for the K14-BMP4 specific transgenic mice. Further screening was carried out with transgene-specific primer pairs: K14-noggin screening, noggin 543 (5′ GGACCTGGCGGAGCT 3′) and K14 (5′ GCCATTGCCGCTAGGTG 3′); K14-BMP4 screening, B4.9 (5′ GTGATGGACTAGTCTGGTGTC 3′) and B4.12 (5′ CACTGTGAGGAGTTTCCATC 3′).

In-situ hybridization and western blot analysis

Whole-mount in situ hybridization analysis was performed on E12.5 mouse embryos with riboprobes prepared against the full length *noggin* or *Bmp4* cDNA using a Digoxigenin labeling kit (Roche). Western Blot analysis was carried out on lysates from 1-month-old mouse back skin from both the transgenic and corresponding wild-type littermates. noggin protein was identified by rat monoclonal antibody against mouse noggin, clone RT57-16, a kind gift from Regeneron pharmaceuticals. BMP4 protein was identified using a BMP4-specific monoclonal antibody (Masuhara et al., 1995).

Processing of adult mouse tissues

Anesthetized animals were perfused with 4% paraformaldehyde (4% PF) in 0.1M phosphate-buffered saline (PBS) pH 7.4 at 4°C and the mystacial pads and the trigeminal ganglia were postfixed by immersion for 4 hours in 4% PF at pH 7.4 at 4°C. Following fixation, specimens were rinsed and stored in PBS at 4°C. Mystacial pads were cryoprotected by overnight filtration in 30% sucrose in PBS and frozen sectioned (14 μm) by cryostat in a plane perpendicular to the skin surface and parallel to the central rows of vibrissa follicles. The sections were directly thawed in an alternating series onto 5-10 slides coated with chrome-alum gelatin and air dried overnight.

Immunofluorescence

Immunofluorescence analyses and controls were performed as described previously (Rice et al., 1997; Paré et al., 2001) with polyclonal primary antibodies against pan-neuronal protein gene product 9.5 (PGP) (rabbit polyclonal, 1:1000, UltraClone); calcitonin gene-related peptide (CGRP) (rabbit polyclonal, 1:800, Chemicon; sheep polyclonal, 1:800, Infiniti); neurofilament 200 (NF) (rabbit polyclonal, 1:800; Chemicon).

Quantitation of neurons and innervation

Quantitative microscopic analyses of ganglion cells and their cutaneous innervation were assessed with NeuroLucida software (MicroBrightField, Colchester, VT). 4% PF fixed trigeminal ganglia were dehydrated in graded series of alcohol, cleared with histoclear and embedded in paraffin. Entire ganglia were serial sectioned at 8 μm and stained with Cresyl Violet. Cell counts were obtained using a modified Abercrombie method as described previously (Albers and Davis, 2001; Davis et al., 1996).

For total neuronal counts in embryonic stages, E12.5 embryos were fixed in 4% PF for 18-24 hours. Embryos were dehydrated in graded series of alcohol, cleared in histoclear and embedded in paraffin wax to cut 5 μm transverse sections upwards from neck. Every tenth section of the serially sectioned trigeminal ganglion was counted and the total number of neurons [cells immunoreactive for β-III tubulin (Chemicon, Temecula, CA)] in 10 sections was multiplied by the interval.

To quantify the cutaneous innervation, the following data were obtained from five sections of whisker pads in three transgenic and three wild-type littermates: (1) epidermal thickness at 20 equally spaced intervals, (2) the length of the epidermal surface, (3) the location of each sensory ending in the underlying epidermis, and (4) the location, outer contour and length of each piece of the nerves in the underlying dermis. Data 1-3 were used to calculate average epidermal thickness and epidermal innervation density. Because the nerves are cylindrical and the plane of sectioning was parallel to their long axis, the average diameter of each nerve profile was determined from the fourth set of data by dividing the area of each profile by the length of its long axis. The average diameter of each nerve was used to calculate the average cross-sectional area.

BrdU immunohistochemistry

Pregnant females at E12.5 gestational day were injected with 5-bromodeoxyuridine (BrdU, Sigma Chemicals, St Louis, MO) at 100 μg/g body weight and sacrificed 1 hour later, embryos collected and immersion fixed for12 hours in 4% PF in 0.1M PBS, pH 7.4. Embryos were washed in PBS, dehydrated in a graded series of alcohol, paraffin wax embedded and 5 μm thick serial sections cut transversely through the head. Sections containing the trigeminal ganglia were stained for BrdU immunohistochemistry with the BrdU in-situ detection kit (BD Pharmingen, San Diego, CA).

Trigeminal ganglion cell culture

E10 trigeminal ganglia were cultured on poly-D-lysine/laminin coated

culture dishes in defined medium containing BDNF (10 ng/ml) following the protocol of Davies et al. (Davies et al., 1993). Six-hundred cells were plated per each 35 mm culture dish. At 6 hours, the number of attached cells in each plate was counted twice in 10×10 mm grids and the number of cells for the whole area of the dish was calculated and used as the initial number of cells for the experiment. Additional growth factors [BMP4 (30 ng/ml), NGF (10 ng/ml), or BDNF (10 ng/ml)] were added at this time. After 72 hours, the number of surviving cells was counted twice in 10×10 mm grids and the overall number of surviving cells for the whole area of the dish was calculated.

Neurotrophin protein quantitation

NGF, BDNF and NT3 protein quantitation was determined by ELISA on lysates prepared from the mystacial pads of 3-day-old mice using the corresponding E_{max} Immunoassay System (Promega Corporation, Madison, WI).

Quantitative real time PCR (QRT-PCR) to estimate levels of neurotrophin transcripts

QRT-PCR was performed using Perkin-Elmer's ABI mPrism 7700 Sequence Detector System. Total RNA was extracted from the mystacial pads of E14.5 and P2 wild-type and K14-noggin transgenic littermates using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was prepared using the thermoscript RT-PCR kit (Invitrogen). QRT-PCR was performed with an initial denaturation of 10 minutes at 95°C followed by 40 cycles of 15 seconds denaturation at 95°C and 1 minute annealing and elongation at 60°C. SYBR green 1 dye was used to produce the fluorescent signal, which was detected at the annealing phase. As SYBR green 1 binds to double stranded DNA nonspecifically, the specificity of the reaction was confirmed by running the PCR products on 2% agarose gel and detecting a single specific band of the right size. Two replicates were run for each cDNA sample and five animals of each genotype were used.

Results

Generation of K14-noggin and K14-BMP4 transgenic mice

The generation of K14-noggin mice has been described before (Guha et al., 2002). Two independent K14-noggin transgenic lines and three independent K14-BMP4 lines were obtained. In both types of transgenic mice, the transgene was overexpressed in the mystacial pad, supplied by the trigeminal nerve, as early as E12.5 (Fig. 1). Expression continued to increase over the next few days and persisted in adult mice (not shown).

