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Noggin Is Required for Correct Guidance of Dorsal Root Ganglion Axons

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Members of the bone morphogenetic protein family of secreted protein signals have been implicated as axon guidance cues for specific neurons in *Caenorhabditis elegans* and in mammals. We have examined axonal pathfinding in mice lacking the secreted bone morphogenetic protein antagonist Noggin. We have found defects in projection of several groups of neurons, including the initial ascending projections from the dorsal root ganglia, motor axons innervating the distal forelimb, and cranial nerve VII. The case of the dorsal root ganglion defect is especially interesting: initial projections from the dorsal root ganglion enter the dorsal root entry zone, as normal, but then project directly into the gray matter of the spinal cord, rather than turning rostrally and caudally. Explant experiments suggest that the defect lies within the spinal cord and not the dorsal root ganglion itself. However, exogenous bone morphogenetic proteins are unable to attract or repel these axons, and the spinal cord shows only very subtle alterations in dorsal–ventral pattern in *Noggin* mutants. We suggest that the defect in projection into the spinal cord is likely the result of bone morphogenetic proteins disrupting the transduction of some unidentified repulsive signal from the spinal cord gray matter.

Key Words: **Noggin; BMP; axon guidance.**

INTRODUCTION

The mammalian nervous system exhibits a tremendously complex and highly conserved pattern of connectivity. During development, many neurons project axons simultaneously, to many different targets: if axons are to be guided by target- and pathway-derived factors, then many different factors must be present, and different kinds of neurons must be responsive to different factors. In keeping with this apparent complexity, a large number of factors have been implicated as axon-guidance cues in recent years, including Ephrins, Semaphorins, Slits, and Netrins (Mueller, 1999). Generally speaking, these signals act by inducing local changes in the actin cytoskeleton, resulting in local actin polymerization and cytoskeletal protrusions toward sources of attractants, and local actin depolymerization followed by protrusions falling back from sources of repellents (Mueller, 1999). Different neurons respond differently to different cues, even cues within the same family (e.g., de Castro *et al.*, 1999).

One group of proteins which has received attention recently as potential axon guidance cues is the bone morphogenetic proteins (BMPs). BMPs were first isolated biochemically as activities that could induce ectopic bone formation in rat soft tissues (Wang *et al.*, 1988). When the active proteins were purified and sequenced, and the genes encoding them cloned, they were found to be homologous to the *Drosophila* gene *decapentaplegic* (*dpp*), a member of the transforming growth factor β (TGFβ) superfamily of secreted signals (Gelbart, 1989; Wozney *et al.*, 1988). The *Drosophila* BMP family members *dpp*, *screw*, and *glass-bottomed boat/60A* play roles in a wide variety of developmental processes, including the establishment of initial dorsal–ventral polarity in the *Drosophila* ectoderm and development of various imaginal disc-derived structures (Gelbart, 1989). This family of proteins has undergone dramatic evolutionary expansion: vertebrate genomes contain 20 or more BMPs (Neuhaus *et al.*, 1999). Loss-of-function mutants in the vertebrate BMPs display a tremen-

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dous variety of defects, and BMP overexpression can cause a wide variety of developmental defects (Hogan, 1996).

In addition to their many activities in cell-type specification, BMPs have also been implicated as regulators of axonal growth. In *Caenorhabditis elegans,* the BMP family member unc-129 is involved in proper guidance of motor axons (Colavita *et al.*, 1998). In the rat, BMP7 is a chemorepellent for dorsal commissural axons (Augsburger *et al.*, 1999). Other TGFβ superfamily members are also able to signal to the cytoskeleton: most notably, TGFβ1 itself is a homing factor for many immune cell types (Adams *et al.*, 1991; Brandes *et al.*, 1991). The mechanism by which TGFβ superfamily members modulate the cytoskeleton remains to be elucidated.

It is possible that axon guidance may be regulated not only by BMPs, but also, indirectly, by their antagonists. Work on the BMP antagonist DAN brought this issue into focus because *Dan* mRNA is selectively localized to projecting axons, where we concluded it might play some role in axon guidance (Dionne *et al.*, 2001). Although we were unable to find any guidance defects in *Dan* mutants, we also examined *Noggin* mutants and *Dan/Noggin* compound mutants for defects in axon guidance. Here, we describe defects found in *Noggin* mutant mice. *Noggin* was originally isolated in an overexpression screen for proteins able to induce dorsal cell fates in the early *Xenopus* embryo (Smith and Harland, 1992). *Noggin* encodes a secreted protein which binds to BMP family members and prevents them from activating their receptors (Zimmerman *et al.*, 1996). Mice lacking *Noggin* have previously been extensively characterized with regard to defects in somite and neural fates and skeletal pattern (Brunet *et al.*, 1998; McMahon *et al.*, 1998). *Noggin* mice had not been examined for defects in neural connectivity. We reasoned that the loss of *Noggin* activity might result in axonal misguidance due to excessive BMP signals, either directly (with BMPs acting as guidance cues) or indirectly (with BMPs disrupting other guidance cues).

MATERIALS AND METHODS

Mice

The *Noggin* mutant mice used were those previously described (McMahon *et al.*, 1998). Genotyping was done by PCR, as previously described (McMahon *et al.*, 1998). The *Noggin* animals were at least eight generations backcrossed onto a C57Bl6/J background.

Immunofluorescence

Immunofluorescence was carried out as previously described (Dionne *et al.*, 2001). Primary antibodies used included purified rabbit polyclonal anti-MATH1 (Helms and Johnson, 1998), used at 1:200, a gift of J. Johnson; mouse monoclonal anti-MASH1 (Lo *et al.*, 1991), used as an unpurified supernatant at 1:1, a gift of D. Anderson; purified rabbit polyclonal anti-Ephrin-B (C-18) (Santa Cruz), used at 1:100; mouse monoclonal anti-Pax7 and anti-Pax6, used as unpurified supernatants at 1:1 (Developmental Studies Hybridoma Bank); polyclonal rabbit anti-β-galactosidase (5′→3′), used at 1:200; and mouse monoclonal anti-neurofilament 2H3 (Developmental Studies Hybridoma Bank), used as an unpurified supernatant at 1:50. Secondary antibodies used included Texas Red goat anti-rabbit IgG, FITC goat anti-mouse IgG, and Rhodamine Red X goat anti-mouse IgG; all were purchased from Jackson ImmunoResearch.

