Signaling through BMP type 1 receptors is required for development of interneuron cell types in the dorsal spinal cord

Lara Wine-Lee[1,2], Kyung J. Ahn[1], Rory D. Richardson[1], Yuji Mishina[4], Karen M. Lyons[5] and E. Bryan Crenshaw III[1,2,3,*]

[1]Mammalian Neurogenetics Group, Center for Childhood Communication, 712 Abramsom Research Center, The Children's Hospital of Philadelphia, 34th and Civic Center Boulevard, Philadelphia, PA 19104, USA
[2]Neuroscience Graduate Group, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA
[3]Department of Otorhinolaryngology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA
[4]Laboratory of Reproductive and Developmental Toxicology, National Institutes of Environmental Health Sciences, Research Triangle Park, NC 27709, USA
[5]Department of Orthopaedic Surgery, Department of Molecular, Cellular and Developmental Biology and Biological Chemistry, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA
*Author for correspondence (e-mail: crenshaw@email.chop.edu)

Accepted 29 July 2004

Development 131, 5393-5403
Published by The Company of Biologists 2004
doi:10.1242/dev.01379

Summary

During spinal cord development, distinct classes of interneurons arise at stereotypical locations along the dorsoventral axis. In this paper, we demonstrate that signaling through bone morphogenetic protein (BMP) type 1 receptors is required for the formation of two populations of commissural neurons, DI1 and DI2, that arise within the dorsal neural tube. We have generated a double knockout of both BMP type 1 receptors, *Bmpr1a* and *Bmpr1b*, in the neural tube. These double knockout mice demonstrate a complete loss of D1 progenitor cells, as evidenced by loss of *Math1* expression, and the subsequent failure to form differentiated DI1 interneurons. Furthermore, the DI2 interneuron population is profoundly reduced. The loss of these populations of cells results in a dorsal shift of the dorsal cell populations, DI3 and DI4. Other dorsal interneuron populations, DI5 and DI6, and ventral neurons appear unaffected by the loss of BMP signaling. The Bmpr double knockout animals demonstrate a reduction in the expression of Wnt and Id family members, suggesting that BMP signaling regulates expression of these factors in spinal cord development. These results provide genetic evidence that BMP signaling is crucial for the development of dorsal neuronal cell types.

Key words: Bone morphogenetic protein receptor type 1, Cre-mediated conditional *Bmpr1a* knockout, *Bmpr1b* mutant, Dorsal interneuron development, Neural tube patterning, Mouse

Introduction

Cell types arising from progenitor domains in the dorsal half of the spinal cord are dedicated to processing sensory signals between the periphery and brain. The ventral progenitor populations give rise to motoneurons and interneurons essential for control of posture and locomotion. Complex hierarchies of transcription factor interactions regulate this highly stereotyped process of neurogenesis (for reviews, see Helms and Johnson, 2003; Jessell, 2000; Lee and Jessell, 1999). These signals set up networks of transcription factors that are regional and cell-specific in their expression patterns, and ultimately lead to the determination of cell fates.

Roof plate-derived signals establish regional identities in the dorsal neural progenitors of the ventricular zone by inducing expression of proneural basic helix loop helix (bHLH) factors Math1 (Atoh1 – Mouse Genome Informatics), Ngn1/2 and Mash1 (Ascl1) (Timmer et al., 2002; Gowan et al., 2001). Although the mechanisms responsible for establishing the restrictive pattern of bHLH factor expression in the ventricular zone are largely unknown, gene knockout studies have shown that specific bHLH factors are crucial for the formation of subsets of neurons (Gowan et al., 2001). Following exit from the cell cycle, neural progenitors migrate out of the ventricular zone and begin to differentiate. At this time, combinatorial expression of homeodomain transcription factors specifies the emerging populations of interneurons (Thor et al., 1999). To date, six populations of neurons have been characterized that are born within the dorsal murine neural tube between 10 and 12.5 dpc. The six populations can be divided into two classes: class A and class B neurons. Class A neurons, DI1-DI3, give rise to commissural and other interneurons of the deep dorsal horn (Muller et al., 2002), and their generation is largely roof plate dependent (Lee et al., 2000; Millonig et al., 2000). Generation of class B neurons, DI4-DI6, appear to be roof plate-independent and require Lbx1 (Gross et al., 2002; Muller et al., 2002). Although the endogenous mechanisms required for the formation of these distinct classes are still unclear, BMP signaling regulates markers of the class A neurons in a gradient-dependent fashion (Timmer et al., 2002; Panchision et al., 2001). Thus, BMPs appear to mediate both the progenitor populations and the ultimate specification of dorsal cell types.

Multiple BMP family members are expressed in the roof plate and epidermal ectoderm, and in vitro work has suggested that the dorsalizing function of the roof plate is carried by the BMP signal (Liem et al., 1997). The BMPs are members of the TGFβ superfamily of cell signaling molecules that play many important roles throughout embryogenesis (for a review, see Hogan, 1996) and in nervous system development (for reviews, see Mehler et al., 1997; Ebendal et al., 1998). BMP ligands bind to transmembrane serine-threonine kinase receptors. The receptors are composed of type 1 and type 2 subunits, of which there are multiple subtypes for each component (for reviews, see Derynck and Zhang, 2003; Massague, 1996; Massague, 1998). Type 1 and type 2 receptors alone exhibit low-affinity binding, while in combination, high-affinity binding can be achieved. Cooperative binding of ligands to the oligomeric receptor complex leads to phosphorylation of the type 1 component by the type 2 kinase domain. Ligand binding initiates the downstream effects of BMP signaling, namely phosphorylation of SMAD proteins by the type 1 receptor subunit. Translocation of phosphorylated SMAD to the nucleus directs the downstream effects of BMP signaling. Multiple subtypes of BMP receptors are found in the developing neural tube. At the time of critical events for cell type specification, the predominant type 1 receptors are BMPR1A and BMPR1B (for a review, see Ebendal et al., 1998). Because of the multiple BMP family members expressed by the neural tube during development, components of the BMP signaling cascade are excellent targets for manipulation in assessing the roles of BMPs during nervous system development.

BMPs and other TGFβ family members mimic effects of roof plate tissue, while BMP antagonists are inhibitory for dorsal neural marker expression (Liem et al., 1997). More recently, in vivo manipulation of BMP signaling has suggested that these pathways are crucial for dorsal interneuron populations (Nguyen et al., 2000; Panchision et al., 2001; Timmer et al., 2002). Loss-of-function analyses in the mouse have for technical reasons provided little insight into endogenous BMP activity in specification of dorsal cell fate (for a review, see Chang et al., 2002). Functional redundancy of BMP proteins and early lethality of BMP signaling mutations have prevented traditional knockout studies from establishing the role of BMPs in neural tube patterning. A role for a BMP-related protein in dorsal neural tube development has been shown, as loss of *Gdf7* expression leads to a specific loss of DI1 interneurons (Lee et al., 1998). However, the DI1 cells are initially specified in *Gdf7*-null mice, but are subsequently lost suggesting that BMP activity may also play an important role in maintaining cells of the dorsal neural tube. Thus, clear genetic evidence for a role of BMP signaling in dorsal cell fate determination and maintenance is still lacking.

Here, we describe our analysis of the role of BMP signaling in development of dorsal cell phenotypes in the neural tube using conditional and classic knockout approaches to disrupt BMP signaling. We have generated a double knockout of BMP type 1 receptors in the neural tube by using a conditional knockout of *Bmpr1a* (Ahn et al., 2001; Mishina et al., 2002) and a classic knockout of *Bmpr1b* (Yi et al., 2000). Either mutation alone does not abrogate the BMP signal nor yield a dorsal neural tube patterning defect. By contrast, the double knockout eliminates BMP signaling in the neural tube during the period of cell type specification. We demonstrate that loss of BMP signaling in the neural tube leads to disruption of the dorsal cell populations. Specifically, *Math1*-expressing progenitors are lost with a subsequent loss of DI1 interneurons. In addition, a profound reduction in DI2 neurons is observed in double mutant animals. Abrogation of the BMP signal also effects other signaling pathways within the neural tube as seen by changes in the expression of Wnts and Ids, indicating a complex network of interactions is probably responsible for proper dorsal cell specification. In this paper, we provide direct genetic evidence that BMP signaling is necessary for the development of dorsal populations in the developing spinal cord in the mouse.

Materials and methods

Transgenic mouse generation and analysis

The *Bmpr1a* conditional knockout pedigree and the *Bcre32* pedigree have been previously described (Ahn et al., 2001). The alleles of the *Bmpr1a* gene are described in Mishina et al. (Mishina et al., 1995) for the null allele and Mishina et al. (Mishina et al., 2002) for the floxed allele (*Bmpr1a*^{flox}). The *Bmpr1b* mice were a generous gift from K. Lyons (UCLA, CA, USA). The mating scheme used to generate mutant animals and normal littermates is described in Fig. 1. Normal controls discussed throughout refer to the 'normal' phenotype as outlined in Fig. 1B. At least four animals of each genotype were examined. The ROSA reporter pedigree was a generous gift from P. Soriano (Soriano, 1999). Whole-mount X-gal staining was carried out according to published methods (Phippard et al., 1999). Midday of the plug date was designated 0.5 dpc.