Increased epidermal and upper dermal innervation in K14-noggin mystacial pad

K14-promoter induced overexpression of noggin or BMP4 resulted in substantial changes in the innervation of the fur between the whisker follicles and in the Merkel innervation that terminates in the epidermis at the mouth of whisker follicles (Figs 2-4). As shown schematically in Fig. 5, based on previous studies (Fundin et al., 1997b; Rice et al., 1998; Rice et al., 1997; Rice et al., 1993), the inter-whisker fur is supplied by a dermal plexus composed of small nerves that are distributed into four distinct tiers oriented parallel to the skin surface.

Based upon anti-PGP as a pan-neuronal marker that labels all known axons and endings in the normal peripheral nervous system, three types of immunochemical characteristics were assessed among the third and fourth tier dermal plexus innervation: (1) some axons expressed only PGP-IR (i.e. nonpeptidergic), (2) others co-expressed CGRP-IR with little or no NF200-IR (i.e. NF-negative peptidergic), and (3) others co-expressed CGRP-IR with intense NF200-IR (i.e. NF-positive peptidergic). Previous studies in other species have shown that the nonpeptidergic and NF-negative peptidergic innervation consists of C fibers, whereas the NF-positive peptidergic innervation may consist of relatively larger caliber C or Aδ fibers. As shown in Figs 2-4 and the schematic in Fig. 5, the third tier of the dermal plexus contains all three types of axons and is the

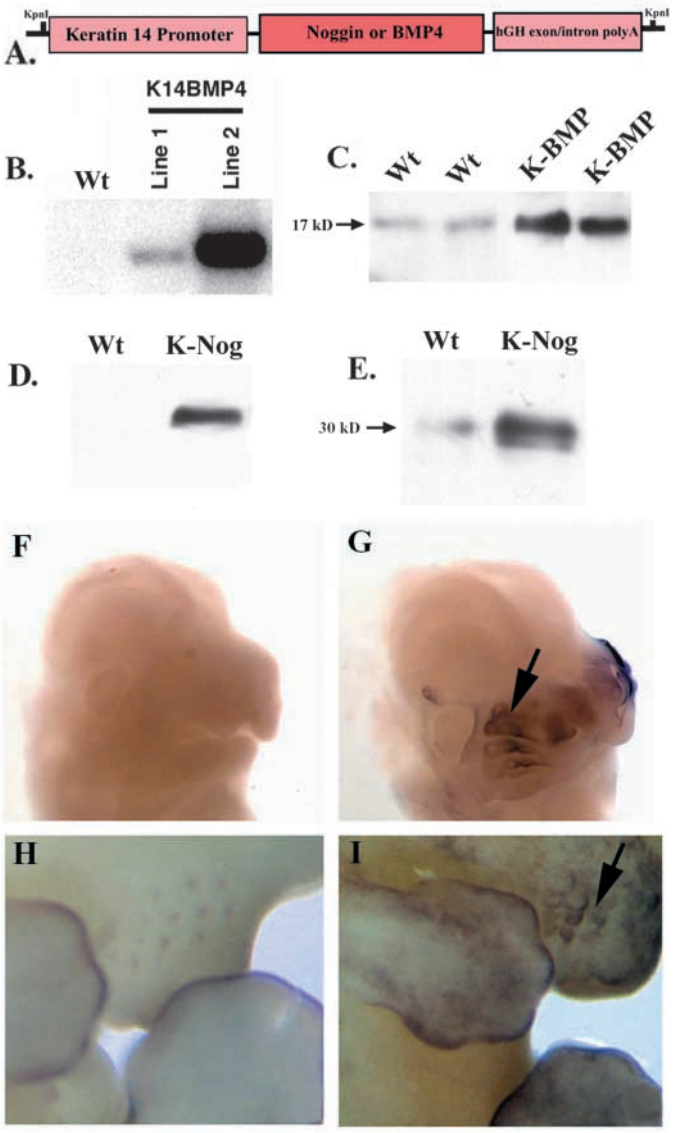

Fig. 1. Generation of K14-BMP4 and K14-noggin transgenic mice. (A) Transgenic constructs as diagrammed were injected into one-cell stage Fvb embryos to generate founders. (B,D) Southern blot analyses of genomic DNA from mouse tails shows the BMP4 and noggin transgenic bands in the respective transgenic founders. (C,E) Western blot analyses of lysates from back skin of one-month-old mice shows that BMP4 and noggin proteins are overexpressed in the skin compared with wild-type (wt) littermates. (F-I) Whole-mount in situ hybridization of E12.5 mouse embryos shows that the *Bmp4* (G) and *noggin* (I) transgenes are overexpressed in the whisker pad (arrows) of the corresponding transgenic embryos compared with wild-type littermates (F,H).