DiI Labeling

Mouse embryos were fixed for at least 12 h, and as long as several weeks, in 4% formaldehyde in PBS at 4°C. Small crystals of DiI (Molecular Probes) were implanted by using glass needles. The DiI was allowed to diffuse for 3 days at room temperature in the dark in 4% formaldehyde/PBS. The embryos were then embedded in 5% low-melt agarose in PBS and sectioned at 100 μm on a vibratome. Sections were collected on Superfrost Plus slides (Fisher), mounted with Vectashield (Vector Research), and immediately examined and photographed.

Cell Culture Methods

Dorsal root ganglia were dissected from e11.5 mouse embryos and cultured as previously described (Keynes *et al.*, 1997), with the following modifications: LIF was added as an unpurified COS supernatant, at a concentration which we had previously determined as sufficient to maintain ES cells in an undifferentiated state; cultures were set up in individual wells of 6- or 12-well tissue culture plates. NGF 7S and ITS-G were purchased from Gibco, BDNF and NT-3 were gifts of Regeneron Pharmaceuticals, and Rat Tail Collagen I was purchased from Collaborative Research/Becton Dickinson.

COS-7 or HEK293 cell pellets were produced as previously described (Shah *et al.*, 1997), with the following modifications: cells were transfected by using Lipofectamine Plus (Gibco) following the manufacturer's instructions; after transfection, cells were cultured in OptiMEM (Gibco) with 4% fetal bovine serum. The following plasmids were used for transfection and expression: *Xenopus* noggin, A3.pMT21 (Smith *et al.*, 1993); *Xenopus* BMP4, pCDM8-XBMP (Nishimatsu *et al.*, 1992), a gift of A. Suzuki; *Xenopus* OP1/BMP7, pcDNAIII-XOP-1H (Wang *et al.*, 1997), a gift of M. Moos; mouse GDF7, BMP4-GDF7/pMT23 (Lee *et al.*, 1998), a gift of T. Jessell. Mouse BMP2, BMP4, BMP7, and eGFP were expressed from plasmids based on pCS2 (Rupp *et al.*, 1994; Turner and Weintraub, 1994). In cases where heterodimer formation was not expected or required, cells were transfected separately for each plasmid and then mixed to make pellets which expressed both proteins. eGFP was used to monitor transfection efficiency and eGFP-transfected cells were used to make control cell pellets. We confirmed that BMP4 and GDF7 proteins were present in the supernatants of transfected HEK293 cells by Western blot analysis. Proteins were initially concentrated from 1 to 2 ml of supernatant using either Centricon YM-10 Centrifugal Filter Devices (Millipore Corporation) or by immunoprecipitation using Fc-tagged human noggin (Zimmerman *et al.*, 1996) crosslinked to Protein A Sepharose (Pharmacia Biotech; Harlow and Lane, 1988). Immunoblotting was carried out after separation of proteins by SDS–PAGE and transfer to nitrocellulose membrane (Schleicher & Schuell, NH). Blots were probed with Fc-tagged noggin to detect BMP/GDF and with a POD-labeled anti-human secondary antibody (Promega); bound antibodies were detected by BM Chemiluminescence (Boehringer Mannheim).

Once the cultures had grown for 24 h at 37° with 5% CO_2, they were fixed for 1–2 h in 4% paraformaldehyde in PBS at room temperature. In situations requiring consistent visualization of axons deep in the embedded tissues, Dent's fixative (80% methanol, 20% DMSO) was used instead of paraformaldehyde. After fixation, each collagen lump was carefully peeled away from the dish and placed in PBS for immunostaining.

In Situ Hybridization

Probes were prepared and *in situ* hybridization was carried out as previously described (Dionne *et al.*, 2001).

Immunostaining of Whole Embryos and Collagen Gel Cultures

Embryos were fixed for 1–2 h at room temperature in Dent's fixative (4:1 methanol:DMSO), rinsed once in 80% methanol, and bleached by incubation for 4–6 h in 80% methanol, 6% H_2O_2. Embryos were then either passed into 100% methanol (for long-term storage at −20°C) or rehydrated through a methanol series, culminating in a PBS rinse. Embryos were then blocked in PBS with 1% heat-inactivated goat serum, 2% BSA, and 0.1% Triton X-100 (PHBT) for 1 h at room temperature. The blocking solution was then replaced with PHBT + antibody and incubated for 4 h at room temperature. The anti-neurofilament antibody 2H3 (Developmental Studies Hybridoma Bank) was applied at 1:50 as an unpurified supernatant. Tissues were then washed at least five times in PHBT, for at least 1 h each at room temperature. Next, tissues were incubated for 4 h at room temperature in PHBT + 1% HRP-coupled goat anti-mouse IgG (Pharmingen) and then washed as before. Embryos were then transferred to PBS + 0.5 mg/ml DAB, incubated for 1 h at 4°C, and then 0.5 μl 30% H_2O_2 was added for each ml PBS/DAB. Staining was allowed to proceed until visible, and then DAB was washed out with PBS, and embryos were mounted in 80% glycerol or 2:1 benzyl benzoate:benzyl alcohol for photography. Collagen cultures were stained similarly, except that they were fixed in 4% paraformaldehyde and were not bleached.