The above alleles were detected using PCR (Ahn et al., 2001) and Southern blot analysis for the *Bmpr1b* allele (Yi et al., 2000). Primers for genotyping *Bmpr1b* alleles were as following: wild-type 3′ GTAAATGCCACCACCACTGT, wild-type 5′ TGCAAAATACTAA-CAATCTC, null 3′ CGTGCTACTTCCATTTGTC and null 5′ TC-CCTGGTTGTTTTCTCTG.

In situ hybridization

In situ hybridization analyses were carried out using 20-25 μm cryosections of embryos fixed overnight in 4% paraformaldehyde (PFA) as described previously (Ahn et al., 2001). The following mouse probes were used: *En1* (A. McMahon), *Foxd3* (P. Labosky), *Lhx2* (J. Botas), *Lhx9* (H. Westphal), *Lmx1b* (R. Johnson), *Math1* (J. Johnson), *Mash1* (D. Anderson), *Ngn2* (D. Anderson), *Wnt1* (A. McMahon) and *Wnt3a* (A. McMahon). Images were taken on a Leica DM-IRBE inverted microscope using a Leica DC500 digital imaging system.

Immunohistochemistry, immunofluoresence and microscopy

Phosphorylated SMAD1 (phospho-SMAD1) immunohistochemistry was performed by modification of previously published methods (Ahn et al., 2001). Briefly, 20 μm cryosections were processed for tyramide amplification (TSA Indirect Tyramide Signal Amplification Kit, Perkin Elmer Life Science) and immunoperoxidase labeling (Vectastain ABC Kit, Vector Labs). Antigen unmasking was performed by heating slides in 10 mM sodium citrate pH 9.0 in an 80°C water bath for 30 minutes. The slides were incubated overnight at 4°C in a 1:10,000 dilution of anti-phospho-SMAD1 (Cell Signaling Technology) in 5% normal goat serum.

Immunofluorescence was performed on 14 μm cryosections using embryos fixed in 4% PFA for 30 minutes. Slides were fixed in cold acetone (–20°C), washed and blocked for 1 hour in blocking solution containing 10% fetal calf serum, 0.5% Triton X-100. Sections were incubated overnight at 4°C in primary antibody diluted in blocking solution. Sections were then washed and incubated with a biotinylated

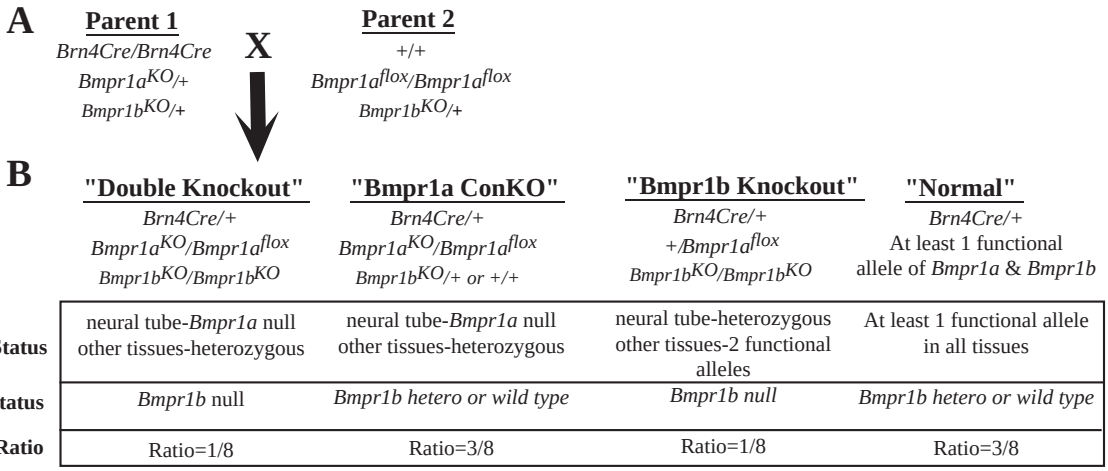

Fig. 1. Mating scheme to generate BMP type 1 receptor (*Bmpr*) double knockouts. (A) Parental genotypes required to generate mutant embryos with a BMP type 1 receptor double knockout. Parent 1 expresses the *Bcre32* transgene and is heterozygous for the *Bmpr1b* knockout allele (*Bmpr1b*^{KO}). *Bmpr1a*^{KO} is a *Bmpr1a* receptor null allele produced by classical knockout technology (Mishina et al., 1995). Parent 2 is homozygous for the floxed *Bmpr1a* allele (*Bmpr1a*^{flox}) and heterozygous at the *Bmpr1b* locus. (B) Four classes of embryos are generated by the parents in A. The genotypes of animals is depicted below the names of each classes, and refer to the genetic composition of the neural tube. Normal embryos have at least one functional allele of *Bmpr1a* and *Bmpr1b* genes in all tissues and do not show any phenotype. The top row of the table depicts the status of the *Bmpr1a* gene in each class. The middle row depicts the status of the *Bmpr1b* gene. The bottom row depicts the expected Mendelian ratios of each phenotypes.

secondary (diluted in blocking solution) at room temperature. After further washes, slides were incubated with a fluorochrome-conjugated avidin. Staining with mouse primary antibodies was accomplished with the M.O.M. Kit (Vector Labs). The following antibodies were used: Pax2 (Zymed), Msx2 (4G1), Isl1 (39.4D5), Lim1/2 (4F2), Pax6 and Pax7 (Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences).

TUNEL analyses were accomplished using 14 μm cryosections and processed according to published protocols (Grinspan et al., 1998). Assays for cell proliferation were carried out by immunolabeling for the mitosis marker, phosphorylated histone H3 (Hendzel et al., 1997; Nowak and Corces, 2000). Cryosections (14 μm) were washed, incubated in 0.5% Triton and blocked in 5% normal goat serum (Vector laboratories). Slides were incubated overnight at 4°C with anti-phospho-histone H3 antibody (Upstate Biotechnology; 1:250). Slides were washed and incubated with secondary antibody (Jackson Laboratories) and nuclei were visualized using 4′,6-diamidino-2-phenylindole (DAPI, Sigma) staining.

Results

Generation of a double knockout of *Bmpr1a* and *Bmpr1b* in the neural tube

The *Bmpr1a* conditional knockout was described previously (Ahn et al., 2001; Mishina et al., 2002). *Cre*-mediated recombination of the *Bmpr1a* gene components located between the loxP (*Bmpr1a*^{flox}) sites leads to excision of the second exon of the *Bmpr1a* gene, which encodes a large segment of the extracellular, ligand-binding domain, and a frame-shift mutation of most of the protein. Tissue-specific recombination of *Bmpr1a* in the neural tube of transgenic embryos is driven by the *Brn4* promoter region of the *Bcre32* [Tg(Pou3f4-cre)32Cren – Mouse Genome Informatics] pedigree (Fig. 1A) (Heydemann et al., 2001). *Bcre32* efficiently induces recombination of floxed genes, including the *Bmpr1a* gene, in the neural tube and its derivative tissue, and has previously shown to completely eliminate the function of floxed alleles in the affected tissue (Ahn et al., 2001; Soshnikova et al., 2003; Zechner et al., 2003).

The spatial and temporal expression of the *Bcre32* transgene was determined in the neural tube by crossing the *Bcre32* strain with the ROSA reporter strain (Soriano, 1999). The resulting *Bcre32*-driven expression of *lacZ* is demonstrated in Fig. 2. Expression is first detected in the anterior neural folds at 8.5 dpc (Fig. 2A) and progresses caudally. As seen in Fig. 2B,D, the entire neural ectoderm of the rostral spinal cord demonstrates *Bcre32*-mediated *lacZ* expression by 9.75 dpc. By 10.5 dpc, *lacZ* expression is detected throughout the neural tube (Fig. 2C).

Additionally, targeted disruption of the *Bmpr1b* (Yi et al., 2000) was used to generate a double knockout of the BMP type 1 receptors in the neural tube as shown in Fig. 1. The resulting double knockout animals died within 1-2 postnatal days. The pups showed truncation of the forelimb digits and gross malformations of the hindlimbs, including agenesis. These phenotypes are characteristic of both the *Bmpr1a* conditional knockout and the *Bmpr1b*-null mice (Ahn et al., 2001; Yi et al., 2000).