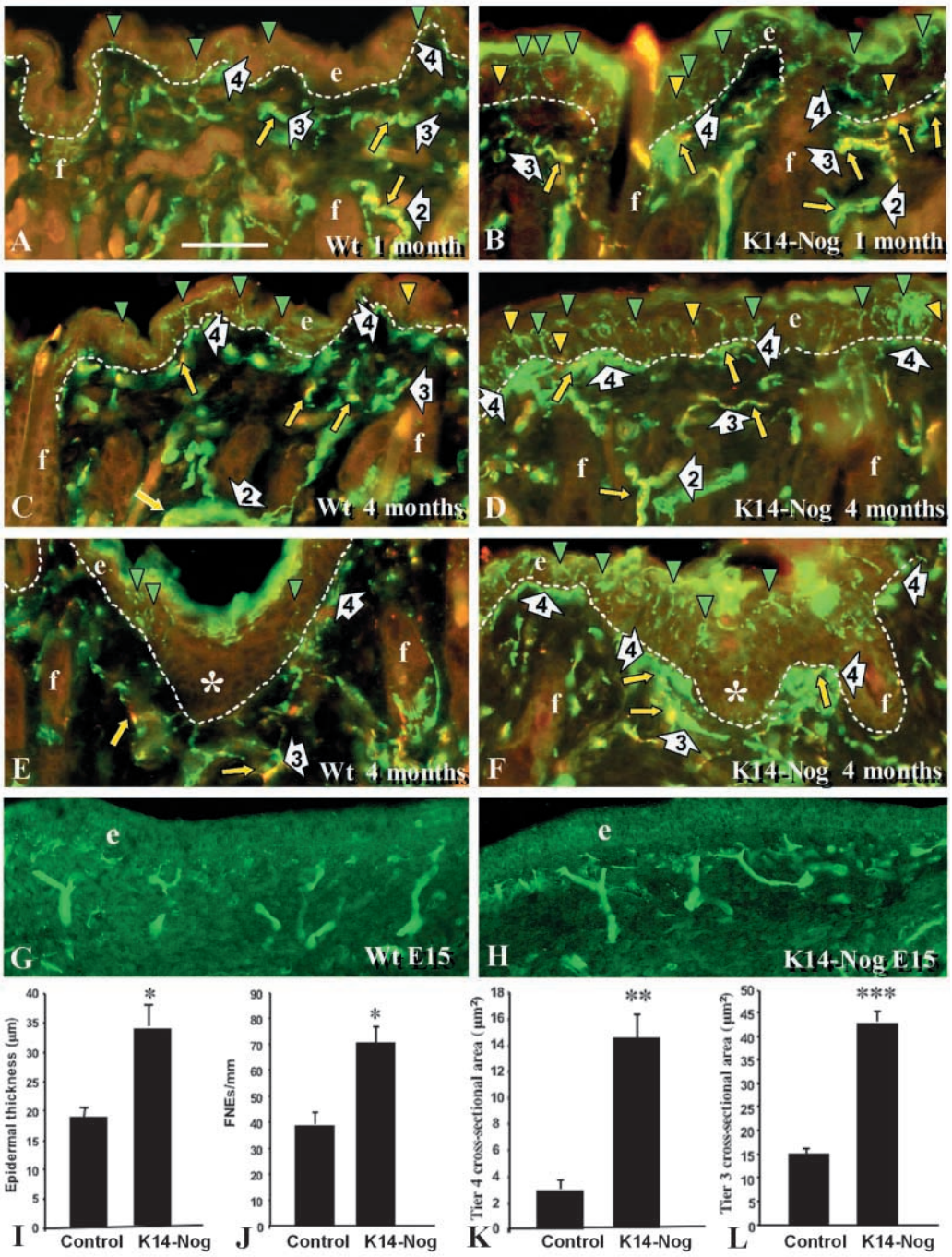

Fig. 2. Hyperinnervation of the skin in postnatal and adult K14-noggin animals. Innervation of the epidermis and upper dermis at comparable locations in the intervibrissal fur (A-D) and at the mouth of vibrissal follicles (E,F) in wild-type and K14-noggin overexpressing mice (K14-Nog) as shown by double-label immunofluorescence with anti-CGRP revealed with Cy3 and anti-PGP revealed with Cy2. e, epidermis; f, hair follicle; *, the mouth of a whisker follicle. The broken line indicates the border between the epidermis and dermis. Green arrowheads indicate examples of FNEs in the epidermis that are labeled only with anti-PGP. Yellow arrowheads indicate examples of epidermal FNEs that also label with anti-CGRP. Note that the epidermis of the K14-Nog mice contains far more FNEs with and without CGRP-IR. Broad arrowheads with numbers 2, 3 and 4 indicate regions of the second, third and fourth tiers of the dermal plexus respectively. In K14-Nog mice, the bundles of axons that compose the fourth tier are much larger and occupy a greater proportion of the epidermal-dermal border. In wild type, axons with CGRP-IR (yellow arrows) are mostly restricted to the second and third tiers. In K14-Nog, numerous axons with CGRP-IR are also located in the fourth tier. However, at E15 there are no detectable differences in PGP immunostaining between transgenic and control mice (G,H). Panels A-H are all mice from an Fvb background. (I-L) Quantification of the innervation to the epidermis and upper dermis in K14-Nog mice. The quantitative analyses were carried out on five sections for each mouse and the mean for each parameter obtained. Mean±s.e.m. of three mice for each genotype are shown (*P<0.05; **P<0.005; ***P<0.0005).

primary source of NF-positive peptidergic axons that ascend to terminate directly as presumptive free nerve endings (FNEs) in the epidermis. The nonpeptidergic and NF-negative peptidergic axons ascend in bundles to form the fourth tier, which is in intimate contact with the deep surface of the epidermal basement membrane. These axons also terminate as FNEs in the epidermis. In wild-type mice, the fourth tier consists almost entirely of nonpeptidergic innervation with few peptidergic axons.

In K14-noggin overexpressing mice, the thickness of the nerves in the third and fourth tiers is 9 and 12 times greater than in wild-type animals, and the density of FNEs in the epidermis is at least twice as great (Fig. 2B). This increase obviously involves the nonpeptidergic and especially the peptidergic innervation. CGRP-IR is far more extensive in the transgenic dermal plexus, especially in the fourth tier where CGRP-IR is rarely detectable in wild-type specimens. Double labeling with anti-CGRP and anti-NF revealed that most of the third tier and virtually all of the increased fourth tier peptidergic innervation had little or no detectable NF200. These results suggest that the noggin overexpression