RT-PCR

RT-PCR was performed largely as described previously (Wilson and Melton, 1994), with the exception that the quantity of DNAse I was doubled, and the reaction time was also doubled, since the mouse tissue samples contained much more genomic DNA than comparably sized *Xenopus* samples. Primers used were: Sema3A, gtg gaa cac gga ttc atg c, ttt gtc ggc gtt gct ttc g; Sema3B, agg cgc atg tgc agt gga c, cac caa ctg cag aaa gtc c; Sema3C, aac cca ctg aca caa tgc c, ggc tat ggt ctg ttt gaa gc; Sema3E, ttg gag gtg gtc gaa gag c, agt gct cag ctt tag agc g; Sema3F, aga tgt ccg tca cgg gaa c, tgc tct gta cgc agg aag c; Sema4A, ctc cta ttg ggt aga cag c, gtt gtt gtc ggc atc tac g; Sema4B, ctt tct cta ccg aca tcg g, gga gac acc tct aca aag c; Sema4C, aga gct aga aaa ggg tgc c, tcg taa ttc atc cgc gag c; Sema4D, aac tgc tac aag ggc tac c, cgt cag cat ccg aat ctg c; Sema4F, ctg gtt ggc ttt ttc ctg g, ata gac gtc tca tcg cac g; Sema4G, tga tgg aga agg aac tgg g, aca agc tgt gtg tgc ttc c; Sema5A, atc tgt ggc aag atc cag c, gcg ttg gaa tag gtc ttc c; Sema5B, tgt cat cct acc tgc ttc c, cag ctg ttg ggg aaa cag c; Sema6A, tgc agg aga aac gga aac c, aca tag ggt gaa ctc tcg c; Sema6B, ttc ttg tga agc cca acg c, cat ctt gct caa acg tgg c; Sema6C, tcc cga tcc att ccc atc c, cag gaa ggt agt gta gag c; Sema7A, cca ttg cag aag gtt tcc c, gac caa gta tga gtg tgg g; EphB2, aac tgt gta tgc cgc aac, ggt cac tga tgt aga tgc; EphB3, tcc tgg gag tta caa agc, act cta cgt tgt cat cgc; EphB4, agt tca cct tgc act acc, aag cag caa cct caa agg; Ornithine Decarboxylase, tca ctc cct ttt acg cag, tgc tgg ttt tga gtg tgg.

RESULTS

Gross Defects in Axon Guidance in Noggin-Mutant Mice

Because BMP-7 acts as a guidance cue for spinal cord commissural axons (Augsburger *et al.*, 1999), we examined axonal projections in mice lacking the secreted BMP antagonist Noggin, reasoning that these animals might display defects in the guidance of other BMP-responsive axonal populations (McMahon *et al.*, 1998). Defects were apparent even at the superficial level in a variety of populations (Fig. 1). All defects discussed were present at 100% penetrance in at least five animals, except as mentioned.

One defect which was immediately apparent was in the projection of motor axons into the most distal portions of the ventral forelimb (Figs. 1A and 1B). In wild-type forelimbs at e13.5, these axons project to the bases of the interdigital spaces and bifurcate, sending fascicles along the edges of each of the digits (Fig. 1A). In contrast, in *Noggin* mutants, these fascicles fail to bifurcate and project directly into the interdigital space (Fig. 1B).

A second defect in the *Noggin* mutant is apparent at e11.5 in the projection of cranial nerve VII into the muscles of the face (Figs. 1C and 1D). In wild-type animals, cranial nerve VII sends a projection deep and rostral to innervate the muscles of the face (arrowhead in Fig. 1C). This projection is present in *Noggin* mutants but projects too far toward the midline (arrowhead in Fig. 1D) and bifurcates to send a projection across the midline (arrow in Fig. 1D).

Finally, *Noggin* mutants displayed aberrant mixing of projections from the dorsal root ganglia (Figs. 1E and 1F). This defect was severe enough that it was difficult to physically separate the dorsal root ganglia for explants. This defect was seen in all *Noggin* animals examined. The severity of this defect and the general increase in axonal staining in the spinal cord inspired us to look more closely at pathfinding by DRG axons in animals carrying only the *Noggin* mutation.

Defects in DRG Axon Pathfinding

When *Noggin* mutant embryos were examined in transverse section at the forelimb level, it became clear that DRG axon pathfinding was severely perturbed in these animals (Fig. 2). We restricted our analysis to the forelimb level because the posterior spinal cord is morphologically disrupted and severely mispatterned (McMahon *et al.*, 1998; and data not shown). At e11.5 in wild-type animals, the dorsal root ganglia have projected into the dorsal root entry zone (DREZ) of the spinal cord (arrowhead in Figs. 2A, 2B, 2D, and 2E). Once in the DREZ, the axons bifurcate and send projections rostrally and caudally along the length of the spinal cord (Ozaki and Snider, 1997). In *Noggin* mu-