BMP signaling eliminated in *Bmpr1a* and *Bmpr 1b* double mutant animals

The loss of BMP receptor signaling was directly assayed by examining the phosphorylation of SMAD1, which is phosphorylated by signaling through the BMP type 1 receptor subunits, BMPR1A and BMPR1B. At 10.0 dpc, high levels of phosphorylated SMAD1 (phospho-SMAD1) immunoreactivity are seen in the dorsal neural ectoderm of normal animals (Fig. 3A). We do not detect any changes in phospho-SMAD1 immunostaining in either the neural tube of *Bmpr1a* conditional knockouts or *Bmpr1b* mutant animals (data not

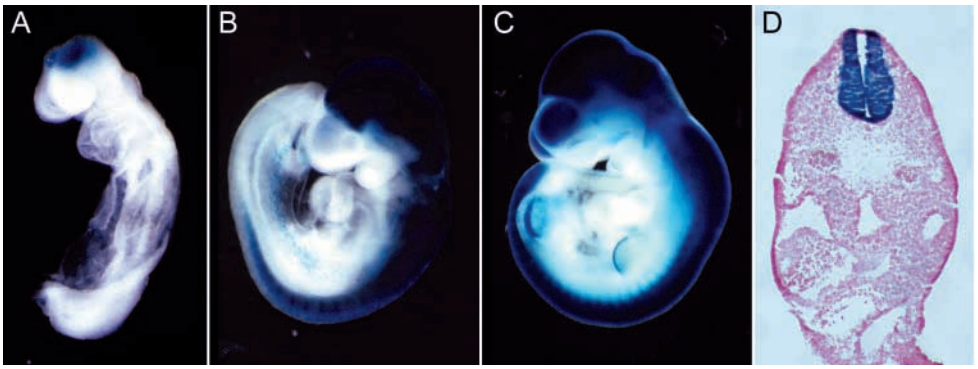

Fig. 2. Spatial and temporal expression of *Bcre-32* using the ROSA reporter demonstrates Cre-mediated recombination in the neural tube. (A) *lacZ* expression at 8.5 dpc in the anlage of the diencephalon and mesencephalon. (B) By 9.75 dpc, *Bcre32*-mediated recombination has targeted the rostral neural tube, including the developing brain and the rostral spinal cord, but largely excluding the telencephalon. (C) *lacZ* expression throughout the neural tube by 10.5 dpc indicating widespread *Bcre32*-mediated recombination, except in the dorsomedial telencephalon and regions of the ventral forebrain (detail not shown). (D) At 9.75 dpc, *lacZ* expression demonstrates *Bcre32*-mediated recombination throughout the neural ectoderm.

shown), suggesting that abrogation of BMP signaling requires the loss of both type 1 BMP receptors. Double mutant animals show a complete loss of immunopositive cells in the dorsal neural tube, except in the specialized tissue of the roof plate where a few phospho-SMAD1-labeled cells remain (arrow, Fig. 3B). Expression in neural crest cells is unaffected (Fig. 3B). These data demonstrate that *Bmpr1a* and *Bmpr1b* are functionally redundant for the phosphorylation of SMAD1 in the dorsal neural tube.

To further demonstrate the loss of the BMP signal, we examined expression of Msx2, which is induced by BMP signaling (Hollnagel et al., 1999; Liem et al., 1995; Pizette and Niswander, 1999; Timmer et al., 2002). Msx2 immunolabeling is detected in the dorsal neural tube at 10.0 dpc and persists in the roof plate at 10.5 dpc in normal animals (Fig. 3C,E). In double mutant embryos, Msx2 immunostaining is detected at 10.0 dpc (Fig. 3D). However, by 10.5 dpc, Msx2 expression in double mutants is lost, as shown by the absence of Msx2 immunoreactivity in the roof plate (Fig. 3F, arrowhead). Msx2 expression is maintained in the epidermal ectoderm of mutant animals (Fig. 3F, arrow). Msx2 expression remains intact in single mutant animals (data not shown). The loss of BMP signaling through the type 1 receptors in the neural tube does not, however, affect the expression of BMP family members, such as *Bmp6* and *Bmp7* (Fig. 3G,H; data not shown).

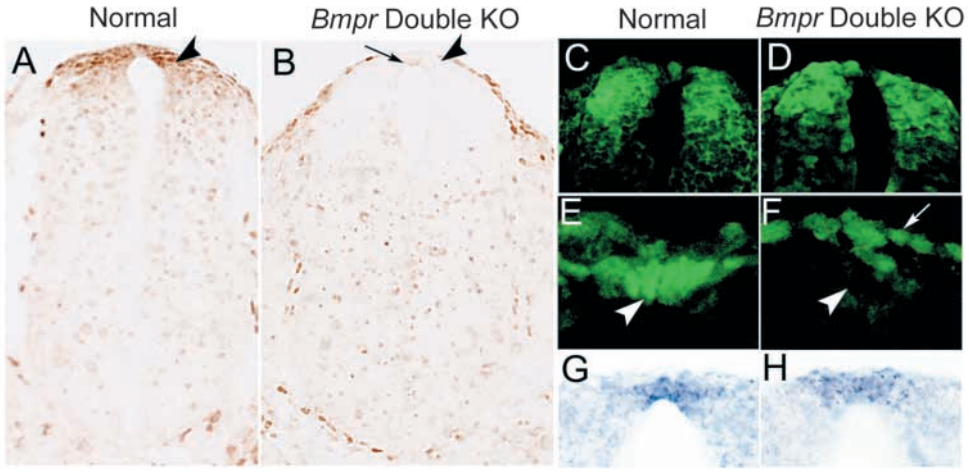

Fig. 3. Loss of BMP signaling in dorsal neural tube of *Bmpr* double knockout mice. (A) Immunostaining for phoshorylated-SMAD1 (phospho-SMAD1) in a normal embryo at 10.0 dpc. Immunoreactive cells are found in the roof plate and the adjacent dorsal neural tube (arrowhead). (B) Phospho-SMAD1 immunostaining in double mutant embryos demonstrates loss of immunoreactivity in the dorsal neural tube (arrowhead). A few phospho-SMAD1 positive cells remain in the roof plate (arrow). (C) Msx2 immunostaining in the dorsal neural tube of a 10.0 dpc normal embryo. (D) Msx2 immunostaining is intact at 10.0 dpc in *Bmpr* double knockout embryos. (E) Msx2 expression in the roof plate (arrowhead) and epidermal ectoderm of normal animals. (F) Msx2 expression is lost in the roof plate of mutant animals (arrowhead), although expression in the epidermal ectoderm is intact (arrow). (G) *Bmp6* is expressed in the roof plate and immediately adjacent tissue at 10.5 dpc in normal embryos. (H) *Bmp6* expression is intact in the *Bmpr* double knockout animals.

BMP signaling is required for development of the DI1 population of sensory interneurons in the dorsal neural tube

The effects of BMP signaling loss in the neural tube were studied by examining the expression of proneural bHLH transcription factors. *Math1*, *Ngn2* and *Mash1* expression marks distinct populations of neuronal progenitors in the developing neural tube (Gowan et al., 2001). The most dorsal population of *Math1*-expressing neural progenitors gives rise to the DI1 population of sensory interneurons (Bermingham et al., 2001). Although *Math1* expression is detected at 10.0 dpc, by 10.5 dpc double mutant animals demonstrate a complete loss of *Math1* expression (data not shown, Fig. 4A,B). The loss of BMP signaling also results in a dorsal shift of the dorsal precursor populations expressing *Ngn2* and *Mash1* (Fig. 4). *Ngn2*-expressing cells now occupy the most dorsal aspect of the neural tube, adjacent to the roof plate (Fig. 4C,D). Although *Mash1* expression is also shifted, the broad region of *Mash1* expression remains intact in the mutant animals (Fig. 4E,F). *Ngn2* also marks a broad domain of

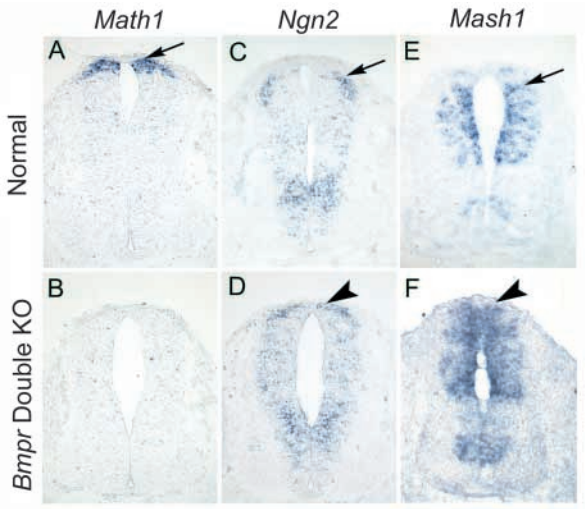

Fig. 4. Expression of bHLH factors shows loss of *Math1* expression, and a dorsal shift of *Ngn2* and *Mash1* expression in *Bmpr* double knockout animals. (A) *Math1* is expressed in the ventricular zone of the dorsal neural tube (arrow) in normal animals at 10.5 dpc. (B) *Math1* expression is lost in *Bmpr* double knockout animals. (C) *Ngn2* expression in a subset of neural progenitors (arrow) is found ventral to *Math1* expression in normal animals and (D) is intact but dorsally shifted (arrowhead) in *Bmpr* double knockout animals at 11.0 dpc. (E) In normal animals, *Mash1* expression marks the remainder of the dorsal ventricular zone (arrow) at 11.0 dpc. (F) In mutant animals, *Mash1* expression is dorsally shifted (arrowhead).