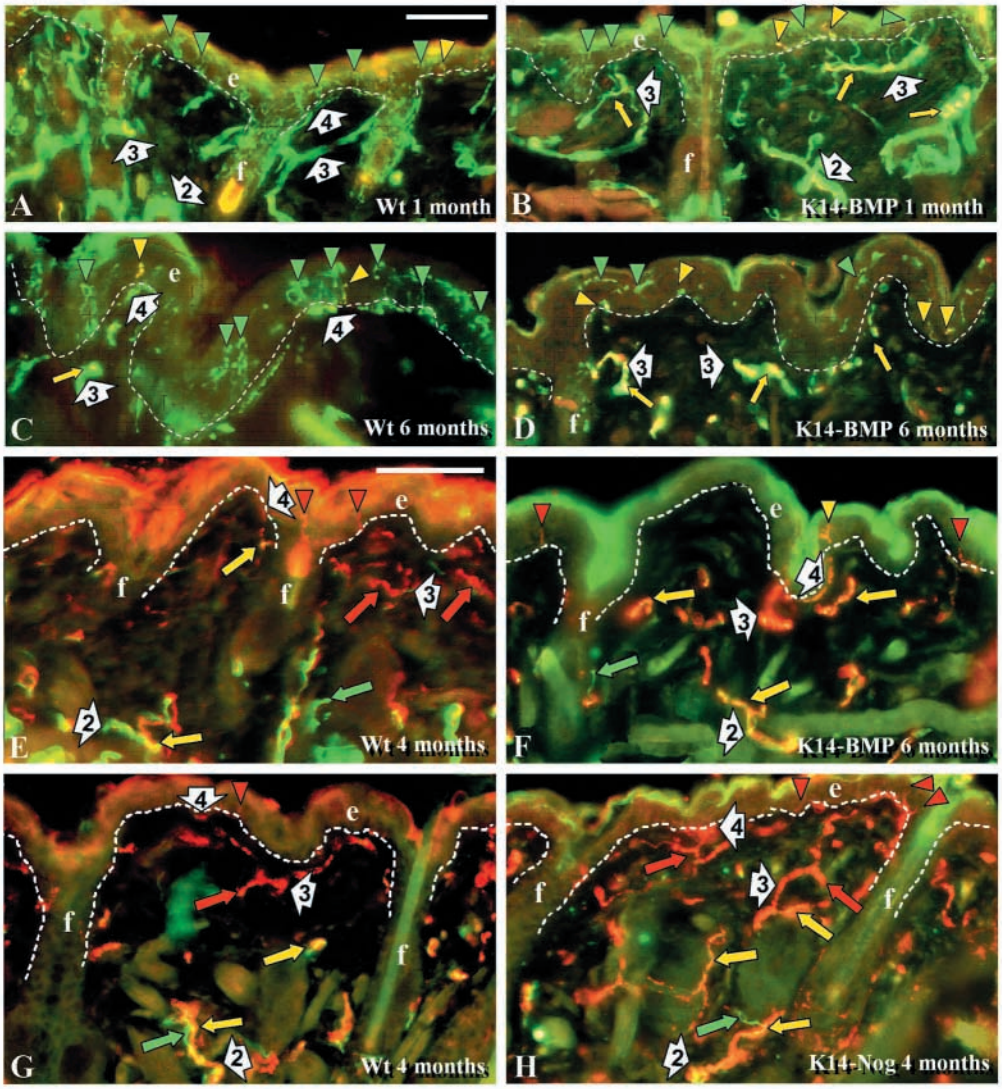

Fig. 3. Diminished innervation of the skin in K14-BMP4 mice. Innervation of the epidermis and upper dermis at comparable locations in the intervibrissal fur in wild type and K14-BMP4 mice. Labels are similar to those in Fig. 2. (A-D) Double-label immunofluorescence with anti-CGRP (Cy3) and anti-PGP (Cy2). At 1 month, the innervation to the epidermis appears comparable in wild type and K14-BMP4 mice. At 6 months, innervation in the K14-BMP4 mice has become reduced and fragmented. However, most of the remaining innervation expresses CGRP-IR, which may be more than in wild type. Yellow arrows indicate sites where axons express CGRP-IR. In contrast to wild type, in K14-BMP4 transgenics most epidermal innervation seems to be supplied directly from the third tier of the dermal plexus. Axon bundles at the epidermal-dermal border, indicative of the fourth tier, are readily encountered in wild type. The fourth tier was rarely detected in K14-BMP4 intervibrissal fur. A-D are all mice from CB6F1 background. (E-H) Comparison of the innervation to the epidermis and upper dermis at comparable locations in the intervibrissal fur and in wild-type, K14-BMP4 and K14-noggin mice as shown by double-label immunofluorescence with anti-CGRP (Cy3) and anti-NF200 (Cy2). E,F are from a CB6F1 background; G,H are from a Fvb background. Red arrows indicate axons labeled only with anti-CGRP, green arrows labeled only with anti-NF200, yellow arrows labeled with both anti-CGRP and anti-NF. Note the massive increase in CGRP-positive innervation at all levels of the dermal plexus in the K14-noggin fur. In both wild-type and K14-noggin mice, the third and fourth tiers of the dermal plexus contain anti-CGRP labeled axons that are mostly NF200 negative (red arrows). Likewise, the anti-CGRP labeled endings in the epidermis lack NF200-IR (red arrowheads). A few axons in the third tier contain NF200 but most of the CGRP-positive/NF200-positive axons are in the second tier. By contrast, in the K14-BMP4 fur, most of the CGRP positive innervation at all levels co-expresses NF200-IR. This includes an ending in the epidermis that co-expresses CGRP-IR and NF200-IR (yellow arrowhead), which is never seen in the wild-type epidermis.

preferentially increased the nonpeptidergic and NF-negative peptidergic innervation to the epidermis. The increases in the epidermal innervation were as evident in 1-month-old noggin transgenics as in mature adults. However, there was no detectable change in the density of innervation of the skin at E15. Thus, the changes in innervation occurred after the major period of naturally occurring cell death in the trigeminal ganglion (Enokido et al., 1999; Davies, 2003).

In contrast to the results in the noggin-overexpressing mice, the innervation of the epidermis is reduced in the BMP4 transgenic animals, especially related to the fourth tier. Interestingly, the epidermis is somewhat innervated in the 1-month-old BMP transgenics but the innervation is severely depleted in the mature adult. However, at both stages, virtually all of the epidermal innervation appears to be supplied by individual axons ascending directly from the third tier of the dermal plexus. Bundles of axons were rare against the deep surface of the basement membrane where the fourth tier is normally located. Compared with wild-type specimens, a relatively high proportion of the third tier epidermal innervation expresses CGRP-IR, especially in the mature adult. Double labeling with anti-CGRP and anti-NF revealed that most of this third tier CGRP innervation was NF positive. Importantly, the epidermis of BMP4 overexpressers also contained endings that co-express CGRP-IR and NF-IR, which are lacking in wild-type epidermis (Fig. 3). In wild-type epidermis, only CGRP-IR is expressed in the epidermal endings of axons that co-express CGRP-IR and NF-IR. These results suggest that the overexpression of BMP4 has a detrimental effect on the fourth tier nonpeptidergic and NF-

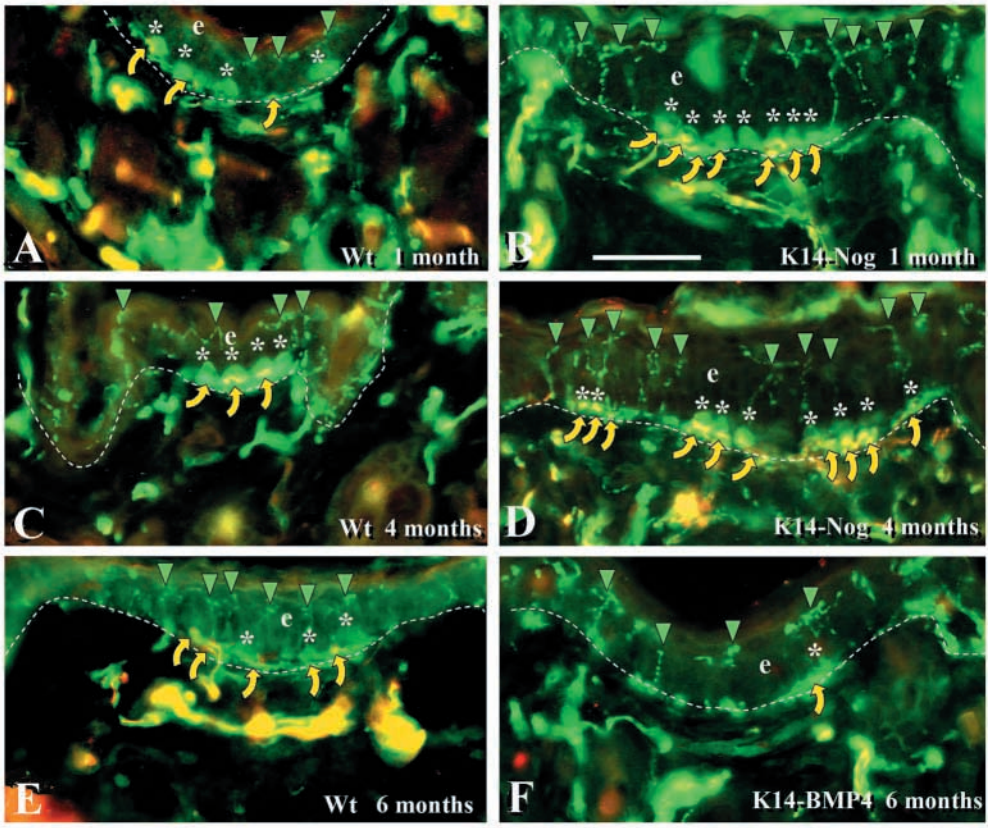

Fig. 4. Merkel cells are increased in K14-noggin and decreased in K14-BMP4 mice. Innervation to the mouth of whisker follicles in wild-type, K14-noggin and K14-BMP4 mice as shown by double-label immunofluorescence with anti-NF200 (Cy3) and anti-PGP (Cy2). e, epidermis at the mouth of a whisker follicle. A-D are from an Fvb background; E,F are from CB6F1 background. The broken line indicates the border between the epidermis and dermis. Green arrowheads indicate examples of FNEs in the epidermis which are labeled only with anti-PGP. Note that the epidermis of the K14-noggin mice contains far more FNEs and that the K14-BMP4 mice have fewer FNEs than their corresponding wild-type littermates. Yellow curved arrows indicate Merkel endings labeled with anti-NF200 and anti-PGP that terminate on Merkel cells (asterisks) labeled only with anti-PGP. Note that the Merkel cells and innervation are increased in K14-Nog and decreased in K14-BMP4 mice.

negative peptidergic innervation, especially over time, but may favor the presence of NF-positive peptidergic innervation.