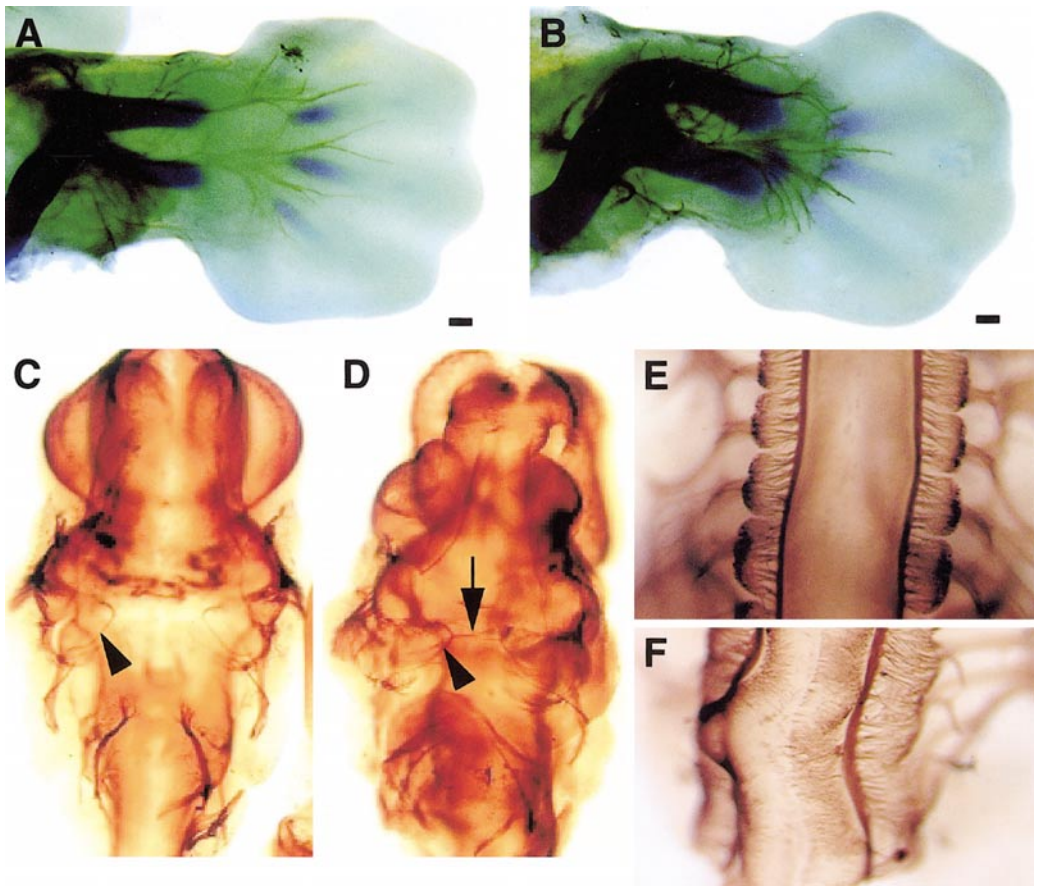
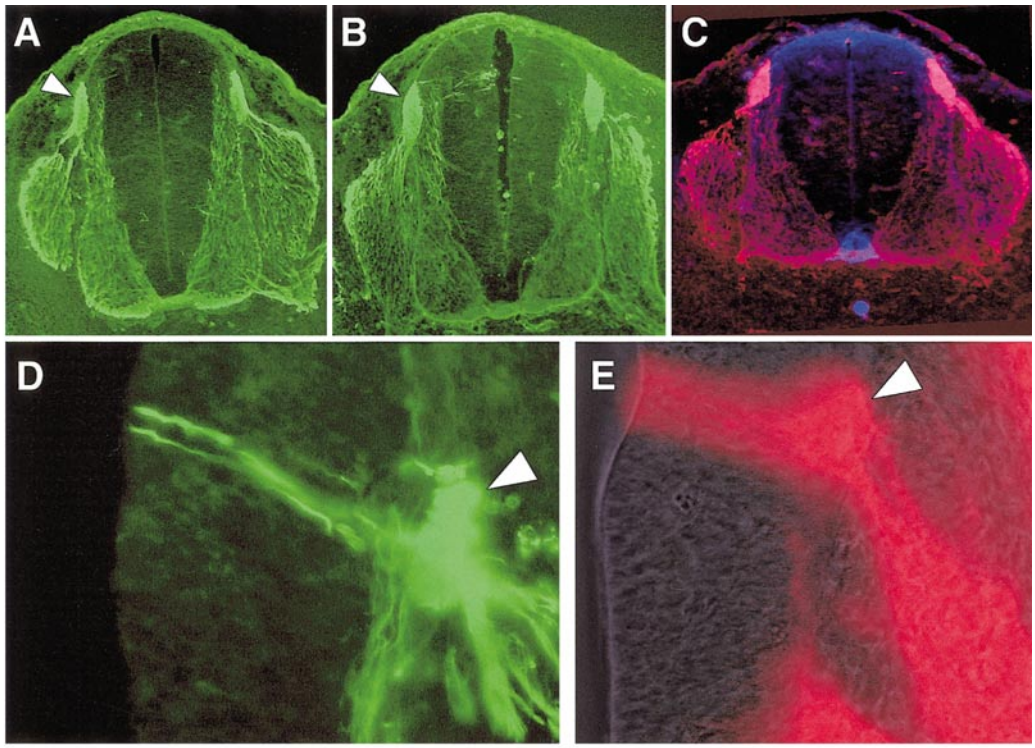


FIG. 1. Defects in axon guidance in *Noggin* mutants. (A, B) Defects in projections of motor axons to the distal part of the forelimbs. Mouse forelimbs stained with the anti-neurofilament antibody 2H3 and also with Alcian blue (to detect condensing cartilage). (C, D) Defects in projection of cranial nerve VII, visualized by whole-mount staining with 2H3. Viewed from the anterior–ventral side; anterior is to the top. In a wild-type animal (C), the anteriormost projection from CN VII projects anteriorly and medially before turning laterally (arrowhead). In *Noggin* mutants (D), some axons follow the correct path (arrowhead), while others continue medially across the midline (arrow). (E, F) Gross defects in dorsal root ganglia, visualized by staining with 2H3. Dorsal views, at the level of the forelimbs; anterior is up. Note the discrete ganglia present in the wild-type (E), as compared with the mixing of projections between the dorsal root ganglia in the *Noggin* mutant (F).

FIG. 2. Closer analysis of defects in projections from the dorsal root ganglion; *Noggin* expression in dorsal spinal cord. (A, B, D) Sections through the spinal cord at forelimb level, stained with 2H3. In wild-type animals (A), few or no axons emerge from the DREZ (indicated by the arrowhead); in contrast, in *Noggin* mutant embryos (B, D), there are many projections from the DREZ into the deep zone of the spinal cord. In (D), medial is to the left, lateral to the right. (C) β-Galactosidase (blue) and 2H3 (red) expression in the spinal cord and DRG at e11.5. Sections were taken and processed as above. (E) DiI tracing of projections to the forelimb in a *Noggin* mutant, demonstrating that the ectopic projections in the spinal cord are derived from the DREZ and hence presumably from the DRG. The DREZ is again indicated by the arrowhead. Medial is to the left, lateral to the right.