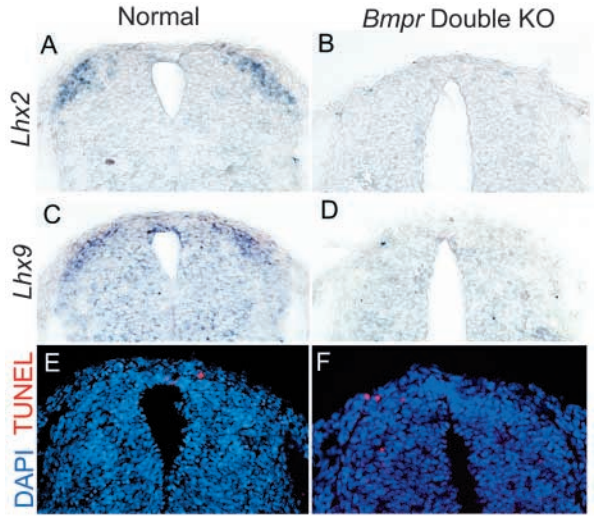

Fig. 5. Loss of DI1 interneurons in *Bmpr* double knockout animals. (A) At 10.5 dpc, *Lhx2* is expressed by the dorsalmost, DI1A, population of sensory interneurons, arising adjacent to the roof plate in normal animals. (B) This population is absent in *Bmpr* double mutant animals, as shown by loss of *Lhx2* expression. (C) *Lhx9* marks the DI1B population of sensory interneurons in the dorsalmost neural tube at 10.5 dpc. (D) This population is completely absent in the *Bmpr* double knockouts, as shown by loss of *Lhx9* expression. (E,F) TUNEL staining (red) at 10.5 dpc, shows no difference in TUNEL-positive cells in the dorsal neural tube of normal (E) and *Bmpr* double knockout (F) animals.

ventral neuronal precursors and this expression remains unaffected in double mutant animals (Fig. 4C,D).

Previous studies have demonstrate that the progenitor population expressing *Math1* gives rise to two distinct neuronal subtypes, DI1A and DI1B (Bermingham et al., 2001; Helms and Johnson, 1998). These subtypes migrate ventrally and contribute to the commissural interneuron population, and are marked by the expression of the LIM homeodomain factors *Lhx2* and *Lhx9* (Helms and Johnson, 1998; Liem et al., 1997). The DI1 neurons are found at these stages in the most dorsal mantle layer of the neural tube (Fig. 5). The loss of *Math1*-expressing dorsal progenitors in the *Bmpr1a;Bmpr1b* double mutant animals is accompanied by a loss of DI1 cells (Fig. 6I), expressing *Lhx2* and *Lhx9* (Fig. 5B,D). These results demonstrate that BMP signaling is required for the specification of the DI1 population of sensory interneurons.

The absence of these dorsal populations is not accompanied by any apparent increases in cell death in the areas of the cell population losses, as demonstrated by the low levels of TUNEL-positive cells in both normal and mutant animals at 10.5 dpc (Fig. 5E,F). TUNEL assays at 10.0 and 11.5 dpc similarly yielded no significant differences in the dorsal neural tube of normal and mutant animals (data not shown). Thus, it does not appear that BMP signaling regulates apoptosis of the dorsal neuronal populations.

BMP signaling is important for specification of DI2 interneurons but not mid-dorsal or ventral populations

The neurons that arise from the next most dorsal aspect of the neural tube, the DI2 population, is another population of ventrally migrating sensory interneurons (for a review, see Helms and Johnson, 2003). The DI2 neurons express markers for the LIM homeodomain factor, Lim1/2 and the winged-helix factor, Foxd3. To determine whether the DI2 population of sensory interneurons was affected in the double mutant animals, we examined the expression patterns of these markers. Dorsal *Foxd3* expression is markedly decreased, while ventral *Foxd3* expression is intact (Fig. 6A,B,E,F). In our mutant animals, a few cells in the dorsal-most aspect of the neural tube retain expression of *Foxd3* (Fig. 6E,F), and these are located more dorsally than normal, adjacent to the roof plate, in the region that is normally occupied by DI1 interneurons (Fig. 6E,F).

We further characterized the loss of DI2 neurons by examining the expression of Lim1/2. Lim1/2 is expressed in multiple regions, marking three distinct dorsal populations: DI2, DI4 and DI6 (for a review, see Helms and Johnson, 2003; Gross et al., 2002; Muller et al., 2002). DI4 and DI6 populations additionally express Pax2. Therefore, Lim1/2-positive, Pax2-negative immunolabeling is indicative of the DI2 population, while Lim1/2-positive, Pax2-positive staining marks the mid-dorsal populations (Fig. 6C). In our double mutant animals, very few Lim1/2-positive, Pax2-negative cells are observed (Fig. 6D,1). In addition, those that are seen are located in the dorsal-most region of the neural tube, adjacent to the roof plate. This further indicates the loss of the DI2 population and a dorsal shift to areas normally expressing markers of DI1 neurons.

To further understand the effect of BMP signaling on development of dorsal cell types, we examined the DI3 and

DI4 populations, located just ventral to the DI2 cells. DI3 cells are marked by expression of Isl1/2. In double mutant animals, Isl1/2-positive cells are shifted, such that some cells are found almost adjacent to the roof plate (arrowhead, Fig. 6H). DI4 cells are marked by the expression of Pax2, and are intermingled with, yet distinct from, DI3 cells, as demonstrated by no co-labeled cells (Fig. 6G,H). The dorsal shift in the DI4 population is also seen in Fig. 6D as the Lim1/2 positive, Pax2-positive immunoreactive cells are more dorsally located. Thus, it is evident that the abrogation of the BMP signal leads to a loss of DI1 and DI2 sensory interneurons, and an associated dorsal expansion of DI3 and DI4 populations. The dorsal expansion is also accompanied by an increase in the number of cells expressing markers for DI3 and DI4 neurons (Fig. 6I).

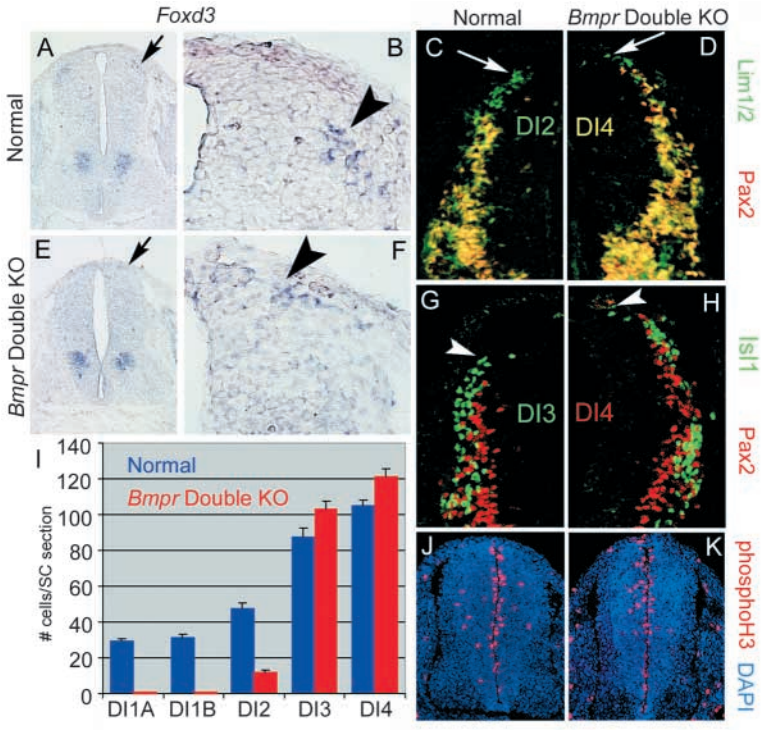

Fig. 6. Loss of DI2 interneurons in *Bmpr* double knockout animals. (A,B,E,F) *Foxd3* expression at 11.0 dpc. (A) *Foxd3* marks dorsal DI2 neurons (arrow), and ventral V1 neurons in normal animals. (B) Higher magnification of DI2 cells from A (arrowhead). (E) Dorsal *Foxd3* expression is reduced in *Bmpr* double knockouts with a few cells found adjacent to the roof plate (arrow). Ventral expression is unaffected. (F) Magnification of remaining DI2 cells (arrowhead). (C) Lim1/2-positive, Pax2-negative cells of the dorsal neural tube indicate the DI2 (green) population. (D) These cells are markedly reduced in the mutant animals, and are found adjacent to the roof plate (arrow). Lim1/2-positive, Pax2-positive, DI4 (yellow) cells are dorsally expanded. (G,H) Sections from 10.5 dpc mouse embryo labeled with antibodies against Isl1 (green) and Pax2 (red). (G) Double immunostaining demonstrates that Isl1-positive DI3 cells (green) and Pax2-positive DI4 cells (red) are intermingled but represent separate populations. (H) In mutant animals, both of these populations are dorsally expanded, and are found adjacent to the roof plate (arrowhead). (I) Double knockout animals show a complete loss of DI1 neurons and a fourfold decrease in DI2 cells ($P<0.001$). DI3 and DI4 cells are increased ($P<0.01$). Single knockout animals do not demonstrate significant differences (data not shown). (J,K) Sections from 11.5 dpc mouse embryo labeled with antibody against phosphorylated histone H3 (phosphoH3, red) and DAPI (blue), demonstrating no change in cell proliferation in mutant animals (K).

