Development 129, 2459-2472 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV9785

BMP signaling patterns the dorsal and intermediate neural tube via regulation of homeobox and helix-loop-helix transcription factors

John R. Timmer, Charlotte Wang and Lee Niswander*

Molecular Biology Program and Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA

*Author for correspondence (e-mail: l-niswander@ski.mskcc.org)

Accepted 19 February 2002

SUMMARY

In the spinal neural tube, populations of neuronal precursors that express a unique combination of transcription factors give rise to specific classes of neurons at precise locations along the dorsoventral axis. Understanding the patterning mechanisms that generate restricted gene expression along the dorsoventral axis is therefore crucial to understanding the creation of diverse neural cell types. Bone morphogenetic proteins (BMPs) and other transforming growth factor β (TGFβ) proteins are expressed by the dorsal-most cells of the neural tube (the roofplate) and surrounding tissues, and evidence indicates that they play a role in assigning cell identity. We have manipulated the level of BMP signaling in the chicken neural tube to show that BMPs provide patterning information to both dorsal and intermediate cells. BMP regulation of the expression boundaries of the homeobox proteins Pax6, Dbx2 and Msx1 generates precursor populations with distinct developmental potentials. Within the resulting populations, thresholds of BMP act to set expression domain boundaries of developmental regulators of the homeobox and basic helix-loop-helix (bHLH) families, ultimately leading to the generation of a diversity of differentiated neural cell types. This evidence strongly suggests that BMPs are the key regulators of dorsal cell identity in the spinal neural tube.

Key words: Neural tube, Bone morphogenetic protein, Dorsal-ventral patterning, Basic Helix-Loop-Helix, Homeobox, Chick

INTRODUCTION

Patterning information along the dorsoventral axis of the spinal neural tube can be viewed as being generated by two processes: the assignment of regional identity and the division of these regions into discrete domains of gene expression. The early expression of developmental regulators by broad areas of the neural tube suggests that regional identity acts to restrict the potential fates adopted by cells within that region. Although the expression of individual genes may act to mark a particular region, multiple factors may contribute to the establishment and maintenance of regional identity over the course of neural tube development. Large regions in the neural tube, however, give rise to many classes of terminally differentiated neurons, suggesting that these regions are subdivided into discrete cell populations. Overlapping and exclusive expression of specific combinations of developmental regulators within a given region has been shown to generate discrete populations (Briscoe et al., 2000; Ericson et al., 1996; Pierani et al., 1999). Although the regionalization and subdivision of the neural tube can be viewed as separate processes, there can clearly be some overlap between them. Pax3 and Pax7, which are believed to contribute to a dorsal identity, also act to subdivide a population of intermediate cells into two distinct populations (Mansouri and Gruss, 1998; Pierani et al., 1999).

Understanding the generation of regional identity and the subdivision of these regions is crucial to an understanding of neural patterning. There is substantial evidence that the sonic hedgehog protein (SHH) controls both regional identity and cell fate in the ventral neural tube. Elimination of *Shh* gene function results in severe abnormalities in the spinal neural tube, including the failure to generate many ventral cell types (Chiang et al., 1996; Ericson et al., 1996; Marti et al., 1995). Activation of the *Shh* signaling pathway in dorsal neural tissue blocks the expression of dorsally restricted proteins and imposes a ventral fate on dorsal cells (Epstein et al., 1996; Hynes et al., 2000; Roelink et al., 1995). In addition to providing a ventral identity to cells in the developing neural tube, SHH has been shown to participate in the division of the ventral neural tube into discrete populations of ventral neuronal precursors (Briscoe et al., 2000). SHH acts to generate a morphogen field in the ventral neural tube, and several transcriptional regulators of the homeobox gene family have expression boundaries set by specific thresholds of SHH signaling (Ericson et al., 1997a; Ericson et al., 1997b). As a result, small populations of proliferating neuronal precursors express specific combinations of homeobox proteins; each population then gives rise to a single class of differentiated neurons.

It has been suggested that members of the TGFβ superfamily, including several of the BMPs, play a similar role in the dorsal neural tube (Lee and Jessell, 1999). BMP gene expression begins

in tissue juxtaposed with the dorsal neural tube prior to neural tube closure. After closure of the neural tube, several TGFβ superfamily members are expressed by the roofplate and surrounding tissues (Basler et al., 1993; Lee et al., 1998; Liem et al., 1995). Moreover, exposure of cultured explants of undifferentiated neural tissue to BMPs elicits changes in the expression of many developmental regulators known to be expressed by cells of the dorsal neural tube (Lee et al., 1998; Liem et al., 1997; Pierani et al., 1999). These experiments have also indicated that the expression of several of these genes is sensitive to the concentration of BMPs, suggesting that BMPs can also act as morphogens in generating patterning information. In intact embryonic neural tissue, however, the activity of BMPs is likely to be influenced by the expression of BMP antagonists and ligands of other signaling pathways, both by surrounding tissues and within the neural tube itself (Bertrand et al., 2000; Ericson et al., 1995; Hollyday et al., 1995; Liem et al., 2000; McMahon et al., 1998; Pierani et al., 1999; Pituello et al., 1995; Williams et al., 1995). In addition, the Shh signaling pathway has also been shown to both influence the expression of BMP-responsive genes (Liem et al., 1997; Liem et al., 1995; Selleck et al., 1998) and to be influenced by BMP signaling(Liem et al., 2000). Thus, there remains a need to identify the role of BMP signaling in its proper context.

Clarifying the precise role of BMP signaling in neural tissue in vivo has proven difficult. The large number of TGFβ ligands expressed in neural tissue suggests a degree of functional redundancy, and gene knockout experiments targeting the BMPs or their receptors in mice have in large part generated mice that either lacked a neural phenotype or died at early stages of development (Beppu et al., 2000; Dudley and Robertson, 1997; Dunn et al., 1997; Luo et al., 1995; Solloway et al., 1998; Yi et al., 2000). The single exception, the GDF-7 knockout, affects only a subpopulation of the neurons generated from the dorsal-most population of neural precursors (Lee et al., 1998). Elimination of the roofplate genetically (Millonig et al., 2000) or via the roofplate-specific expression of a toxin (Lee et al., 2000) has supported a role for the roofplate and the BMPs it expresses in generating some dorsal neural cell types. These experiments, however, differ in the severity of their effects on gene expression, possibly as a result of either differences in the timing of roofplate elimination or due to alteration of surrounding tissues, which express TGFβ ligands or other factors.