FIG. 3. Noggin is required in the spinal cord, not the dorsal root ganglion, for proper guidance of DRG axons. Explant cultures in which mutant or wild-type dorsal root ganglia have been apposed to mutant or wild-type spinal cords were cultured for 24 h, and then sectioned and stained with 2H3 (green) and with Hoechst dye (blue; to indicate the location of living tissues). In each picture, the ectopic dorsal root ganglion is separated from the spinal cord by a white dotted line. The ectopic dorsal root ganglion was identified before sectioning in each case by examination of the explant; the explanted pieces of spinal cord adopted a stereotypical shape, which made identification easy. Mutant spinal cord permits ingrowth by wild-type dorsal root ganglia (A; the ectopic wild-type DRG is indicated by the white arrowhead, and the black arrowhead indicates the projection from the wild-type DRG into the mutant spinal cord). In contrast, wild-type DRG will not grow into wild-type spinal cord (B; again, the ectopic DRG is indicated by the arrowhead); axons from mutant DRGs also cannot enter wild-type spinal cord (C; the mutant DRG is indicated by the arrowhead).



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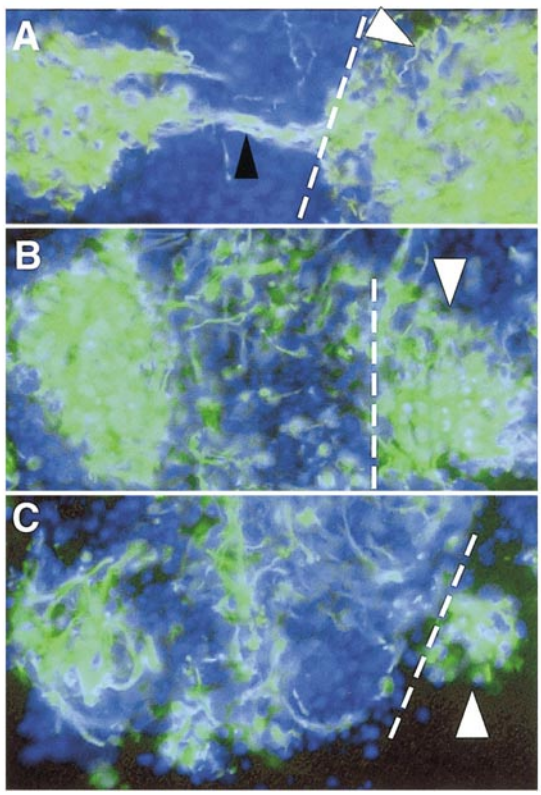

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tants, many of these axons project properly, as can be seen by the many axons present in the DREZ (Figs. 1F and 2B); however, a subset of axons project from the DREZ into the deep spinal cord, passing through the gray matter of the cord and often reaching the lumen (Figs. 2B and 2D). This defect was seen in all *Noggin* mutant animals examined, and was never present in heterozygotes or wild-type animals (the wild-type example in Fig. 2A, showing a single misprojection, is typical; no wild-type section displayed more than two such misprojections at the level of the DREZ). The cases shown are typical in only showing this misprojection on one side of the spinal cord: most sections showed misprojection only on one side or the other, but examination of multiple sections from the same animal revealed that these misprojections were present on both sides without any clear bias to the right or left, as is evident from the staining of axons penetrating the spinal cord seen in Fig. 1F.

Because of these observations, we examined expression of *Noggin* in the dorsal spinal cord by staining animals heterozygous for the knockout allele for β-galactosidase expression. β-Galactosidase antibody staining confirmed previously observed expression in the roofplate, floorplate, and notochord and revealed strong expression along the entire dorsal margin of the spinal cord and low-level expression in the gray matter of the dorsal spinal cord and the dorsal root ganglion at e11.5 (Fig. 2C). These observations were confirmed by staining for β-galactosidase activity (data not shown).

Because the 2H3 antibody stains all axons, it was not possible to tell with certainty that the axons present in the spinal cord gray matter originated in the DRG. Accordingly, we labeled projections which had reached the limb at e11.5 with DiI. Although most of the labeled axons were from motor neurons (data not shown), the DRG was labeled as well and was clearly the origin of the ectopic spinal projections (Fig. 2E).

Noggin Is Required in the Spinal Cord, Not the DRG, for Correct Pathfinding

Since Noggin is expressed in both DRG and spinal cord, we wished to determine whether Noggin protein was required in the projecting axons, or in the target tissue, for axon pathfinding. We cultured mutant and wild-type e11.5 dorsal root ganglia adjacent to the ventricular surface of mutant and wild-type e10.5 spinal cord, sectioned the explants, stained with the anti-neurofilament antibody 2H3, and scored them for growth into the spinal cord. Wild-type DRGs grew into mutant spinal cords (4/5) (Fig. 3A); in contrast, wild-type DRGs did not grow into wild-type spinal cords (1/7; Fig. 3B) and mutant DRGs did not grow into wild-type spinal cords (0/3; Fig. 3C). These data localize the requirement for Noggin to the spinal cord.

Dorsal–Ventral Pattern in the Spinal Cord Is Generally Normal at Forelimb Level

Several groups have reported that dorsal and ventral spinal cord have differing activities on guidance of DRG axons *in vitro*. Ventral spinal cord is strongly repellent in these assays, while dorsal spinal cord is neutral or weakly repellent (Keynes *et al.*, 1997; Nakamoto and Shiga, 1998). We reasoned that our results might reflect dorsalization of the spinal cord and consequent reduction of repulsion. In order to test this hypothesis, we examined the expression of the markers MATH1, MASH1, PAX6, and PAX7 in forelimb-level mutant and wild-type spinal cord. MATH1, which marks the most dorsal pool of neural progenitors in the spinal cord at this stage (Helms and Johnson, 1998), was mildly expanded (Figs. 4A and 4B, red staining; and data not shown). MASH1, PAX6, and PAX7, which mark more ventral pools of neural precursors, were unaffected (MASH1, green in Figs. 4A and 4B; PAX7, Figs. 4C and 4D; PAX6, data not shown). We conclude that, although the dorsalmost fates are mildly expanded, the spinal cord does not show the global dorsalization which might be expected to generate the projection defects present in the *Noggin* mutant.