The Merkel innervation at the mouth of whisker follicles and occasional guard hair follicles is supplied from the second tier of the dermal plexus, and consists of Aβ fibers that co-label with anti-PGP and anti-NF200. The Merkel endings penetrate the basement membrane of the epidermis at the mouth of the follicle to terminate on anti-PGP immunoreactive Merkel cells in lamina basalis. As seen in Fig. 4A-D, the mouths of comparable whisker follicles have far more Merkel cells and Merkel endings in noggin overexpressers than wild-type mice. By contrast, BMP4 overexpressing mice have fewer Merkel endings seen in the whisker pads (Fig. 4E,F). These results indicate that the overexpression of noggin promotes the development of Merkel cells and their endings whereas BMP4 is detrimental. (See Fig. 5 for a summary of the data presented in Figs 2-4.)

Increased neuron number in adult K14-noggin transgenic trigeminal ganglia

To determine whether BMP signaling also influences neuron numbers in the peripheral nervous system, we quantified the total number of neurons in adult trigeminal ganglia. As shown in Fig. 6A, there was a 35% increase in total number of neurons in the trigeminal ganglia of K14-noggin mice compared with the wild type. There were 42194±1684 neurons (±s.e.m.; n=7) in the noggin transgenic trigeminal ganglia compared with 31209±2888 (±s.e.m.; n=9) neurons in control ganglia. Moreover, the increase was specifically in the smaller size neuronal population as shown by size distribution of the neurons in adult trigeminal ganglia (Fig. 6B). This is

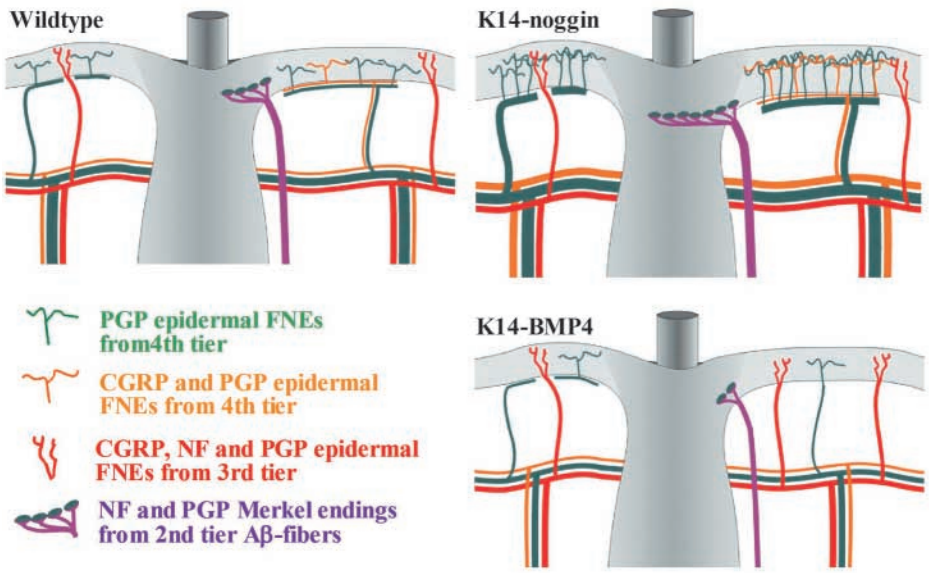

Fig. 5. Summary of changes in the innervation within the intervibrissal fur and the mouth of the whisker follicles of the K14-noggin and K14-BMP4 mice.

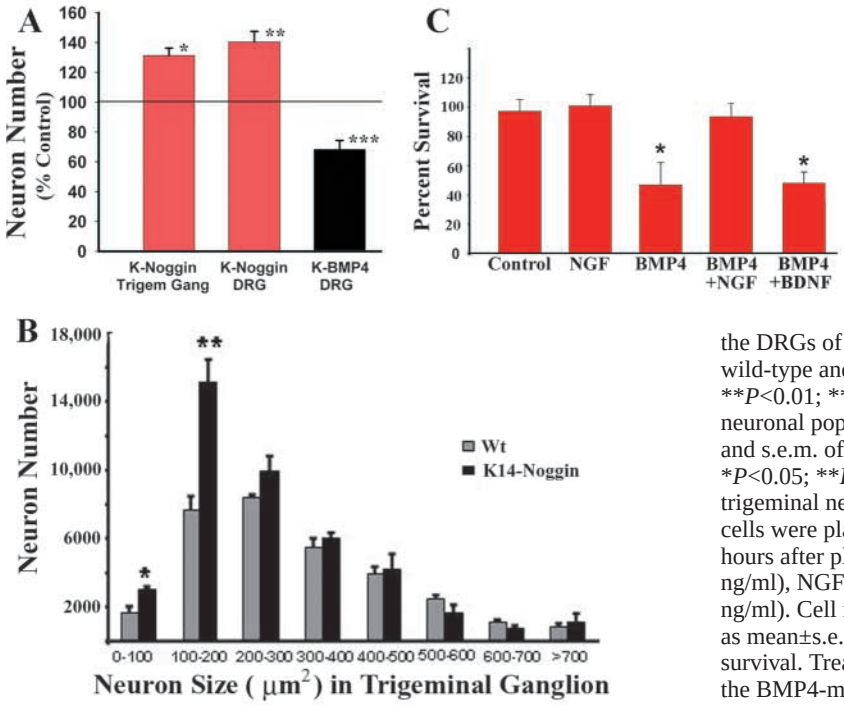

Fig. 6. BMP signaling decreases neuron numbers in vivo and in vitro. (A) Neuron numbers in the trigeminal ganglion and DRG of K14-noggin animals and the DRG of the K14-BMP4 animals normalized to their respective wild-type controls for each ganglion. Neuron numbers were significantly increased in the trigeminal and dorsal root ganglia of the K14-noggin animals, but significantly reduced in the DRGs of K14-BMP4 animals. The mean and s.e.m. of seven wild-type and seven transgenic ganglia are shown. **P*<0.02; ***P*<0.01; ****P*<0.015. (B) Preferential increase of the smaller size neuronal population in K14-noggin trigeminal ganglia. The means and s.e.m. of three wild-type and four transgenic ganglia are shown. **P*<0.05; ***P*<0.005. (C) Effects of BMP4 and NGF on cultured trigeminal neurons. E10 trigeminal ganglia were dissociated, and 600 cells were plated per well in the presence of BDNF (5 ng/ml). Six hours after plating, some cultures were treated with BMP4 (30 ng/ml), NGF (10 ng/ml), or both, or with additional BDNF (10 ng/ml). Cell numbers were counted 72 hours later and are expressed as mean±s.e.m. per dish. Note that BMP4 treatment reduced cell survival. Treatment with NGF but not additional BDNF prevented the BMP4-mediated cell death (**P*<0.04 using ANOVA).

consistent with the increase in the peptidergic and the nonpeptidergic free nerve endings in the epidermis that are normally derived from the smaller neuronal population in the ganglion. There was also a 40% increase in the number of neurons in the C6 DRG of the K14-noggin animals (Fig. 6A). By contrast, there was a 33% reduction in neuron numbers in the K14-BMP4 transgenic animals (Fig. 6A).