We have taken an opposite approach to study the role of BMPs in patterning the neural tube. We have activated the BMP signaling pathway within cells of the developing neural tube in a cell autonomous manner via the use of mutated BMP receptor (BMPR) constructs that are active in the absence of ligand. We have found that BMP signaling regulates patterning genes that promote regional identity, as well as those that divide these regions into discrete cell populations. Our results also suggest that thresholds of BMP signaling activity regulate the proteins that ultimately generate a diversity of dorsal neuronal cell types.

MATERIALS AND METHODS

Embryo manipulations

Fertilized White Leghorn eggs were obtained from SPAFAS (Norwich, Conn.) and incubated at 39°C. Two methods were used to express the activated BMPR and other proteins in the developing chicken neural tube: retroviral infection (Fekete and Cepko, 1993) and electroporation (Muramatsu et al., 1997). In both cases, procedures were timed so that construct expression would begin shortly after neural tube closure in the caudal regions of the embryo. Viral infections were carried out at Hamburger and Hamilton (Hamburger and Hamilton, 1992) stage 10-12 and electroporations were performed at stages 14-16. Embryos were dissected approximately 48 hours after these manipulations, at stages 22-24, fixed in 4% parafomaldehyde in PBS for 1 hour, washed extensively in PBS, embedded in OCT, and cryosectioned at 10 μm. Sections were taken from the region between the fore- and hind-limbs. All results described here were obtained in at least four embryos.

Purified, replication-competent retroviruses were injected directly into the lumen of the neural tube. Viral infection was assayed via immunofluorescence using an antibody to the viral gag protein, as shown in Fig. 1A. For electroporations, DNA was injected into the lumen of the neural tube and introduced into cells by three pulses of 25 mV (50 ms each). Electroporated cells were marked by the co-electroporation of a vector expressing EGFP (Clontech, for example, see inset of Fig. 1C). Cell autonomy was correlated with phenotype in electroporated samples; virally infected samples generally had uniform expression of viral antigen, owing to the continual spread of RCAS. In some samples electroporated with activated BMPR-Ib, extensive construct expression resulted in apoptosis, disrupting the morphology of the neural tube (data not shown). Electroporated samples shown here (except Fig. 1H) had limited expression and no phenotype indicative of apoptosis.

BMPR and homeobox expression constructs

Point mutations have been generated in both the type Ia and Ib BMPRs that mimic ligand driven phosphorylation and cause these receptors to be active in the absence of ligands (Wieser et al., 1995). BMPR constructs were expressed from either pMiW III (Muramatsu et al., 1997), pCAGGS (Niwa et al., 1991) or the Clontech EGFP-N1 vector (CMV promoter; GFP coding region was replaced by that of activated BMPR-IB). Levels of expression driven by these vectors correlated with the strength of phenotypes as detailed in Table 1. All samples shown here were generated with activated BMPR-Ib (except Fig. 1K,L).

RCAS viruses expressing activated BMPR-Ib (Zou et al., 1997), Msx1 (Bendall et al., 1999) or Dbx2 (Pierani et al., 1999) have been described. RCAS-Msx1 infection was carried out solely by viral injection; RCAS-Dbx2 infection was carried out both by viral injection and by electroporation of viral DNA. No difference in the results generated by the two methods was apparent.

Protein and transcript detection

Immunofluorescent detection of proteins on cryosections was carried

Table 1

Construct	Activity	Assays
PMiwIII-caBMPR-Ib	+++++	1,2,3,5,6
pCAGGS-caBMPR-Ia	++++	1,2,4,5,6
RCAS-caBMPR-Ib	++/–*	1,2,3
pCMV-caBMPR-Ib	+	1,2,4,5

Assays: (1) induction of Msx protein expression in its endogenous domain; (2) induction of Msx protein expression in ventral cells; (3) induction of apoptosis (measured by TUNEL), resulting in altered neural tube morphology; (4) generation of a morphological change similar to that in assay 3; (5) expression of construct transcript (higher level of expression correlates with higher construct activity); (6) higher activity of caBMPR-Ib versus caBMPR-Ia was also seen in other tissues (Zhou, 1995).

*Activity in infected cells varied according to the timing of infection and viral spread.

out using standard methods (Yamada et al., 1993). The following antibodies were obtained from the Developmental Studies Hybridoma Bank (the Developmental Studies Hybridoma Bank was developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242): AMV viral gag protein (Potts et al., 1987), En1 (Ericson et al., 1997b), Isl1 (Ericson et al., 1992), Lim1/2 (Tsuchida et al., 1994), Msx (Liem et al., 1995), Pax6 (Ericson et al., 1997b) and Pax7 (Ericson et al., 1996). Dbx1, Dbx2, Evx1 (Pierani et al., 1999), and LH2A/B (Lee et al., 1998) have been described previously and were kindly provided by Tom Jessell. Mash1 (Horton et al., 1999) and Math1 (Helms and Johnson, 1998) were kindly provided by Jane Johnson. Species appropriate secondary antibodies conjugated to either FITC or Cy3 were obtained from Jackson ImmunoResearch.

In situ hybridization on cryosections was performed using established procedures (Holmes and Niswander, 2001). Probes for Ngn1 and Ngn2 (Perez et al., 1999) were a gift from D. Anderson. Probes for Msx1 and Msx2 (Chan-Thomas et al., 1993) were a gift from B. Robert.