In order to further analyze spinal-cord pattern, we assayed expression of all 17 known mouse Semaphorins (Sema3A–3C, 3E, 3F, 4A–4D, 4F, 4G, 5A, 5B, 6A–6C, and 7A) in e10.5 and e11.5 forelimb-level spinal cord and DRG by RT-PCR. Of these, 11 (3A, 3C, 3F, 4A–4D, 4F, 5A, 5B, and 6B) were expressed at significant levels; none of these were reduced in *Noggin* mutant animals (data not shown). We also examined expression of Ephrin-B1, -B2, and -B3 by *in situ* hybridization and immunofluorescence. Ephrin-B1 and Ephrin-B2 were both expressed in the dorsal spinal cord gray matter; their expression was unchanged in *Noggin* mutants, as was expression of all three Ephrin-B's as revealed by immunofluorescence (Figs. 4E–4J). RT-PCR analysis revealed that EphB2, EphB3, and EphB4 are all expressed in dorsal root ganglia, and are not disrupted in *Noggin* mutants (Fig. 4K).

BMPs Fail to Attract or Repel DRG Axons in Vitro

Since we had begun these experiments in part to find axons which might be guided by BMP signals, we wished to see whether or not DRG axons would respond to BMP gradients *in vitro*. Accordingly, we dissected dorsal root ganglia from wild-type e11.5 embryos and cultured them as previously described (Keynes *et al.*, 1997), in combination with HEK293 cell pellets expressing BMP2, BMP4, BMP7, or GDF7. We chose these BMPs because BMP2 and BMP4, and the GDF7-related protein GDF6, interact with Noggin with high affinity (Chang and Hemmati-Brivanlou, 1999; Zimmerman *et al.*, 1996), while BMP7 has been shown to be able to act in guidance of commissural neurons (Augsburger *et al.*, 1999). In our hands, BMP2, BMP4, BMP7, and GDF7-transfected cell pellets had no effect on outgrowth from cultured DRGs (Figs. 5A–5D). We confirmed that our

cells produced BMPs by immunoprecipitation followed by Western blot (data not shown).

In many assays, BMP heterodimers have been shown to have different signaling properties from homodimers (Aono *et al.*, 1995; Israel *et al.*, 1996). Noggin is able to block signaling by heterodimers as well as by homodimers (Eimon and Harland, 1999). In order to test the chemotactic properties of BMP heterodimers, we cotransfected cells with BMP4 and BMP7, BMP7 and GDF7, and BMP4 and GDF7. None of these combinations were any more effective than BMP4, BMP7, or GDF7 alone (Figs. 5E and 5F; and data not shown).

It was also possible that BMPs might not be chemotactic agents themselves, but might alter the responsiveness of DRG axons to other chemotactic agents. Accordingly, we examined the responsiveness of DRG axons to Sema3A and Sema3F, with and without BMPs. Both of these signals repelled early DRG axons (Figs. 4G and 4I; and data not shown). Coexpression of BMP4, BMP7, or GDF7 did not alter the response to either of these repellents (Figs. 5H and 5J; and data not shown). The response to Slit1 was also unchanged (data not shown).

DISCUSSION

Noggin Is Required for Proper Axonal Pathfinding

We have presented data which show that *Noggin* is required for correct peripheral axonal pathfinding in the mouse embryo (Figs. 1 and 2). We have chosen to focus on the altered pathfinding of centrally projecting DRG axons (Fig. 2). *Noggin* activity is required in the spinal cord and not in the DRG axons themselves (Fig. 3). The nature of the requirement for *Noggin* remains obscure, although we have demonstrated that the defects are not simply a result of altered patterning of the spinal cord (Fig. 4), that BMPs are not themselves guidance cues for DRG axons (Fig. 5), and that BMPs are not able to alter the response to other known cues for DRG axons (Fig. 5). Together, we take these data to indicate that excess BMP signaling in *Noggin* mutant mice disrupts reception of some unknown repulsive cue for DRG axons.

How Are Axons Excluded from the Ventricular Zone?

One important aspect of axon guidance is the prevention of axonal growth into inappropriate areas. Is misprojection of axons into such regions simply the result of the proper action of neuron-specific attractants and repellents, or are there more general axonal repellents expressed in regions where axons should not grow? Our work suggests that the repellents in the ventricular zone of the spinal cord are somewhat specific for different neural types: while the DRG axons misproject into the ventricular zone, the trajectory of commissural axons (which must skirt the ventricular axonal–exclusion zone) is apparently unchanged. This issue has been examined previously in the dorsal root ganglion: previous authors have concluded that the prevention of axonal entry into the ventricular zone is dependent on activities of NCAMs, especially Axonin/SC1, Ng-CAM and Nr-CAM (Shiga *et al.*, 1997). Although our work is generally compatible with these findings, NCAM is not expressed in the deep spinal cord at e11.5 (Lustig *et al.*, 2001), suggesting that there must be other repellents in the gray matter at these early stages. The expression of Ephrin-B1 and Ephrin-B2 in the gray matter of the dorsal spinal cord, and the expression of EphB2, EphB3, and EphB4 in the dorsal root ganglion, suggests that these factors may play roles in preventing ectopic growth of DRG axons into the gray matter. We have been unable to demonstrate repulsion of DRG axons by preclustered Ephrin-B2 in collagen cultures. However, this experimental paradigm may not accurately reflect the *in vivo* case. If B-class Ephrins *are* repellents for DRG axons *in vivo*, the clearest explanation of our data are that BMPs are capable of disrupting EphB signal transduction.

In this context, it may be notable that those axons which misproject into the ventricular zone in *Noggin* mutants almost invariably adopt the apparent shortest path from the DREZ to the lumen. This suggests that there may be some attractant in the ventricular zone, whose effect is normally masked by the ventricular-zone repulsion; this attraction would make ventricular-zone repulsion all the more important.

Sema3A and Sema3F Are Repellents for Early-Emerging DRG Axons

In our assays, Sema3F and Sema3A were both repulsive for early-emerging, NGF-responsive DRG axons. This is in keeping with previous reports for Sema3A (Messersmith *et al.*, 1995). The behavior of these axons in response to Sema3F has not been previously reported; it is intriguing that Sema3A and Sema3F should act similarly, since previous work has shown that these signals have different effects on olfactory bulb neurons and that their effects are likely to be mediated to some extent by different receptors (de Castro *et al.*, 1999; Nakamura *et al.*, 2000).