BMP4 treatment makes E10 trigeminal neurons dependent upon NGF for survival in vitro

As these observations suggested that BMP signaling reduces trigeminal neuron survival in vivo, the effects of BMP4 on survival of E10 trigeminal neuron were examined in vitro. BMP-mediated apoptosis of other populations of neural crest derived neurons can be prevented by neurotrophins in vitro (Song et al., 1998; Gomes and Kessler, 2001; Pisano et al., 2000; Chalazonitis et al., 2003), so the effects of concurrent treatment with NGF were also examined. Because at E10 cultured trigeminal neurons survive in the presence of BDNF and do not require NGF (Enokido et al., 1999), the cultures were all established in the presence of BDNF. During 3 days in vitro there was no significant reduction in neuron numbers in cultures containing only BDNF (control cultures), and addition of NGF did not alter cell numbers (Fig. 6). Addition of BMP4 significantly reduced cell numbers by more than 50%. Treatment with NGF prevented the BMP-mediated cell death, but treatment with equal amounts of additional BDNF did not prevent the cell death. Thus, E10 trigeminal neurons treated with BMP4 in vitro became dependent upon NGF for their survival. Importantly, there was no detectable difference in neurite outgrowth in cultures treated with BMP4 plus NGF versus cultures treated with either NGF or BDNF alone (controls), indicating that BMP4 treatment did not exert a detectable effect on neurite outgrowth in vitro.

Total numbers of neurons and proliferating neuroblasts are unchanged in E12.5 transgenic ganglia

The increased neuronal number in the K14-noggin mice could have resulted from either increased proliferation or increased survival of the developing sensory neurons in the ganglia. Proliferation of neuroblasts in the mouse trigeminal ganglia occurs between E9 and E13. We therefore counted the total number of β-tubulin positive neurons in the trigeminal ganglion at E12.5 day of gestation and found no significant difference in the total number of neurons between the transgenic and wild type littermates (Fig. 7A). There were 39,107±2522 neurons in the noggin transgenic ganglia compared with 37210±3097 neurons in control ganglia. Thus, at this time period, there was not an increase in the number of neurons generated in the K14-noggin animal. Analysis of cell proliferation in the trigeminal ganglia at E12.5 using bromodeoxyuridine (BrdU) labeling also revealed no significant changes. There were 15131±1092 BrdU-positive cells in the noggin transgenic ganglia compared with 13172±716 in the control ganglia (Fig. 7B) indicating that proliferation was not altered. There were also no significant differences in TUNEL labeling, although there was a trend towards a decrease in the K14-noggin animals (Fig. 7C). Comparison of neuron numbers at E12.5 with numbers in the adult ganglion (Fig. 7D) demonstrates that there is the expected loss of about a third of the neurons in the wild-type animals but no loss in the K14-noggin trigeminal ganglia. Hence, the increased neuronal number seen in the adult in the K14-noggin transgenic animals appears to reflect increased survival of neurons that would normally have been destined to die.

Neurotrophin estimation in the mystacial pad

The increased trigeminal innervation and neuron numbers observed in K14-noggin mice and the complementary changes

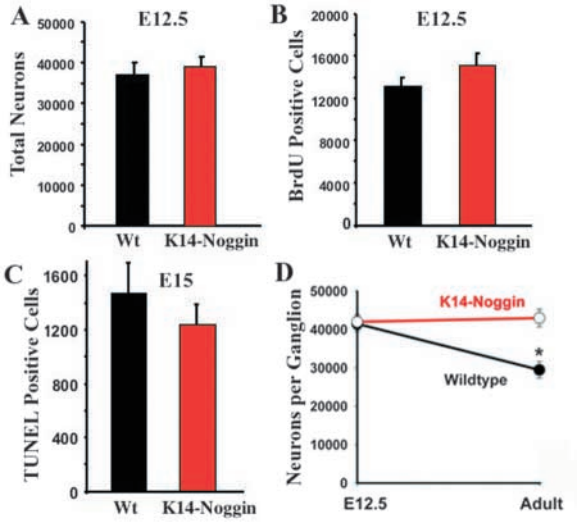

Fig. 7. Neuron numbers are unchanged in E12.5 K14-noggin trigeminal ganglia. (A) Total number of β-tubulin positive neurons in the trigeminal ganglion is unchanged in K14-noggin compared with wild-type littermates. Mean and s.e.m. of four animals of each genotype are shown. (B) Total number of BrdU-positive cells is not significantly different in K14-noggin versus wild-type trigeminal ganglia. Means and s.e.m. of four animals of each genotype are shown. (C) Total number of TUNEL-positive cells is unchanged. Mean and s.e.m. of four animals of each genotype are shown. (D) Neuron numbers in the trigeminal ganglion decrease by about 33% between E12.5 and adult life in wild type but do not change in the K14-noggin animals (*P<0.02).

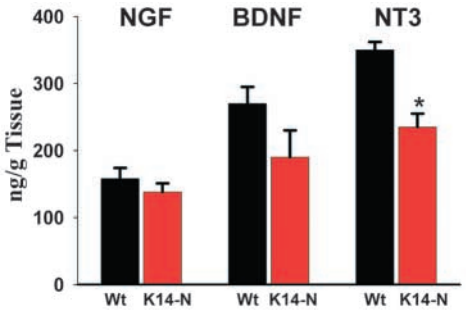

Fig. 8. Neurotrophin levels are not increased in the skin of K14-noggin animals. NGF and BDNF proteins as measured by ELISA of lysates from the whisker pads of 3-day-old mice are unchanged in K14-noggin compared with the wild-type mice and levels of NT3 are actually reduced significantly. Mean±s.e.m. of five animals of each genotype are shown (*P<0.025).

seen in the K14-BMP4 mice could be a result of modulation of neurotrophin expression in the target organ. We therefore measured the abundance of NGF, BDNF and NT3 protein in the mystacial pads of early postnatal mice by ELISA. There were no significant changes in the expression of NGF or BDNF in the mystacial pads of 3-day-old K14-noggin transgenic mice and NT3 protein was actually reduced (Fig. 8). Thus, the density of innervation clearly did not correlate with the levels of any of these neurotrophins. The abundance of neurotrophin transcripts was also examined in the mystacial pads of E14.5 and postnatal day 2 mice using real-time quantitative PCR (Fig. 9). There were no changes in NGF, NT3 or GDNF transcripts at E14.5, while BDNF transcripts were slightly increased about twofold. At PN2 there were no changes in transcript levels of BDNF, NT3 or GDNF, but there was a small (fourfold) increase in NGF transcripts.