RESULTS

An activated BMPR was expressed in the developing chicken neural tube after neural tube closure, either by infection with a modified retrovirus (RCAS; transfected cells were identified by expression of a viral protein; see Fig. 1A) or via

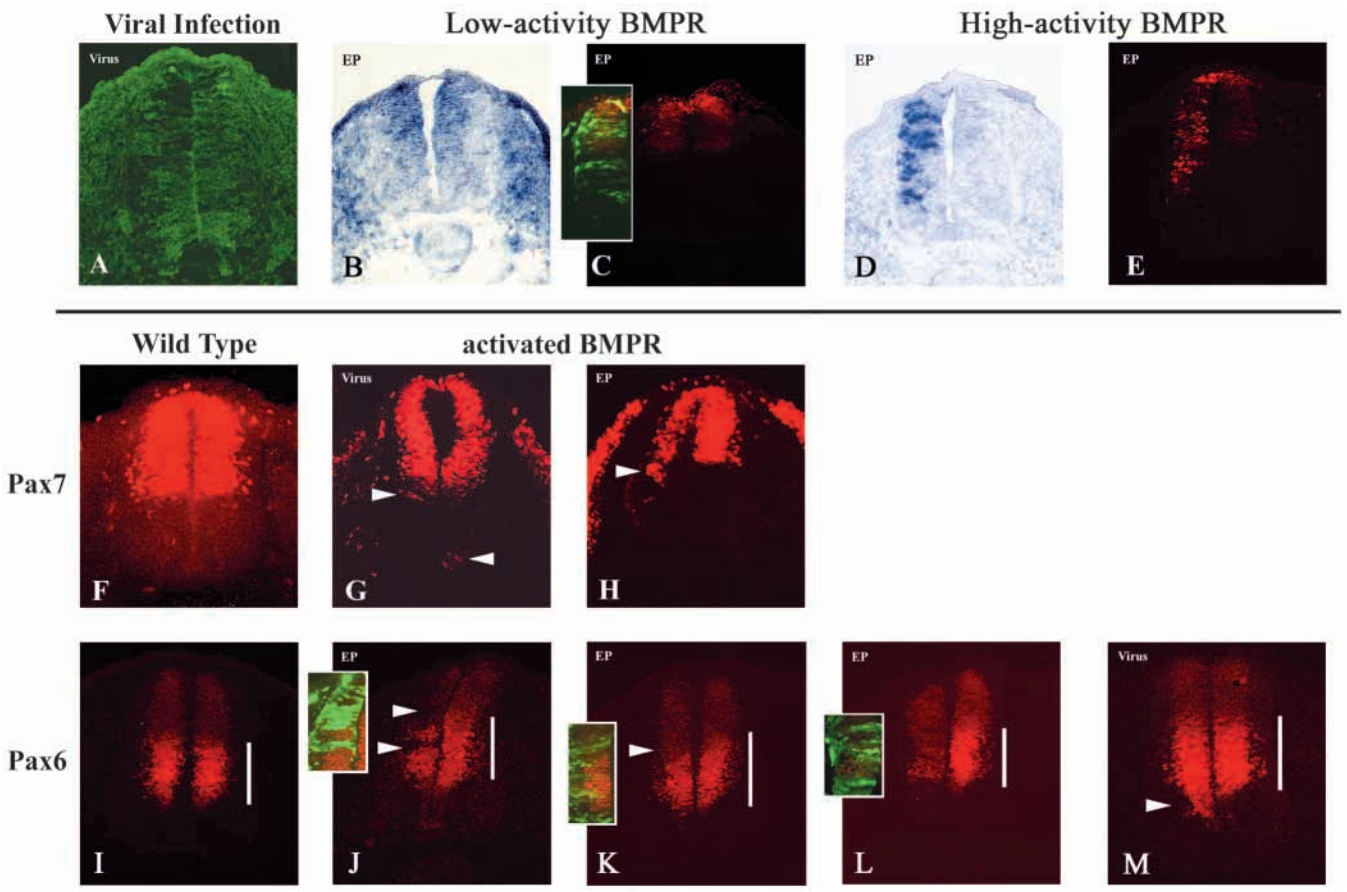

Fig. 1. BMP signaling regulates Pax gene expression in dorsal and intermediate domains. (A) Identification of virally infected cells by immunofluorescence using an antibody to the viral gag protein. (B-E) Electroporation constructs generate different levels of BMP signaling. Electroporations into the left side of the neural tube included a GFP expression plasmid to mark transfected cells. (B,C) Low levels of activated BMPR expression generate weak BMP target gene activation. (B) Expression of activated BMPR-Ib driven by the EGFP vector is barely detectable over endogenous expression of BMPR-Ib (stage 24). An alternate section (C) shows correspondingly weak activation of Msx protein expression, a target of BMP signaling. Inset shows GFP, which labels transfected cells, indicating that the construct is present. (D,E) High levels of activated BMPR expression generate strong BMP target gene activation. (D) Expression of activated BMPR-Ib driven by pMiW-III vector results in expression readily detected above the endogenous expression pattern (stage 24 embryo, detection reaction stopped prior to clear detection of endogenous BMPR-Ib expression). This results in a correspondingly robust MSX response in alternate sections (E). (F-H) Pax7 expression is activated by BMP signaling. (F) Wild-type Pax7 expression in a stage 23 embryo. Ectopic Pax7 expression (arrowheads) is apparent in virally infected (G, stage 24) and electroporated (H, stage 24) embryos. The unusual morphology in H was caused by apoptosis (see Materials and Methods). (I-M) Expression of Pax6 is regulated by BMP signaling. The vertical bar denotes the normal region of high-level Pax6 expression in the intermediate region of the neural tube. (I) Wild-type Pax6 expression in a stage 24 sample. (J) Pax6 expression is repressed by high levels of BMP signaling (arrowheads; inset shows that Pax6 repression is limited to transfected cells). High signaling was generated by electroporation of pMiWIII-activated BMPR-Ib; sample is stage 24. (K,L) High level expression of Pax6 in intermediate cells is downregulated by moderate levels of BMP signaling. Moderate signaling was generated by electroporation of pCAGGS-activated BMPR-Ia; sample is stage 24, insets show correlation of alterations with transfected cells. (M) Ventral Pax6 expression is upregulated in response to BMP signaling (arrowhead; viral infection, stage 23).

electroporation of expression plasmids (transfected cells were marked by co-electroporation of a GFP expression plasmid; see inset in Fig. 1C). The identification of transfected cells indicated that changes in gene expression generated by the activated receptor appear to be cell autonomous (see insets of all electroporated and some viral samples). No qualitative difference between receptors Ia and Ib was apparent; however, quantitative differences could be elicited depending on the choice of receptor and vector used. Accordingly, for some experiments we have generated different levels of BMP signaling as indicated by Table 1 and Fig. 1B-E. Low levels of BMP signaling were generated using a construct that drove weak construct expression (Fig. 1B; correspondingly weak activation of BMP target gene Msx shown in Fig. 1C). High levels of signaling were generated using a stronger expression vector (Fig. 1D; extensive activation of Msx shown in Fig. 1E). Transfection of any of these constructs did not appear to alter the pattern of proliferation in undifferentiated neural cells (data not shown). Although the strongest two constructs could induce apoptosis in neural cells, the apoptosis was unlocalized (data not shown), indicating that alterations in patterning were not mediated by selective cell death. Samples shown here did not have a morphology consistent with significant apoptosis (excepting Fig. 1H).