Assays for BMPs as Chemotactic Agents

In our hands, BMPs failed to affect the outgrowth of axons from dissected dorsal root ganglia *in vitro*. The most obvious explanation for this result is that these neurons are simply unresponsive to BMP signals, *in vivo* as well as *in vitro*. However, an alternative explanation is suggested by results on homing of immune cells to TGFβ1 (Adams *et al.*, 1991; Brandes *et al.*, 1991). TGFβ1 can act as a chemoattractant for monocytes, lymphocytes, and neutrophils, but this attraction is abrogated at high concentrations of TGFβ1. For example, monocytes are maximally attracted at a TGFβ1 concentration of 0.4 fM, an attraction which is lost at levels of 400 fM; neutrophils are maximally attracted

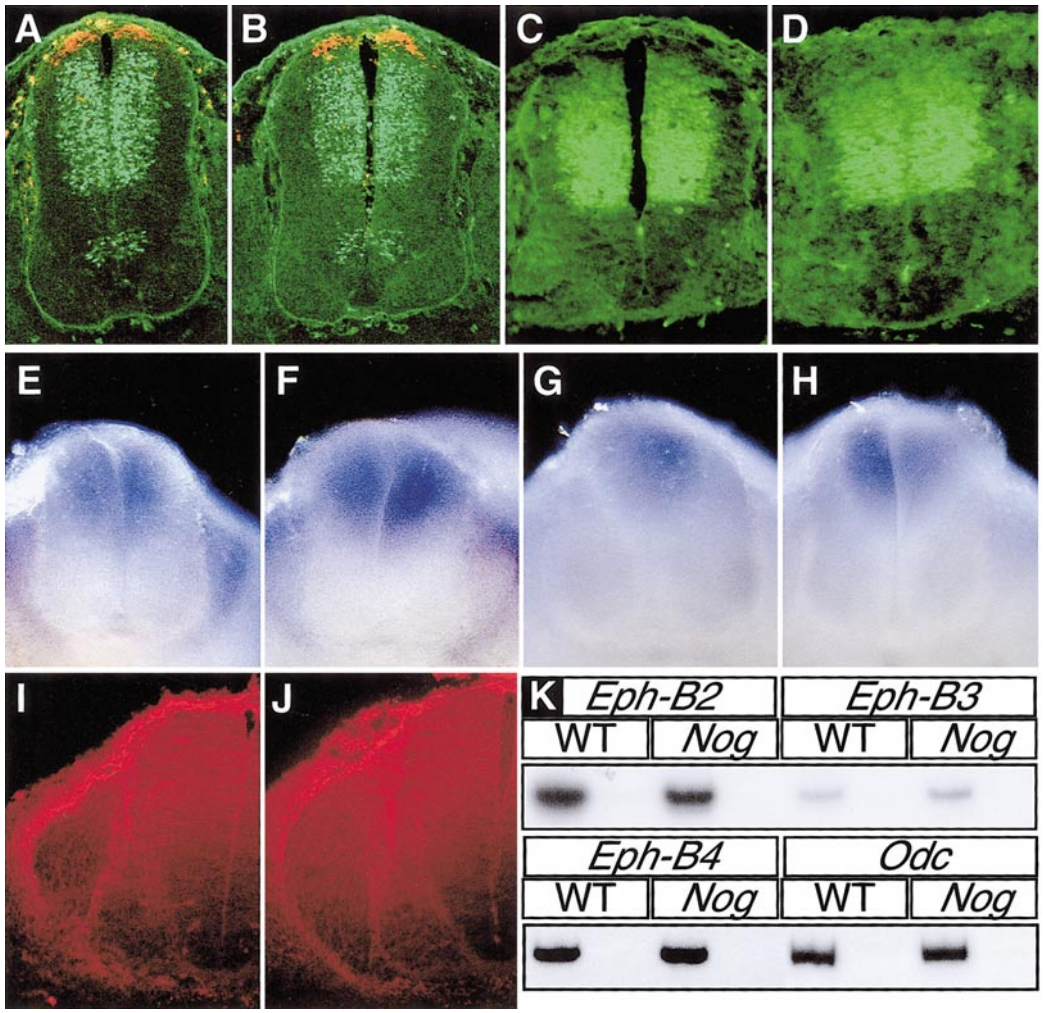

FIG. 4. Pattern of the spinal cord is normal in *Noggin* mutants. (A, B) Expression of MATH-1 (red) and MASH-1 (green) in forelimb-level spinal cord of wild-type (A) and *Noggin*-mutant (B) e11.5 mouse embryos. MATH-1 marks the dorsalmost pool of neuronal precursors, and is mildly expanded; MASH-1 marks a more ventral set of cells, and is apparently unaffected. (C, D) Expression of PAX7 (green) in forelimb-level spinal cord of wild-type (C) and *Noggin*-mutant (D) e11.5 mouse embryos. PAX7 shows little or no change in expression. (E–H) *Ephrin-B1* (E, F) and *Ephrin-B2* expression (G, H) in forelimb-level spinal cord of wild-type (E, G) and *Noggin*-mutant (F, H) e11.5 mouse embryos. (I, J) Immunofluorescent localization of total Ephrin-B protein in forelimb-level spinal cord and dorsal root ganglion of wild-type (I) and *Noggin*-mutant (J) mouse embryos. Again, there is no apparent change in localization. Note the Ephrin-B expression completely surrounding the DREZ and DRG. (K) Expression of *Eph-B2*, *Eph-B3*, and *Eph-B4* in dorsal root ganglia dissected from *Noggin*-mutant and wild-type mouse embryos. *Odc* is Ornithine Decarboxylase, a loading control. In each case, the first lane is the RT-PCR, and the second is a reaction carried out without reverse transcriptase, as a control against contamination with genomic DNA.

at 20 fM, and again, the effect is lost at 400 fM. This effect is probably due to maximal receptor occupation on all sides of the cell at high concentrations of ligand. A similar effect could pertain here. It is notable in this context that previous studies showing an *in vitro* effect from BMPs have involved BMP diffusion through tissue, rather than through collagen (Augsburger *et al.*, 1999); this may result in a lower local concentration of BMP protein, or a steeper gradient.