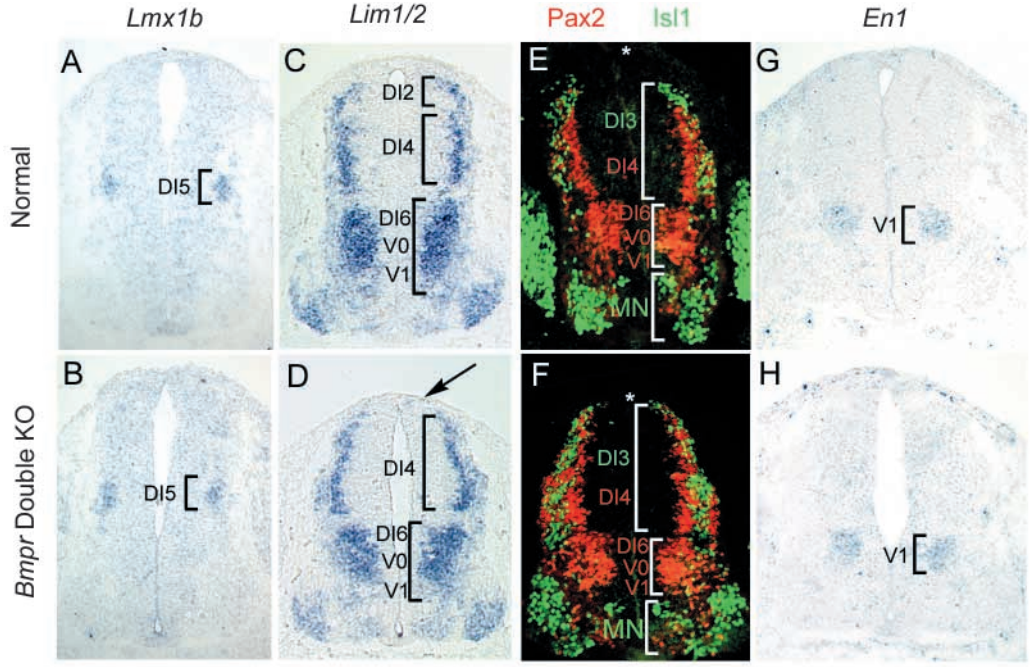

Fig. 7. Mid-dorsal and ventral populations of neurons are not affected in *Bmpr* double knockout animals. (A,B) *Lmx1b* expression labels the DI5 neurons of the dorsal spinal cord at 11.5 dpc normal (A) and mutant animals (B). (C) In normal animals, *Lim1/2* is expressed by three populations of dorsal sensory neurons, DI2, DI4 and DI6, and by two populations of ventral neurons, V0 and V1. (D) In double mutant animals, *Lim1/2* expression is unaffected in the DI6, V0 and V1 populations, but the number of DI2 cells is greatly reduced (arrow). (E,F) Sections from 11.5 dpc spinal cord, labeled with antibodies against Isl1/2 (green) and Pax2 (red). (E) In the neural tube, Isl1/2 marks dorsal DI3 cells and ventral motoneurons (MN). Pax2-positive cell groups include DI4, DI6, V0 and V1. (F) In double knockout animals, DI3 and DI4 cells are shifted dorsally toward the roof plate (*), but are otherwise normal. Other populations marked by Isl1 and Pax2 are unaffected. (G,H) Sections from 11.5 dpc spinal cord, showing expression of *En1* in normal (G) and double knockout (H) animals, indicating V1 populations are unaffected.

The observed expansion of these populations is not accompanied by changes in proliferation. Immunostaining for the mitosis marker phospho-histone H3 in normal neural tubes demonstrates that cells immediately adjacent to the central canal of the spinal cord were undergoing or had recently undergone DNA replication at the time of embryo harvesting. This pattern of proliferation is intact in the mutant animals (Fig. 6G,H). At 10.5 dpc, there are no quantitative differences in the proliferation of neuronal progenitors between normal, single mutant and double mutant animals (Fig. 6G,H; data not shown). Additionally, no differences in cell proliferation were detected at 10.25 or 11.5 dpc (data not shown). Thus, BMP signaling does not appear to regulate neuronal cell proliferation in the developing spinal cord.

Neurons derived from the more ventral regions of the dorsal neural progenitor domains migrate to occupy the upper layers of the dorsal horn (Muller et al., 2002). Further classes of sensory interneurons, DI5 and DI6, are born from *Mash1*-expressing progenitors cells of the dorsal spinal cord. Lmx1b exclusively marks the population of DI5 neurons. As seen in Fig. 7B, *Lmx1b* expression is normal in the double mutant animals. DI6 neurons express the same homeodomain profile as DI4 neurons, but arise at a distinct location along the dorsoventral axis. DI6 interneurons in our mutant animals appear to be intact as well, as indicated by the expression of *Lim1/2* (Fig. 7D). Thus, it is evidenced that mid-dorsal populations of neurons DI3-DI6 are generated independently of BMP signaling in the dorsal neural tube.

Within the ventral spinal cord arise motoneurons (MN) and interneurons (V0-V3) that are important for the control of posture and locomotion (for reviews, see Ericson et al., 1997; Goulding, 1998; Jessell, 2000; Lee and Pfaff, 2001). To determine if BMP signaling modulates or influences ventral specification, we examined markers of the V0-V3 and MN populations. Early postmitotic motoneurons express *Isl1/2*. In mutant animals, ventral *Isl1/2* expression is intact, indicating no effect of BMP signaling loss on MN specification (Fig. 7E,F). In addition, Lim1/2- and Engrailed (En1)-positive cells of the ventral neural tube give rise to the V0-V1 interneurons. Here again, this ventrally derived cell population is unaffected in our mutant animals (Fig. 7D,H). The above data suggests that cell fate specification of cells arising from ventral progenitor domains is independent of BMP signaling from the dorsal neuroectoderm.

Wnt expression is downregulated in *Bmpr* double mutant animals

In addition to the BMP family members, two members of the Wnt family, *Wnt1* and *Wnt3a*, are expressed by roof-plate cells throughout the time of neurogenesis (for reviews, see Hollyday et al., 1995; Parr and McMahon, 1994). To examine the effects of BMP signaling abrogation on Wnt signaling in the dorsal neural tube, we examined the expression patterns of *Wnt1* and *Wnt3a* in our double mutant animals. As seen in Fig. 8, the roof plate and adjacent dorsal neuroepithelium at 10.5 dpc in normal animals express *Wnt1* and *Wnt3a*. The loss of BMP signaling in our mutants leads to a decreased domain of Wnt expression. *Wnt1* and *Wnt3a* become restricted to the roof plate in mutant animals with a complete loss of expression in the adjacent tissue (Fig. 8B,D). This effect is seen as early as 10.25 dpc (data not shown). Again, this loss is not accompanied by changes in cell proliferation in mutant animals (Fig. 6G,H). Thus, the loss of BMP signaling reduces, but does not eliminate, Wnt signaling in the developing spinal cord.

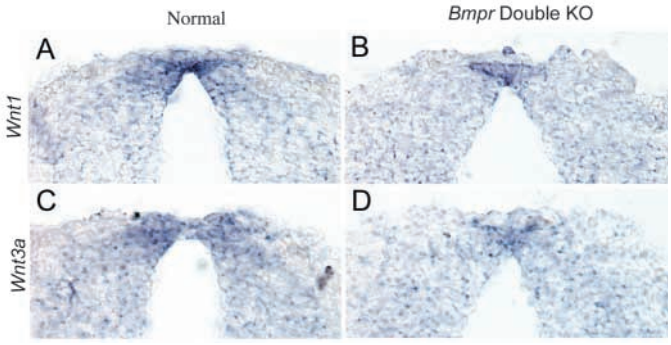

Fig. 8. *Wnt1* and *Wnt3a* expression are decreased in *Bmpr* double knockout animals. (A) In normal animals, *Wnt1* is expressed in the roof plate and adjacent neural ectoderm at 10.5 dpc. (B) In double mutant animals, *Wnt1* expression is restricted to the roof plate. (C) In normal animals, *Wnt3a* is expressed in the roof plate and adjacent neural ectoderm. (D) *Wnt3a* in double mutants is not expressed in the neural ectoderm, but roof plate expression appears unaffected at 10.5 dpc.