BMP signaling regulates Pax gene expression

Some of the earliest indicators of dorsoventral patterning in the spinal neural tube are genes of the Pax family (Chalepakis et al., 1993). In wild-type embryos, expression of Pax7 and the related Pax3 gene is restricted to dorsal cells, prior to closure of the neural tube, at least partly due to repression by SHH (Goulding et al., 1993; Hynes et al., 2000; Liem et al., 1995). At stage 23, expression of Pax7 in untransfected embryos occurs only in cells within the dorsal half of the neural tube (Fig. 1F). As seen in Fig. 1G,H, expression of activated BMPR results in the expression of Pax7 in ventral neural tissue. Ventral Pax7 expression occurred most frequently in those cells in proximity to the normal expression boundary, although $Pax7^+$ cells were detected further ventrally, including in close proximity to the floorplate (arrowheads in Fig. 1G). In electroporated samples, ventral expression of Pax7 was more frequent and robust (Fig. 1H). These data indicate that BMP signaling can promote the expression of Pax7 in ventral cells, but that activation may be limited in embryos with lower levels of BMP signaling. This limited activation may be due to reduced competence of ventral cells to express Pax7, or the presence of other factors, such as SHH, that repress Pax7 expression.

The regulation of Pax6 expression is more complex. Pax6 mRNA is first detectable immediately after neural tube closure (Ericson et al., 1997b; Walther and Gruss, 1991). Ventrally, Pax6 has been shown to respond to specific levels of *Shh* signaling (Ericson et al., 1997b), being repressed near the floor plate by high levels of SHH and activated in the intermediate neural tube at lower levels of SHH. Dorsally, Pax6 is expressed at reduced levels, except in the roofplate, where no protein is detectable at the stages examined here (Fig. 1). Studies in neural explants suggest that the reduced expression of Pax6 in dorsal tissues might be mediated by activin signaling (Pituello et al., 1995).

We have found that BMPs regulate Pax6 expression, and that levels of BMP signaling activity may also play a role in setting expression boundaries of this gene. In embryos with the highest levels of BMP signaling (those electroporated with activated BMPR-Ib), expression of Pax6 was silenced in all cells expressing the construct (Fig. 1J). This suggests that high levels of BMP signaling may also be responsible for the repression of Pax6 expression in roofplate cells. In embryos electroporated with activated BMPR-Ia (which generates lower levels of BMP signaling activity) or low concentrations of BMPR-Ib, Pax6 expression was retained in transfected cells, but cells in the intermediate region of the neural tube had reduced levels of Pax6 expression (Fig. 1K,L). In samples with a moderate number of transfected cells, the normal dorsal border of high-level Pax6 expression is shifted ventrally (arrowhead in Fig. 1K). In more thoroughly transfected embryos, Pax6 was expressed at a uniformly low level, similar to that normally seen in dorsal cells (Fig. 1L). These data suggest that BMPs may act to set the dorsal border of the high-level Pax6 expression.

In the above samples, as well as virally infected samples, a third effect of BMP signaling on Pax6 expression was apparent. As shown in Fig. 1M, cells ventral to the high-level Pax6 expression domain expressed increased levels of Pax6 protein in response to BMP signaling. The reduced expression of Pax6 in this region is thought to be necessary for the proper generation of motoneurons (Briscoe et al., 2000); we have found that motoneuron populations are reduced in embryos expressing activated BMPR, consistent with the results of Basler et al. (Basler et al., 1993). Thus, it appears that levels of BMP signaling regulate many borders of expression of Pax6 in the spinal neural tube.

BMP signaling activates dorsal regulators and represses intermediate regulators

BMP signaling activates Msx expression

The homeobox proteins of the Msx gene family are also expressed early in neural tube development. In many tissues, BMP signaling activates Msx expression (Bei and Maas, 1998; Graham et al., 1994; Maeda et al., 1997; Pizette and Niswander, 1999; Suzuki et al., 1997), leading us to examine the expression of these genes in samples expressing activated BMPR. In mice, the Msx gene family consists of three members. In the mouse spinal neural tube, Msx1 and Msx2 are expressed in the roofplate and its overlying mesenchyme and ectoderm, beginning at times similar to the Pax genes. Msx3 is expressed in a broader region that encompasses the dorsal third of the neural tube (Shimeld et al., 1996; Wang et al., 1996). In the chicken spinal neural tube, Msx protein expression follows a similar time course, but there are differences in expression patterns. Msx2 RNA is expressed in the roofplate (Fig. 2A), while Msx1 RNA is expressed in a manner reminiscent of murine Msx3 (Chan-Thomas et al., 1993; Liem et al., 1995).

Previous reports have shown that BMPs can induce Msx expression in cultured neural tube explants (Liem et al., 1995; Shimeld et al., 1996). Fig. 2A-D shows that activated BMPR causes a similar activation in the intact neural tube. Electroporation of activated BMPR results in expression of Msx2 in all regions of the neural tube, including ventral cells (Fig. 2B). Although only cells of the roofplate normally express Msx2, the ectopic expression does not appear to reflect the