BMPs as Signal Disruptors

Despite the argument above on the possibility that BMP gradients may be difficult to assay in collagen cultures, we interpret our results as suggesting that overactive BMP signals may disrupt responses to some other axon-guidance cue. It is clear from our data that the pattern of the spinal cord is essentially normal, and yet in our explant assays, the

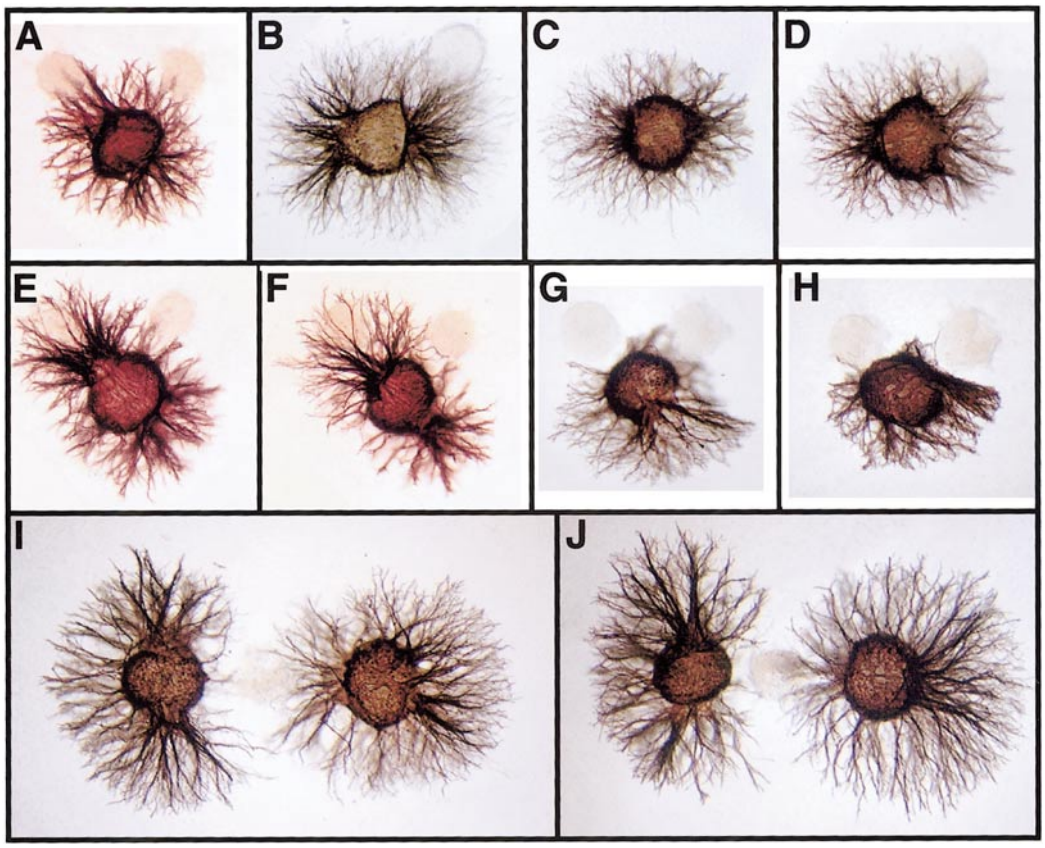

FIG. 5. BMPs cannot guide DRG axons or change responses to Semaphorin 3A or 3F *in vitro*. (A–F) DRG axons do not obviously respond to BMP homodimers or heterodimers. GFP-transfected 293 cells (A) were not significantly attractive or repulsive for DRG axons in collagen cultures; the same was true of cells expressing BMP4 (B), BMP7 (C), GDF7 (D), BMP4 and BMP7 (E), or GDF7 and BMP7 (F). (G–J) Sema3A (G) and Sema3F (I) both repel DRG axons; BMP4 does not alter the response to Sema3A (H), and BMP7 does not alter the response to Sema3F (J). (I) and (J) show two DRGs with each pellet to show an effect which was persistent with the semaphorins: the semaphorins were persistently more efficient at preventing growth in the anterior–posterior direction relative to the DRG axis than in the dorsal–ventral direction (in each case, the left-hand DRG is oriented with the AP axis pointing toward the pellet, and the right-hand explant is orthogonal to this, within the plane of the paper.) Each of these experiments was performed at least nine times, with consistent results.

defect localized to the spinal cord; this suggests that the signal being affected must be more or differently sensitive to BMP signals than is overall spinal cord pattern. This leaves two possibilities: either the expression of some guidance molecule is significantly disrupted in the absence of other detectable fate defects, or BMP signals can directly disrupt reception of some other axonal chemorepellent. The first possibility cannot be absolutely excluded on the basis of our data, but seems unlikely based on the large number of spinal cord markers examined. The second possibility, that BMPs must be blocked to present direct disruption of other signals, emerges as a more plausible explanation.

There are many examples of this kind of signal competition in the literature. The best-studied cases involve disruption of TGFβ class signals by EGF or HGF, in which cases, MAP kinase activation or Ras activation prevent BMP/TGFβ responsiveness (Kretzschmar *et al.*, 1997, 1999). Conversely, TGFβ is able to block proliferation in response to EGF in many cell lines (Like and Massague, 1986). To our knowledge, this kind of effect has not previously been demonstrated to be important in development.

This point raises an interesting subtlety in the biological action of BMP antagonists. It has often been asserted that the role of BMP antagonism in various biological processes is simply to prevent BMP signals from being received and hence to permit tissues to follow "default" pathways of differentiation (Hemmati-Brivanlou and Melton, 1997). However, our data suggest that the role of BMP antagonism may be not only to simply block BMP signals but also to *permit* the activity of other signals which would otherwise be directly suppressed by BMP signal activation.

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