Discussion

As homozygous deletion of *Bmp2* or *Bmp4* or their receptors causes lethality in early development (Winnier et al., 1995; Zhang and Bradley, 1996; Beppu et al., 2000; Mishina et al., 1995), we chose a strategy of overexpressing an inhibitor of BMP signaling, noggin (Zimmerman et al., 1996), in skin, in order to define the role of BMP signaling in the development of peripheral sensory neurons in vivo. In prior studies, we demonstrated that BMP signaling (SMAD phosphorylation and translocation) is inhibited in the skin and interdigital mesenchyme of these animals (Guha et al., 2002). We also overexpressed BMP4 in different transgenic mice in the same domain as noggin to conversely augment the effects of endogenous BMPs on sensory neurons. Inhibition of BMP signaling resulted in an increase in neuron numbers in the trigeminal and dorsal root ganglia and hyperinnervation in the epidermis and upper dermis both in young adult and mature adult mice, while increasing BMP4 in the skin had converse effects on the sensory innervation in the mystacial pad and neuron numbers in the dorsal root ganglion.

BMPs including BMP2, BMP4 and BMP7 are expressed in the developing epidermis (Bitgood and McMahon, 1995; Jones et al., 1991; Lyons et al., 1989; Lyons et al., 1990; Takahashi and Ikeda, 1996; Winnier et al., 1995). Moreover, embryonic ganglia throughout the PNS including the trigeminal ganglion express both type I and type II BMP receptors (Zhang et al., 1998), indicating their potential for BMP sensitivity. The responsiveness of sensory neurons to BMPs is further evidenced by prior studies indicating that BMP signaling regulates neuropeptide expression by developing peripheral sensory neurons (Ai et al., 1999; Hall et al., 2001). Furthermore, treatment of E10 trigeminal neurons with BMP4 in vitro promoted cell death (Fig. 6), indicating responsiveness of the neurons to BMP4. Hence, the various components of the BMP pathway are present in embryonic neurons for target-derived BMPs to have a direct effect on neuron survival and on the innervation of the skin. However the BMP receptors, BMPR-IA and BMPR-IB are also expressed in the basal layer of the epidermis and the keratinocytes of the suprabasal layer, respectively, at E16.5 (Botchkarev et al., 1999), and the signal transducers of the BMP pathway, Smad1 and Smad5 are also expressed in the developing murine epidermis (Dick et al., 1998; Flanders et al., 2001). Furthermore, substantial evidence indicates that BMP signaling plays a crucial role in the development of both skin and hair follicles (for a review, see Botchkarev, 2003). It is therefore possible that some of the changes in the sensory innervation occur indirectly because of BMP-mediated changes in the target tissue.

Irrespective of whether the changes observed in the transgenic animals reflect direct or indirect effects of the BMPs on the sensory innervation, these studies indicate that the number of neurons surviving in the innervating ganglion is not coupled exclusively to the level of neurotrophins produced by the target tissue. Levels of NGF and BDNF were unchanged in the transgenic mystacial pads at E14.5 and PN3, and NT3

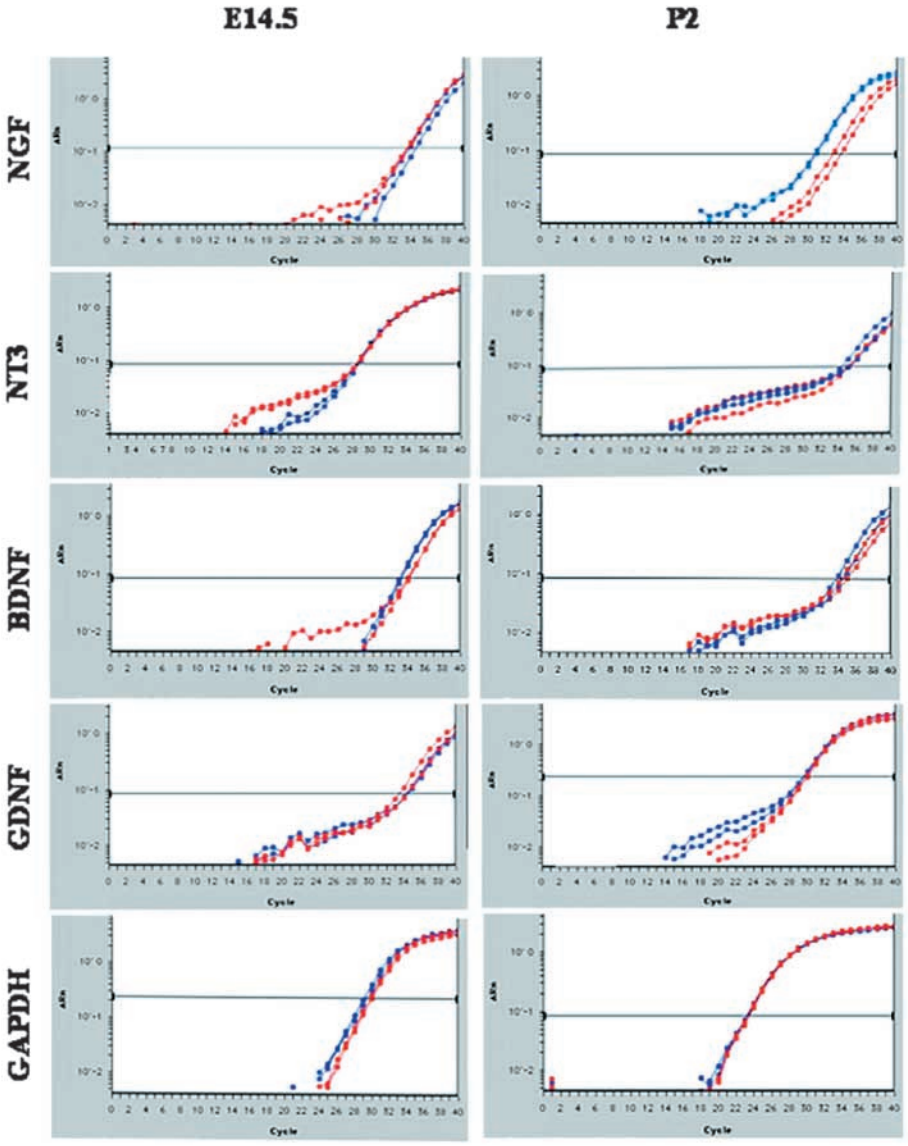

Fig. 9. Neurotrophin transcript expression in the mystacial pads of K14-noggin mice. Expression of neurotrophin mRNAs was analyzed by quantitative RT-PCR performed with mRNA extracted from the mystacial pads of E14.5 and postnatal day 2 (P2) K14-noggin and wild-type littermates. Shown are two curves each for transgenic (blue) and wild-type (red) tissues. At E14.5 there were no changes in NGF, NT3 or GDNF transcripts, while BDNF transcripts were slightly increased about twofold in the K14-noggin animals. At PN2 there were no changes in transcript levels of BDNF, NT3 or GDNF, but there was a small (fourfold) increase in NGF transcripts in the K14-noggin animals.