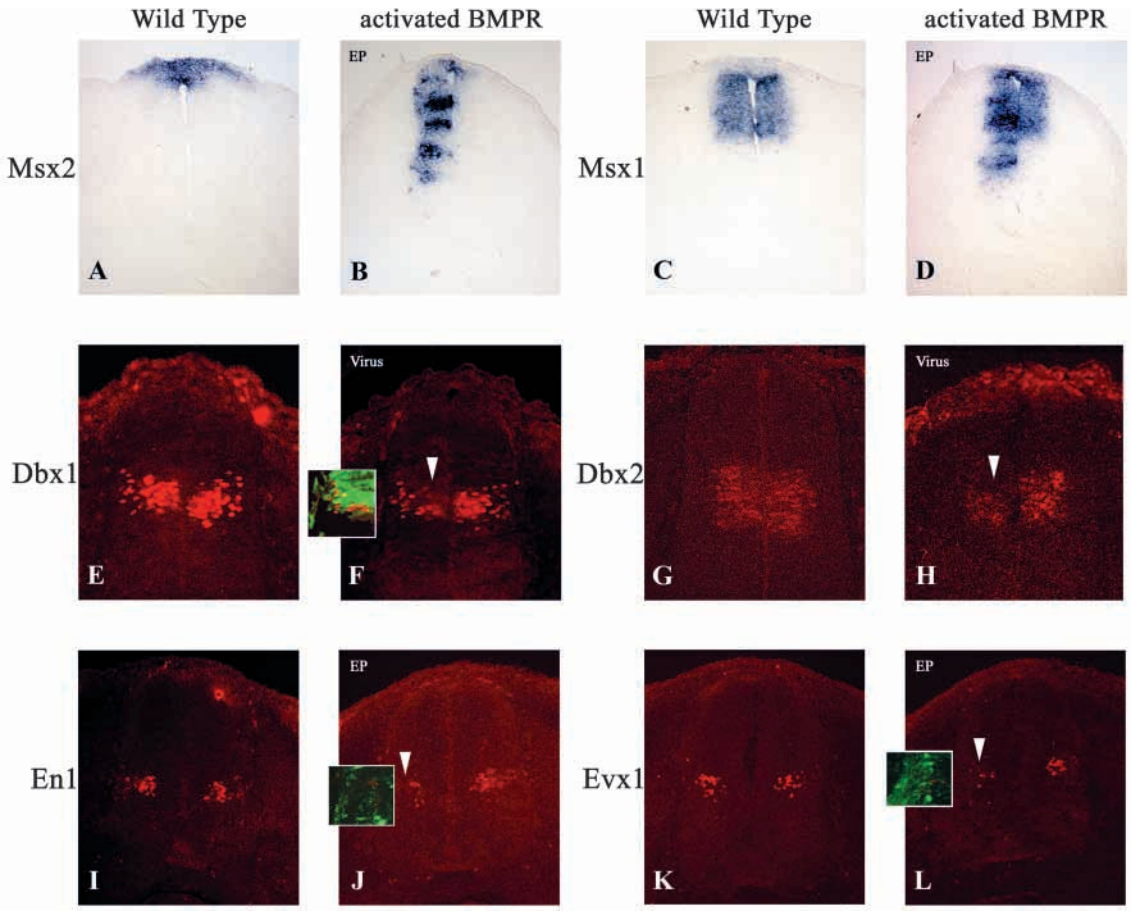

Fig. 2. BMP signaling regulates the expression of homeobox genes in the neural tube. (A-D) Expression of Msx genes is upregulated by BMP signaling. (A,B) Msx2 RNA expression. A stage 24 wild-type sample. (B) Expanded Msx2 expression following electroporation of activated BMPR-Ib (stage 23). (C,D) Msx1 RNA expression. (C) Stage 24 wild-type embryo. (D) Upregulation of Msx1 expression in an electroporated embryo expressing activated BMPR-Ib (stage 23). Expression is detected in the ventral neural tube and at higher levels within the endogenous expression domain. (E-H) Dbx protein expression is repressed by BMP signaling. (E,F) Dbx1 protein expression. (E) Stage 23 wild-type sample. (F) Cells expressing activated BMPR cease to express Dbx1 (stage 23, viral infection; arrowhead denotes region of BMP-driven repression). Inset shows viral infection in green; the absence of Dbx1 correlates with viral infection. (G,H) Dbx2 protein expression. (G) Stage 24 wild-type sample. (H) Repression of Dbx2 expression in a stage 23 sample expressing activated BMPR-Ib following viral infection. Arrowhead indicates a group of cells that no longer express Dbx2. (I-L) Differentiated cell types of the intermediate neural tube are altered in response to BMP signaling. (I,J) En1 protein expression, which marks the interneurons derived from the ventral-most Dbx2-expressing cells. (I) Wild-type expression in a stage 24 sample. (J) Arrowhead indicates that this population is greatly reduced in samples expressing activated BMPR (stage 24, electroporation). Inset shows that the absence of En1 expression correlates with extensive transfection (marked by GFP). (K,L) Evx protein expression, which marks the interneurons derived from $Dbx1^+$, $Dbx2^+$, $Pax7^-$ cells. (K) Wild-type expression in a stage 24 sample. (L) Arrowhead indicates that this population is greatly reduced in samples expressing activated BMPR (stage 24, electroporation). GFP fluorescence indicates extensive transfection of cells in the region that would normally express Evx.

generation of ectopic roofplate tissue, as cells within these regions continue to express markers of other neural fates (i.e. LH2A/B, data not shown), and display normal morphology and migration. Msx1 is also activated in cells ventral to its normal border of expression (Fig. 2D). In cells where Msx1 is normally expressed, activated BMPR appears to increase the levels of Msx1 transcript, as in situ hybridization analysis revealed groups of cells with darker staining than normal within the dorsal third of the neural tube (Fig. 2D). Similar results were obtained with an antibody that detects both Msx1 and Msx2 (Fig. 3A,B and data not shown). In the presence of activated BMPR, high levels of Msx protein detected outside of the roofplate are likely to reflect both the activation of Msx2 and increased or ectopic Msx1 expression. Combined with the activation of Pax7 described above, our results indicate that markers of dorsal cell fates can be activated in ventral neural cells by BMP signaling.