Expression of Id proteins is downregulated upon loss of BMP signaling

Id proteins are promoters of cell growth and inhibitors of cell differentiation (for a review, see Norton, 2000). They are members of the HLH family of transcription factors that antagonize bHLH factors during development to maintain progenitor populations (Norton, 2000; Ross et al., 2003). The delicate balance between Ids and proneural bHLH factors is believed to be an essential part of neural tube development (for a review, see Ross et al., 2003). In addition, Id genes are responsive to BMP (Hollnagel et al., 1999) and Wnt signaling (Rockman et al., 2001). Because of the potential importance of Id factors in spinal cord development and the possible role for BMPs in regulating Id proteins, we examined the expression of Ids in the *Bmpr1a;Bmpr1b* double mutant animals.

Id1 and *Id3* are expressed in the neural progenitors adjacent to the roof plate at 10.0 dpc in normal animals (Fig. 9A,E). By contrast, *Id2* shows a low level of expression throughout the progenitor domain of the dorsal spinal cord with higher expression in the dorsal midline (Fig. 9C). In our mutant animals, *Id1* and *Id3* are greatly reduced at 10.0 dpc (Fig. 9B,F). The dorsal expression is lost by 10.5 dpc (Fig, 9G,H, data not shown). Ventral *Id1* and *Id3* expression is intact (arrowhead, Fig. 9B,F). *Id2* expression appears unaffected at 10.0 dpc, but is lost dorsally by 10.5 dpc (Fig. 9C,D; data not shown). Thus, the expression of Id family members is affected by the loss of BMP signaling in the *Bmpr1a;Bmpr1b* double mutant animals.

Discussion

We have demonstrated that signaling through BMPR1A and BMPR1B receptors is crucial for proper development of sensory interneurons in the dorsal neural tube. The observed loss of dorsal cell types requires complete abrogation of the BMP signal, because the loss of a single receptor subtype

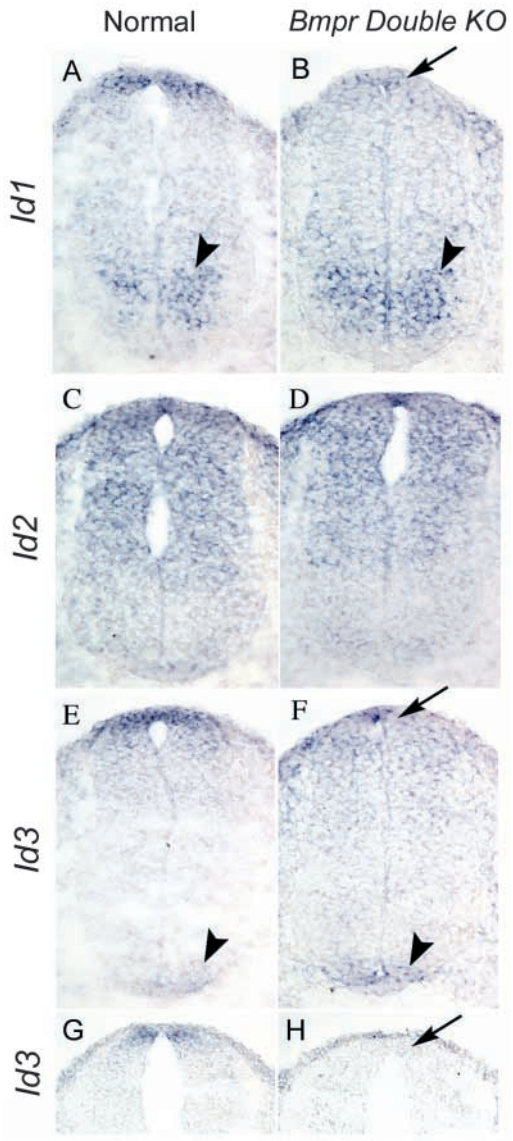

Fig. 9. Id gene expression is decreased in *Bmpr* double knockout animals. (A) *Id1* expression in 10.0 dpc spinal cord in the roof plate and adjacent neural ectoderm of normal animals. There is also a region of ventral expression (arrowhead). (B) In double knockout animals, *Id1* expression is greatly reduced in the dorsal neural tube (arrow). The ventral domain of expression (arrowhead) is unaffected. (C) In normal animals, *Id2* is expressed diffusely throughout the dorsal half of the neural tube at 10.0 dpc. (D) Expression of *Id2* appears unaffected at 10.0 dpc in mutant animals. (E) The roof plate and dorsal neural ectoderm express *Id3* in normal animals. (F) In mutant animals, expression of *Id3* in the neural ectoderm is diminished (arrow) at 10.0 dpc. (G) *Id3* expression at 10.5 dpc in normal embryos. (H) By 10.5 dpc, *Id3* expression is abolished in double mutant embryos.

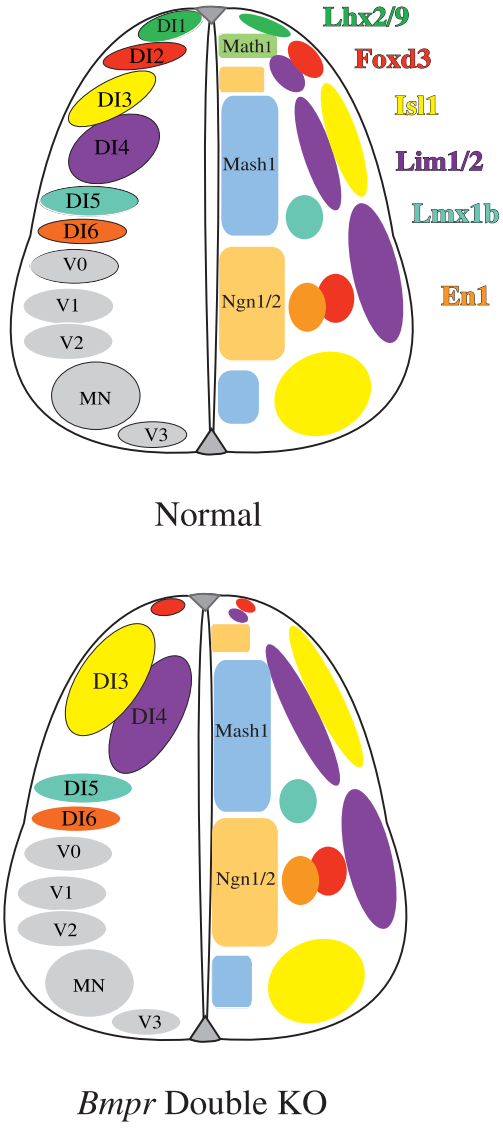

Fig. 10. Summary of the phenotype of dorsal neural tube development in *Bmpr1a*;*Bmpr1b* double knockout animals. (A) On the left, the normal distribution of dorsal interneurons, DI1-DI6, are schematically illustrated. Ventral subtypes, V0-V3 and MN are shown in grey. The neuron populations are characterized by expression of LIM-homeodomain factors, shown on the right. The ventricular zone of the developing spinal cord is subdivided by the expression domains of the bHLH factors, as shown. (B) In the *Bmpr* double knockout animals, the DI1 population is absent, as shown by the loss of *Lhx2* and *Lhx9*. Furthermore, *Math1* expression in the ventricular zone is also lost. The other bHLH domains are intact but shifted dorsally. Only a small number of DI2 cells remain with decreased expression of *Foxd3* and *Lim1/2*. The DI3 and DI4 populations are shifted dorsally as well.

yields no differences in dorsal spinal cord patterning. This further demonstrates that the receptors are functionally redundant for dorsal cell development. Knockout of *Bmpr1a* and *Bmpr1b* in the neural tube results in loss of the two dorsal-most populations of sensory interneurons, DI1 and DI2 (Figs 5, 6). These findings are summarized schematically in Fig. 10. Furthermore, BMPs are not required for the specification of DI3 neurons nor for the formation of Class B neurons (Fig. 7). Our results provide definitive genetic evidence that BMP signaling is essential for dorsal spinal cord patterning.

BMP signaling is required for specification of the dorsal commissural neurons

We have demonstrated that loss of the BMP signaling in vivo

eliminates expression of *Math1* by 10.5 dpc. Math1 is a member of the bHLH family of proteins, which have been shown to be crucial for proliferation, specification and differentiation in a variety of cell lineages (for reviews, see Norton, 2000; Ross et al., 2003). The *Math1*-expressing progenitor cells give rise to the dorsal commissural neuronal populations, DI1A and DI1B, marked by their expression of *Lhx2* and *Lhx9*, respectively. Consequently, expression of *Lhx2* and *Lhx9* is also lost, demonstrating that BMP signaling is necessary for maintaining this dorsal progenitor domain and, ultimately, for differentiation of DI1 neurons. D1 progenitors and DI1 interneurons probably require very high levels of BMP signaling as loss of even one BMP related protein, Gdf7, results in perturbation of this population (Lee et al., 1998). Gdf7 was shown to be important for maintaining late *Math1*-positive progenitor populations and DI1A cells. Furthermore, high levels of BMP signaling directly activate the expression of chicken homolog, *Cath1* (Timmer et al., 2002), supporting the hypothesis that the BMPs establish and maintain DI1 interneurons by establishing a morphogenic gradient. It is possible, however, that specification of D1 progenitors is dependent on earlier BMP activity that is not affected in our mutants, as seen by the initial expression of Msx2 (Fig. 3D).