protein abundance was actually significantly lower in the hyperinnervated skin of the noggin transgenic animals, possibly reflecting increased competition by the larger number of nerve fibers for a limited supply of NT3. It is interesting that NGF transcripts were increased fourfold in the transgenic skin at PN2. However, more importantly there was no change in transcript expression of NGF at E14.5 mystacial pads, the time period when apoptosis in the trigeminal ganglion is at its peak. BDNF transcript expression is also increased twofold at E14.5 mystacial pads in the transgenic mice. However, most neurons in the trigeminal ganglion have switched their dependence from BDNF to NGF by this time period. The mechanisms by which target-derived BMPs alter the requirements for neurotrophins are unclear. BMP treatment of MAH cells, a sympathoadrenal cell line, promotes exit from cell cycle and induces apoptosis and neurotrophin dependence (Song et al., 1998). Furthermore, treatment of cultured embryonic sympathetic neuroblasts with BMP4 induces premature dependence of the neurons on neurotrophins for survival (Gomes and Kessler, 2001), suggesting that the BMPs might be crucial factors that induce neurotrophin dependence during development. The findings in this study are consistent with this hypothesis. In control mice there was a decrease in neuron number of about 30% between E12.5 and the adult. By contrast, there was no decrease in neuron number in the noggin transgenic animals during this period of time, indicating that the normal amount of programmed cell death did not occur in the absence of BMP signaling in the target. There was no significant difference in TUNEL-positive apoptotic cells at E15.5 though the mean number of those cells was less in the transgenic. It is possible that there is a small decrease of apoptotic cells in the transgenic ganglia over a prolonged period of embryonic development when normal cell death occurs. A role of increased proliferation of neuroblasts in the transgenic ganglia followed by reduced neurotrophin dependence is possible. However, at E12.5, both the total neuron number and BrdU-positive proliferating cells in the trigeminal ganglia are unchanged in the noggin-transgenic mice. Interestingly, once sensory neurons have become dependent upon neurotrophins for survival, BMPs have been shown to augment neuronal survival when neurotrophins are present (Farkas et al., 1999). This is also consistent with the hypothesis that the pro-apoptotic effects of the BMPs on embryonic neuroblasts reflect induction of neurotrophin dependence rather than a direct toxic/pro-apoptotic effect of the BMPs on sensory neurons. This conclusion is also supported by evidence that overexpression of noggin in embryonic gut dramatically increases the density of the neural crest-derived enteric neurons in both myenteric and submucosal plexuses in the gut of 4-week-old animals (Chalazonitis et al., 2003). Furthermore, treatment of E10 trigeminal neurons with BMP4 in vitro resulted in neuronal death that could be rescued by NGF but not BDNF (Fig. 6). Trigeminal ganglion neurons normally switch their dependence from BDNF to NGF between E10 and E13 through

unknown mechanisms (Enokido et al., 1999). However dissociated E10 neurons do not develop NGF dependence in vitro and survive well in the presence of BDNF (Enokido et al., 1999). By contrast, exposure of E10 trigeminal neurons to BMP4 resulted in dependence upon NGF for their survival, i.e. they died unless NGF (and not BDNF) was added to the medium. This suggests that BMP signaling participates in the acquisition of NGF dependence by trigeminal neurons.

At E15 there was no increase in the density of innervation of the skin in the K14-noggin transgenic animals (Fig. 2). Because this corresponds to the normal period of maximal neuronal death in the trigeminal ganglion, enhanced sprouting of nerve fibers in the target did not mediate the enhanced neuronal survival. However, the increase in the density of innervation could conversely reflect, in part, the increase in the number of trigeminal neurons that survived. Nevertheless the magnitude of the increase in innervation was much greater than the 40% increase in neuron numbers in the K14-transgenic animals, suggesting that other mechanisms were also involved. Treatment of cultured sympathetic or cortical neurons with BMP7 promotes dendritic rather than axonal outgrowth (Lein et al., 1996; LeRoux et al., 1999), and BMP signaling exerts repulsive effects on axonal outgrowth by roof-plate commissural neurons (Augsburger et al., 1999; Butler and Dodd, 2003). These observations raise the possibility that BMP signaling directly represses trigeminal innervation of the skin. However, treatment of cultured trigeminal neurons with BMP4 did not alter process outgrowth, and treatment with BMP7 and other BMPs also did not alter trigeminal outgrowth in culture (Le Roux et al., 1999). This suggests that the changes in the density of innervation do not solely reflect direct effects of BMP signaling. NGF signaling regulates the density and pattern of innervation of target tissues independent of effects on sensory neuron survival (Patel et al., 2000). Although there was no change in the level of neurotrophins in the skin at the ages examined, it is possible that there were changes in other elements of the neurotrophin signal transduction pathway (Kobayashi et al., 1998; Zhang et al., 1998) or changes in neurotrophins at other ages. In addition to an increase in the density of innervation in the skin of the noggin transgenic animals, there was a marked change in the pattern of innervation by different types of fibers. The most striking observations in the K14-noggin transgenic mice are the increase in the epidermal free nerve endings, the nonpeptidergic penicillate endings in the epidermis, along with a hypertrophic fourth tier of innervation that abuts the basement membrane of the epidermis. There is also some increase in the peptidergic innervation labeled with CGRP in the third and fourth tier as well as the unmyelinated CGRP FNEs in the epidermis. Interestingly, this excess innervation and number of sensory endings increases with age in the noggin transgenic animals. By contrast, in the K14-BMP4 transgenic mice, the fourth tier of innervation is significantly reduced even in one-month-old mice and there is deterioration of the fourth tier and epidermal free nerve endings with increasing age. However, there is a marked increase in CGRP-immunoreactive processes in the epidermis, consistent with known effects of BMP signaling on expression of CGRP (Ai et al., 1999; Hall et al., 2001). The complementary findings in the K14-noggin and K14-BMP4 animals suggest that endogenous BMPs in the skin regulate the maintenance of epidermal and upper dermal innervation apart from a possible effect on sensory neuron number.

Another component of epidermal innervation, the Merkel innervation and the Merkel cells at the mouth of the whisker follicle are also increased in K14-noggin mice. Recently, it has been shown that the Merkel innervation has two components, one dependant on trkA and the other on trkC. In mice mutant for both these receptors, the Merkel innervation is completely lost (Cronk et al., 2002). However, levels of NT3 are reduced in the skin of the K14-noggin animals, and levels of NT3 transcripts are unchanged. Further levels of NGF are also unchanged in the skin, and levels of NGF transcripts are unchanged at E14.5, the time during which the Merkel cells are specified. Hence, it is unlikely that the effects on Merkel innervation and the Merkel cells occur indirectly because of changes in neurotrophin expression.

In summary, these findings indicate that target-derived BMPs limit the final number of neurons in sensory ganglia as well as the extent and pattern of innervation of targets. Coupled with prior observations, these findings suggest that BMP signaling may regulate the acquisition of dependence of neurons on neurotrophins for survival, as well as their dependence on target-derived neurotrophins for determining the density of innervation of the target.

This work was supported by NIH grant NS20778 to J.A.K. and NS34692 to F.L.R. We thank Dr Aris Economides and Regeneron Pharmaceuticals for the noggin antibody.

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