BMP signaling represses Dbx protein expression

The Dbx homeodomain proteins are expressed in nested domains in the intermediate region of the neural tube (Pierani et al., 1999) (chicken Dbx2 is also known as ChoxE) and are necessary for the development of specific classes of interneurons. In explant experiments, their expression is activated by retinoic acid and low concentrations of SHH and repressed by BMPs, suggesting a similar regulation in the neural tube (Pierani et al., 1999). We have found that BMP signaling represses Dbx protein expression in intact neural tissue. Fig. 2F,H show samples infected with activated BMPR;

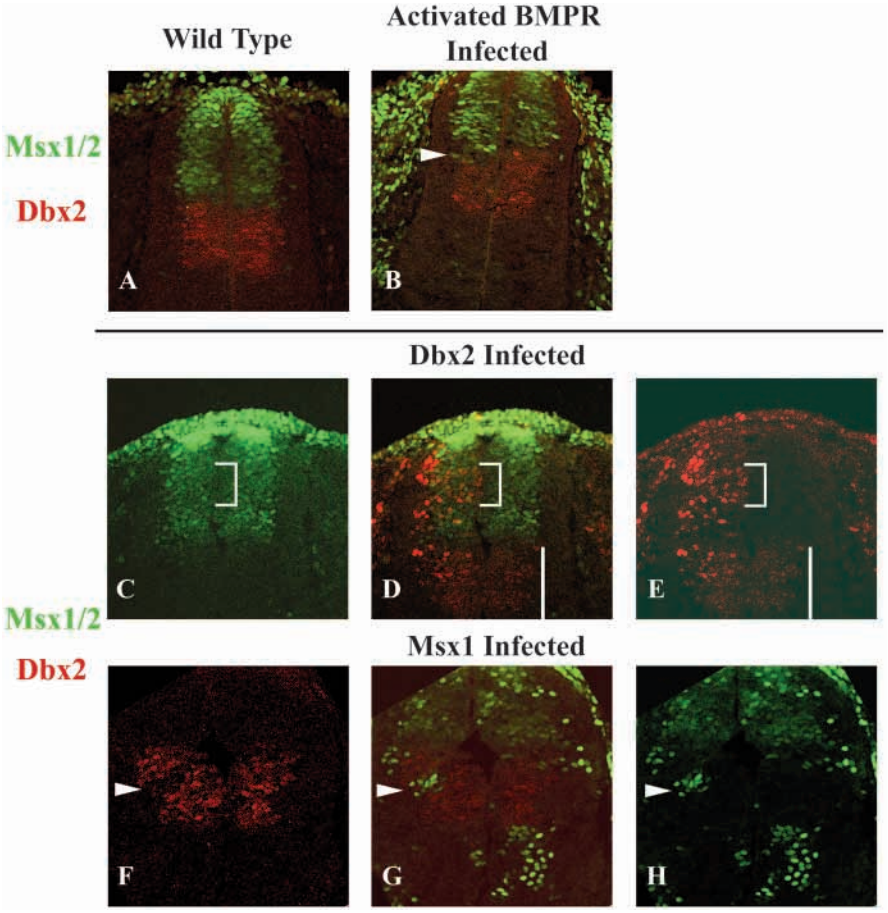

Fig. 3. Msx1 mediates the repression of Dbx2 by BMPs. (A,B) Dbx2 and Msx1 expression domains are juxtaposed. (A) Expression of Dbx2 and Msx1 in a stage 24 wild-type sample. Confocal microscopy reveals that cells do not co-express these proteins. (B) The mutually exclusive expression of these proteins is retained in samples expressing activated BMPR (viral infection, stage 24). Arrowhead indicates cells expressing Msx1 ventral to its normal expression border. Confocal microscopy reveals that these cells do not express Dbx2. (C-E) Mis-expression of Dbx2 does not affect Msx1 expression. Samples were generated by electroporation of viral DNA that drives Dbx2 expression and analyzed at stage 23. Brackets indicate a region of cells expressing Dbx2 within the normal Msx1 domain; bars represent endogenous Dbx2 expression domain. Virally infected cells express Dbx2 at levels equal to or higher than those in the endogenous domain. (C) Expression of Msx1 is unaltered by ectopic expression Dbx2. Co-expressing cells are apparent in D; E shows that many cells in the endogenous Msx1 domain are expressing Dbx2. (F,G) Mis-expression of Msx1 represses Dbx2 expression. Embryos were injected with an Msx1-expressing virus and analyzed at stage 24. Arrowhead denotes a region of ectopic Msx1 expression. (F) Gaps in the Dbx2 expression domain are apparent in the Msx1-infected samples. (G,H) These gaps correspond precisely with those cells that ectopically express Msx1.

arrowheads indicate regions of reduced Dbx1 and Dbx2 protein expression. These data show that BMP signaling represses the expression of both of these proteins and suggests that it normally acts to set the dorsal border of their expression domains.

BMP signaling alters the generation of intermediate cell types

The BMP regulation of homeobox genes shown above suggested that embryos expressing activated BMPR would have reduced populations of some classes of intermediate interneurons. $Dbx2^+$ cells ventral to the Pax7 expression boundary generate two types of interneurons: those cells that express Dbx1 generate Evx1-expressing neurons, while the more ventral, $Dbx1^-$ population generates neurons that express En1 (Briscoe et al., 2000; Pierani et al., 1999). We have found that the generation of both Evx1- and En1-expressing interneurons is severely reduced in embryos that express activated BMPR (Fig. 2J,L). It is not clear whether the primary cause of their reduction is the loss of Dbx protein expression or the expanded expression of Pax7.

Msx1 mediates the BMP driven repression of Dbx2

We noted that the ventral boundary of Msx1 expression corresponded to the dorsal boundary of Dbx2 expression. Confocal microscopy revealed that these proteins were expressed in a mutually exclusive manner (Fig. 3A). This relationship was retained in embryos where Msx1 expression was expanded because of the expression of activated BMPR (arrowhead in Fig. 3B), suggesting a regulatory interaction between these genes. To examine potential interactions between Msx1 and Dbx2, we used RCAS vectors to express these genes in the developing neural tube. Fig. 3C-E shows that, even in the presence of high levels of Dbx2 (compare ectopic expression with endogenous expression), Msx1 expression remains uniform in the dorsal third of the neural tube, indicating that Dbx2 is not capable of regulating Msx1 expression. By contrast, when Msx1 expression occurs in cells within the normal domain of Dbx2 expression, gaps appear in the otherwise uniform field of Dbx2 expression (Fig. 3F-H). Fig. 3F,G (note arrowheads) shows that those cells that fail to express Dbx2 correspond precisely to those cells ectopically expressing Msx1. These data strongly suggest that BMP signaling acts through Msx1 to repress Dbx2 expression.