Genetic ablation of the roof plate, as seen in the *dreher* mutant mice, also leads to a decrease in DI1 populations (Millonig et al., 2000). However, the progenitor population is intact, suggesting that the BMP signal that is crucial for maintaining progenitor populations may be extrinsic to the roof plate, possibly from earlier BMP activity. Although our studies do not elucidate the mechanisms of initial establishment of the dorsal progenitor domains, we clearly show that BMP signaling is directly or indirectly necessary for maintenance of the D1 precursor cells. However, proper specification of the differentiated DI1 cells requires BMP signaling, as seen in our studies. Moreover, Math1 is necessary, but not sufficient, for DI1 differentiation (Gowan et al., 2001), further suggesting that BMPs, in addition to Math1, may be necessary for specification of the postmitotic DI1 population. In the absence of *Math1* expression, DI1 precursors neither differentiate nor migrate (Bermingham et al., 2001). Thus, in our mutant animals, it is not possible to determine whether the observed loss of *Lhx2*- and *Lhx9*-expressing cells is only secondary to the loss of *Math1* expression. Further studies will be needed to determine if loss of BMP signaling in the presence of normal Math1 activity also affects DI1 specification. Nonetheless, our studies, in conjunction with previous work, indicate that BMP signaling is necessary for expression and maintenance of the D1 precursor.

Our analyses demonstrate that other dorsal progenitor domains remain intact in double knockout, as seen by the continued expression of *Ngn2* and *Mash1*. This suggests that BMP signaling, at this stage, is not necessary for establishment of the remaining dorsal ventricular zone areas. Toxin-mediated roof plate ablation does affect the D2 precursors, as shown by loss of *Ngn1* expression (Lee et al., 2000). These results, taken with ours, suggest that other factors originating from the roof plate may be essential for formation of D2 precursor populations. Our mutant animals demonstrate that the formation of DI2 interneurons is affected by loss of BMP signaling, indicating that specification of postmitotic DI2 cells is BMP dependent.

The bHLH proteins, Math1, Mash1, Ngn1 and Ngn2 establish and maintain the ventricular zone regions necessary for correct specification of neuronal fates (for a review, see Gowan et al., 2001; Ross et al., 2003). Id proteins, negative regulators of bHLH proteins, are expressed within the ventricular zone of the developing neural tube. Members of the Id protein family have been implicated as crucial for maintenance of neural progenitor populations by antagonizing proneural bHLH factors, and downregulation of Ids is hypothesized to be a crucial molecular switch for terminal differentiation of neural precursors cells (for reviews, see Norton, 2000; Ross et al., 2003). *Id1*, *Id2* and *Id3* expression has been shown to be directly induced by BMP activity in a variety of cell lines (Hollnagel et al., 1999). Thus, Ids are candidates for mediators of the actions of BMPs in neural patterning. Although we have observed alterations in *Id* expression in mutant animals (Fig. 9), these changes do not appear to be profound enough to fully account for the observed patterning defects in our mutant animals. Some *Id* expression remains when losses of neural markers have already been detected. In addition, we suggest that the dorsal patterning defects seen in our double mutants are independent of the *Id* expression changes, as *Id1;Id3* double knockout animals show expanded *Math1* expression (Lyden et al., 1999).

In contrast to the DI1 and DI2 cell populations, we observed an expansion of DI3 and DI4 cells in the double knockout animals. Toxin-mediated roof-plate ablation leads to loss of DI3 interneurons (Lee et al., 2000). Thus, roof-plate signals other than BMPs are likely to be important. The increased number of DI3 and DI4 cells suggests that these cells have switched from a more dorsal fate in the absence of BMP signaling, although the absolute number of cells lost can not be fully accounted for by the DI3 and DI4 expansion. Other dorsal and ventral populations are unchanged, consistent with other reports that class B neurons and ventral populations are roof plate independent (Gross et al., 2002; Muller et al., 2002).

The *Bmpr1a* conditional knockout and the *Bmpr1b* null animals alone do not show any of the neural tube defects observed in the double mutant animals. Therefore, our studies demonstrate that, in the mouse, these two BMP type 1 receptor subtypes are functionally redundant in dorsal patterning of the neural tube. This differs from the results obtained by Panchision et al. (Panchision et al., 2001), who observed sequential and differential actions of *Bmpr1a* and *Bmpr1b* activated constructs in transgenic animals. The contradictory nature of our findings suggests that the use of constitutively activated receptors in this context does not accurately mimic endogenous roles of the BMP type 1 receptors. However, both reports do show a clear role for BMPs in dorsal cell type specification. Their report also suggests a role for BMP signaling in proliferation and apoptosis. Again, we do not see such differences in double knockout animals. Furthermore, BMPs probably activate proliferation through observed induction of Wnt signaling, and in our animals we have some remaining Wnt expression.

BMPs and Wnts may act cooperatively in dorsal cell type specification

It is likely that other non-TGFβ related proteins are involved in dorsal spinal cord development. Such candidates for these BMP-independent roof plate activities are the Wnt family

members. There are many examples throughout embryogenesis of the important interactions between Wnt and BMP family members (Ahn et al., 2001; Roelink, 1996; Soshnikova et al., 2003) and Wnts have been shown to be directly inducible by BMP signaling (for reviews, see Roelink, 1996; Panchision et al., 2001). We see some initial expression of *Wnt1* and *Wnt3a*, with subsequent loss of expression in the dorsal neural ectoderm (Fig. 8, data not shown). However, roof plate expression remains intact. It is thus possible that BMP signaling could induce and/or maintain *Wnt* expression specifically in the dorsal neural ectoderm. Alternatively, the loss of Wnt expression could be secondary to the general perturbation of cell specification seen in the double mutant animals.

The role of Wnts in dorsal cell specification is still not completely understood. *Wnt1;Wnt3a* double knockouts demonstrate losses in D1 and D2 precursor and decreased DI1 and DI3 cells without accompanying alterations in BMP expression (Muroyama et al., 2002). These defects are similar to those seen in the roof plate ablation studies (Lee et al., 2000). The losses seen in the Wnt double knockouts are, however, not complete, suggesting that Wnts and BMPs may act cooperatively for dorsal cell specification. Furthermore, Wnt3a-conditioned media induced expression of DI1 and DI3 markers (Muroyama et al., 2002). It is thus possible that a Wnt gradient works in conjunction with or is downstream of BMP signaling to specify dorsal cell types. However, Wnt signaling through the β-catenin pathway has been shown to regulate cell cycle progression and negatively regulate cell cycle exit in neural cells (Chenn and Walsh, 2002; Megason and McMahon, 2002; Soshnikova et al., 2003; Zechner et al., 2003). *Bmpr* double knockouts do not show any alterations in cell proliferation despite the perturbation in Wnt expression. It is thus likely that either the changes we see in Wnt expression are too late to alter cell proliferation or that the residual expression in the roof plate is sufficient to maintain the mitogenic effects of Wnts.

In conclusion, we have demonstrated that signaling through the predominant BMP type 1 receptors, *Bmpr1a* and *Bmpr1b*, is crucial for maintenance of dorsal progenitor cells and for the specification of dorsal neural cell types. As summarized in Fig. 10, the double knockout animals demonstrate losses of DI1 and DI2 sensory interneurons and their accompanying molecular markers. The role of BMPs in the neural tube is complex, as other signaling pathways, such as Wnts and Ids, are also affected. Further work is needed to understand fully the complexity of these interactions and their role in neural tube patterning.

We gratefully acknowledge Drs. D. Anderson, J. Botas, J. Johnson, R. Johnson, A. McMahon, P. Labosky and H. Westphal for probes used in these studies. We also thank Drs J. Davis, J. Germiller, J. Grinspan, J. Golden, M. Mullins and N. Solowski for critical discussion and comments on the manuscript. We further appreciate the technical assistance that was provided by Drs P. Bannerman, J. Grinspan, J. Golden and S. Scherer. Work from the laboratory of E.B.C. is supported by the NIH. L.W.L. is supported by a fellowship under the Training in the Neurobiology of Otorhinolaryngology grant.

References

Ahn, K., Mishina, Y., Hanks, M. C., Behringer, R. R. and Crenshaw, E. B., III (2001). BMPR-IA signaling is required for the formation of the apical ectodermal ridge and dorsal-ventral patterning of the limb. *Development* **128**, 4449-4461.

Bermingham, N. A., Hassan, B. A., Wang, V. Y., Fernandez, M., Banfi, S., Bellen, H. J., Fritzsch, B. and Zoghbi, H. Y. (2001). Proprioceptor pathway development is dependent on Math1. *Neuron* **30**, 411-422.