Within the dorsal neural tube, the expression of developmental regulators responds to discrete levels of BMP signaling

Several genes of the bHLH family of transcription factors are expressed in well-defined domains within the Msx1 expression domain in the dorsal third of the neural tube, including the acheate-scute homolog Cash1 (Guillemot and Joyner, 1993), the chicken atonal homolog 1, Cath1 (Lee et al., 1998), neurogenin 1 (Ngn1), and neurogenin 2 (Ngn2) (Ma et al., 1996; Sommer et al., 1996). We have observed changes in the expression of all of these genes in response to BMP signaling;

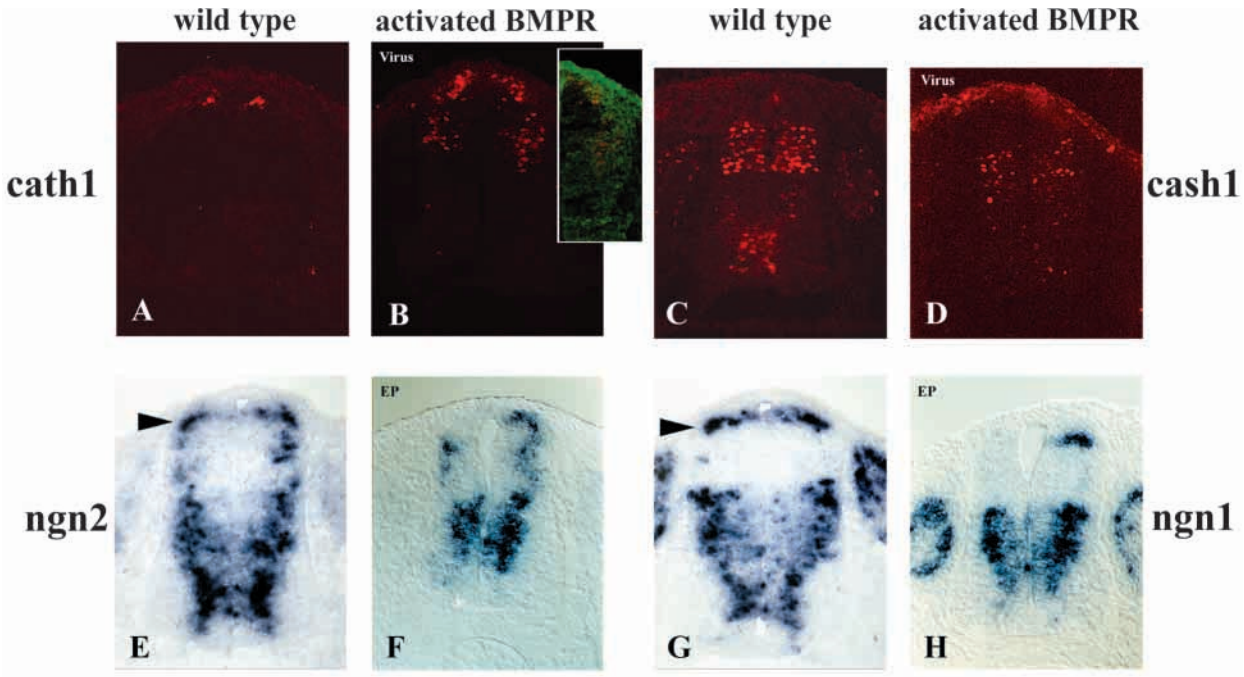

Fig. 4. Expression of activated BMPR alters the expression of bHLH proteins in the dorsal neural tube. (A,B) Cath1 expression expands ventrally in response to BMP signaling. (A) Wild-type expression of Cath1 protein occurs in cells immediately ventral to the roofplate (stage 23 sample). (B) Expression of Cath1 expands dramatically in response to expression of activated BMPR (stage 24, viral infection). Note that the expanded expression remains restricted to dorsal neural tissue. Inset shows viral antigen expression, indicating infected cells; expanded Cath1 correlates with areas of extensive viral infection. (C,D) Cash1 expression is repressed by BMP signaling. (C) Wild-type expression of Cash1 protein includes a broad band of cells in the dorsal neural tube (stage 24). (D) The Cash1 domain is reduced in samples expressing activated BMPR (stage 23 viral infection). (E,F) Dorsal Ngn2 expression is reduced by BMP signaling. (E) Wild-type expression of Ngn2 RNA is found in ventricular and subventricular cells in the dorsal half of the neural tube, as well as a large population of ventral cells (stage 24; arrowhead indicates dorsal expression domain). (F) Expression of Ngn2 in most dorsal cells is extinguished by the expression of activated BMPR (stage 24 electroporation); ventral expression remains unaffected. (G,H) Dorsal expression of Ngn1 is repressed by BMP signaling. (G) At stage 24, Ngn1 RNA is expressed by cells of the dorsal root ganglia (DRG), proliferating ventral cells and a narrow band of cells in the dorsal neural tube (arrowhead). (H) Ngn1 expression in the dorsal neural tube is absent in samples expressing activated BMPR (stage 24 electroporation), while ventral and DRG expression remains normal.

combined with the results of others, these results indicate that BMPs act at specific levels of activity to pattern the dorsal neural tube.

BMP signaling activates cath1 throughout the dorsal neural tube

The *Cath1* gene is expressed by the cells of the ventricular zone that reside between the roofplate and an Ngn1-expressing dorsal cell population (Lee et al., 1998). Cath1 protein expression has been shown be activated by BMPs in explants and partially dependent upon the BMP relative GDF7 in mice. In samples expressing activated BMPR, Cath1 is activated in most cells of the dorsal neural tube (Fig. 4B); no expression was detected in ventral cells. This result suggests that the activated BMPR converted dorsal neural cells to a more dorsal fate, marked by Cath1 expression.

BMP-induced expansion of Cath1 correlates with the repression of other bHLH genes

The Cash1 protein is expressed in a broad band of cells in the dorsal neural tube with a dorsal border several cell diameters ventral to the roofplate; levels of Cash1 protein expression appear to vary within this domain (Fig. 4C). In experiments where the roof plate was ablated, Lee et al. (Lee et al., 2000) found that the mouse homolog of this gene was expressed uniformly throughout the dorsal third of the neural tube, including the roofplate. This led these authors to conclude that BMP-mediated repression was responsible for setting the dorsal expression border of this gene. Our data support this conclusion; in response to BMP signaling, we have found that dorsal expression of Cash1 is repressed (Fig. 4D), although some expression was always retained near its ventral border. Combined with the previous results, these data imply that the dorsal border of Cash1 expression is set at a threshold of BMP signaling activity.