Chang, H., Brown, C. W. and Matzuk, M. M. (2002). Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocr. Rev.* **23**, 787-823.

Chenn, A. and Walsh, C. A. (2002). Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science* **297**, 365-369.

Derynck, R. and Zhang, Y. E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425**, 577-584.

Ebendal, T., Bengtsson, H. and Soderstrom, S. (1998). Bone morphogenetic proteins and their receptors, potential functions in the brain. *J. Neurosci. Res.* **51**, 139-146.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-180.

Goulding, M. (1998). Specifying motor neurons and their connections. *Neuron* **21**, 943-946.

Gowan, K., Helms, A. W., Hunsaker, T. L., Collisson, T., Ebert, P. J., Odom, R. and Johnson, J. E. (2001). Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. *Neuron* **31**, 219-232.

Grinspan, J. B., Coulalaglou, M., Beesley, J. S., Carpio, D. F. and Scherer, S. S. (1998). Maturation-dependent apoptotic cell death of oligodendrocytes in myelin-deficient rats. *J. Neurosci. Res.* **54**, 623-634.

Gross, M. K., Dottori, M. and Goulding, M. (2002). Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. *Neuron* **34**, 535-549.

Helms, A. W. and Johnson, J. E. (1998). Progenitors of dorsal commisural interneurons are defined by Math1 expression. *Development* **125**, 919-928.

Helms, A. W. and Johnson, J. E. (2003). Specification of dorsal spinal cord interneurons. *Curr. Opin. Neurobiol.* **13**, 42-49.

Hendzel, M. J., Wei, Y., Mancini, M. A., van Hooser, A., Ranalli, T., Brinkley, B. R., Bazett-Jones, D. P. and Allis, C. D. (1997). Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106**, 348-360.

Heydemann, A., Nguyen, L. C. and Crenshaw, E. B., III (2001). A regulatory region of the *Brn4/Pou3f4* promoter directs expression to developing forebrain and neural tube. *Dev. Brain Res.* **128**, 83-90.

Hogan, B. L. (1996). Bone morphogenetic proteins, multifunctional regulators of vertebrate development. *Genes Dev.* **10**, 1580-1594.

Hollnagel, A., Oehlmann, V., Heymer, J., Ruther, U. and Nordheim, A. (1999). Id genes are direct targets of bone morphogenetic protein induction in embryonic stem cells. *J. Biol. Chem.* **274**, 19838-19845.

Hollyday, M., McMahon, J. A. and McMahon, A. P. (1995). Wnt expression patterns in chick embryo nervous system. *Mech. Dev.* **52**, 9-25.

Jessell, T. M. (2000). Neuronal specification in the spinal cord, inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1**, 20-29.

Lee, K. J. and Jessell, T. M. (1999). The specification of dorsal cell fates in the vertebrate central nervous system. *Annu. Rev. Neurosci.* **22**, 261-294.

Lee, K. J., Mendelsohn, M. and Jessell, T. M. (1998). Neuronal patterning by BMPs, a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. *Genes Dev.* **12**, 3394-3407.

Lee, K. J., Dietrich, P. and Jessell, T. M. (2000). Genetic ablation reveals that the roof plate is essential for dorsal interneuron specification. *Nature* **403**, 734-740.

Lee, S. K. and Pfaff, S. L. (2001). Transcriptional networks regulating neuronal identity in the developing spinal cord. *Nat. Neurosci.* **4**, 1183-1191.

Liem, K. F., Jr, Tremml, G., Roelink, H. and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969-979.

Liem, K. F., Jr, Tremml, G. and Jessell, T. M. (1997). A role for the roof plate and its resident TGFbeta-related proteins in neuronal patterning in the dorsal spinal cord. *Cell* **91**, 127-138.

Lyden, D., Young, A. Z., Zagzag, D., Yan, W., Gerald, W., O'Reilly, R., Bader, B. L., Hynes, R. O., Zhuang, Y., Manova, K. et al. (1999). Id1 and

Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. *Nature* **401**, 670-677.

Massague, J. (1996). TGFbeta signaling, receptors, transducers, and Mad proteins. *Cell* **85**, 947-950.

Massague, J. (1998). TGF-beta signal transduction. *Annu. Rev. Biochem.* **67**, 753-791.

Megason, S. G. and McMahon, A. P. (2002). A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* **129**, 2087-2098.

Mehler, M. F., Mabie, P. C., Zhang, D. and Kessler, J. A. (1997). Bone morphogenetic proteins in the nervous system. *Trends Neurosci.* **20**, 309-317.

Millonig, J. H., Millen, K. J. and Hatten, M. E. (2000). The mouse Dreher gene Lmx1a controls formation of the roof plate in the vertebrate CNS. *Nature* **403**, 764-769.

Mishina, Y., Suzuki, A., Ueno, N. and Behringer, R. R. (1995). Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. *Genes Dev.* **9**, 3027-3037.

Mishina, Y., Hanks, M. C., Miura, S., Tallquist, M. D. and Behringer, R. R. (2002). Generation of Bmpr/Alk3 conditional knockout mice. *Genesis* **32**, 69-72.

Muller, T., Brohmann, H., Pierani, A., Heppenstall, P. A., Lewin, G. R., Jessell, T. M. and Birchmeier, C. (2002). The homeodomain factor lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. *Neuron* **34**, 551-562.

Muroyama, Y., Fujihara, M., Ikeya, M., Kondoh, H. and Takada, S. (2002). Wnt signaling plays an essential role in neuronal specification of the dorsal spinal cord. *Genes Dev.* **16**, 548-553.

Nguyen, V. H., Trout, J., Connors, S. A., Andermann, P., Weinberg, E. and Mullins, M. C. (2000). Dorsal and intermediate neuronal cell types of the spinal cord are established by a BMP signaling pathway. *Development* **127**, 1209-1220.

Norton, J. D. (2000). ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. *J. Cell Sci.* **113**, 3897-3905.

Nowak, S. J. and Corces, V. G. (2000). Phosphorylation of histone H3 correlates with transcriptionally active loci. *Genes Dev.* **14**, 3003-3013.

Panchision, D. M., Pickel, J. M., Studer, L., Lee, S. H., Turner, P. A., Hazel, T. G. and McKay, R. D. (2001). Sequential actions of BMP receptors control neural precursor cell production and fate. *Genes Dev.* **15**, 2094-2110.

Parr, B. A. and McMahon, A. P. (1994). Wnt genes and vertebrate development. *Curr. Opin. Genet. Dev.* **4**, 523-528.

Phippard, D., Lu, L., Lee, D., Saunders, J. C. and Crenshaw, E. B., III (1999). Targeted mutagenesis of the POU-domain gene, *Brn4/Pou3f4*, causes development defects in the inner ear. *J. Neurosci.* **19**, 5980-5989.

Pizette, S. and Niswander, L. (1999). BMPs negatively regulate structure and function of the limb apical ectodermal ridge. *Development* **126**, 883-894.

Rockman, S. P., Currie, S. A., Ciavarella, M., Vincan, E., Dow, C., Thomas, R. J. and Phillips, W. A. (2001). Id2 is a target of the beta-catenin/T cell factor pathway in colon carcinoma. *J. Biol. Chem.* **276**, 45113-45119.

Roelink, H. (1996). Tripartite signaling of pattern, interactions between Hedgehogs, BMPs and Wnts in the control of vertebrate development. *Curr. Opin. Neurobiol.* **6**, 33-40.

Ross, S. E., Greenberg, M. E. and Stiles, C. D. (2003). Basic helix-loop-helix factors in cortical development. *Neuron* **39**, 13-25.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.

Soshnikova, N., Zechner, D., Huelsken, J., Mishina, Y., Behringer, R. R., Taketo, M. M., Crenshaw, E. B., III and Birchmeier, W. (2003). Genetic interaction between Wnt/beta-catenin and BMP receptor signaling during formation of the AER and the dorsal-ventral axis in the limb. *Genes Dev.* **17**, 1963-1968.

Thor, S., Andersson, S. G., Tomlinson, A. and Thomas, J. B. (1999). A LIM-homeodomain combinatorial code for motor-neuron pathway selection. *Nature* **397**, 76-80.

Timmer, J. R., Wang, C. and Niswander, L. (2002). BMP signaling patterns the dorsal and intermediate neural tube via regulation of homeobox and helix-loop-helix transcription factors. *Development* **129**, 2459-2472.

Yi, S. E., Daluiski, A., Pederson, R., Rosen, V. and Lyons, K. M. (2000). The type I BMP receptor BMPRIB is required for chondrogenesis in the mouse limb. *Development* **127**, 621-630.

Zechner, D., Fujita, Y., Hulsken, J., Muller, T., Walther, I., Taketo, M. M., Crenshaw, E. B., III, Birchmeier, W. and Birchmeier, C. (2003). beta-Catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system. *Dev. Biol.* **258**, 406-418.