The bHLH gene Ngn2 is expressed by most ventral cells. In dorsal cells, Ngn2 transcripts are detected in a narrow band of cells in the ventricular zone, similar to those that express Ngn1, as well as a larger population of subventricular cells (Perez et al., 1999). Fig. 4F shows that BMP signaling represses the dorsal expression of Ngn2. Some dorsal cells retained normal expression of Ngn2 in all samples examined. As fluorescence from the co-electroporated GFP does not survive in situ analysis, it is not clear whether this reflects a tolerance of high levels of BMP signaling by a subdomain of Ngn2-expressing cells or the lack of uniform activated BMPR expression in the cells of the dorsal neural tube. The related gene *Ngn1* is also expressed broadly in ventral cells; additionally, ngn1 transcripts

are expressed in a narrow band of cells near the roofplate (Fig. 4G). As shown in Fig. 4H, expression of activated BMPR also resulted in the loss of the dorsal Ngn1 expression domain; as with Ngn2, ventral expression was unaffected.

Ngn1 expression is regulated by thresholds of BMP signaling

In the roofplate ablation experiments (Lee et al., 2000), absence of BMP signaling resulted in the loss of the dorsal domain of Ngn1. As Ngn1 both requires BMP signaling for its expression and is repressed by high levels of BMP signaling, BMPs may regulate Ngn1 expression at specific thresholds of activity. In order to test this, we used a construct that expresses extremely low levels of activated BMPR (see Materials and Methods for a description). If BMP signaling is also capable of activating Ngn1, the low level of BMP signaling generated by this construct, when combined with endogenous BMP signaling from the roofplate, should be capable of activating Ngn1 transcription in cells ventral to its endogenous expression domain. As shown in Fig. 5A, these samples frequently had ventral expansion of Ngn1 expression on the electroporated side of the neural tube. This expansion correlated with an increase in the generation of terminally differentiated neurons that arise from cells near the endogenous ngn1 domain (Fig. 5D, discussed below). These results suggest that genes within the dorsal neural tube respond to specific thresholds of BMP signaling.

BMP regulation of dorsal patterning genes controls the generation of populations of differentiated neurons

Previous work has identified several populations of interneurons that are derived from the cells of the dorsal neural tube. Cath1-expressing cells give rise to terminally differentiated cells that express either LH2A or LH2B and migrate to ventral positions (Helms and Johnson, 1998; Liem et al., 1997). More ventral cell types generate differentiated cells that express Islet1 (Isl1) or Lim1 and Lim2, which also migrate to ventral positions in the neural tube (Fig. 5D,G). We have found that altering levels of BMP signaling dramatically alters the generation of these classes of dorsal neurons.

As expression of ventral markers is replaced by Cath1 expression in samples expressing activated BMPR, we sought to determine if there was a corresponding increase in the generation of Cath1 derivatives, which express LH2A or LH2B. Normal expression of LH2A/B is shown in Fig. 5B. As would be predicted, in samples expressing activated BMPR, cells expressing LH2A/B develop throughout the dorsal neural tube (arrowhead in Fig. 5C). As with Cath1, the expanded expression is limited to cells in the dorsal third of the neural tube.

Cells expressing Lim1/2 are generated at various locations along the dorsoventral axis of the neural tube. In addition to ventral populations, terminally differentiated neurons arising from two dorsal locations express this marker (bars in Fig. 5D). Expression of activated BMPR in dorsal cells frequently reduced and occasionally eliminated these dorsal populations (arrowhead in Fig. 5E). Cells generated at more ventral locations continue to express these proteins, and some Lim1/2^{+} cells were generated at ectopic, ventral positions within the ventricular zone of undifferentiated cells. The significance of the ectopic generation of Lim1/2 cells is unclear at this time.

The Isl1 protein is expressed by a variety of neural cell types, including neural crest derivatives in the dorsal root ganglia, differentiated motoneurons and a population of dorsal interneurons (Fig. 5G, dorsal interneurons indicated by a bar) (Tsuchida et al., 1994). In samples electroporated with activated BMPR, this population was reduced or absent, depending on the extent of activated BMPR expression (arrowhead in Fig. 5H). As Isl1 cells arise from cells near where Ngn1 is expressed, we decided to examine Isl1 expression in samples electroporated with the low-level activated BMPR expression construct that was shown to expand Ngn1 expression. In many samples expressing this construct, the number of Isl1-expressing dorsal cells was increased. In addition, these cells arise from a broader progenitor domain (bracket in Fig. 5F). Taken together, these data indicate that BMP-driven changes in progenitor cell markers correlate with changes in differentiated cell populations.

These results show that BMP driven changes in gene expression in undifferentiated neurons reflect a change in the identity of a cell. Thus, within the Msx1 expression domain, BMP signaling acts to dorsalize both immature and differentiated neurons. By generating specific cell types at thresholds of activity, the BMPs ultimately regulate the diversity and numbers of dorsal neurons.

DISCUSSION

Our data suggest that BMPs provide patterning information in the dorsal and intermediate regions of the neural tube. In the dorsal neural tube, BMP signaling regulates a series of bHLH proteins in order to generate several distinct populations of neural progenitor cells; in intermediate cells, BMP signaling performs the same function via the regulation of homeodomain proteins. In both cases, the generation of these populations is necessary for generating a diverse population of differentiated neurons. BMPs appear to act in a cell autonomous manner in performing this function; although we cannot rule out a role for secondary signals, the consistent correlation of patterning alterations with transfected cells and absence of changes in neighboring cells argues against a significant role for other signals. The data also indicate that BMPs act instructively, as changes in patterning were not accompanied by regional changes in apoptosis or proliferation.

We have seen only quantitative differences between constructs that express BMPR-Ia and BMPR-Ib. This differs from the results of Panchision et al. (Panchision et al., 2001), who expressed similar constructs in a mouse transgenic system. Although data from both agree on a role for BMPs in dorsalizing neural tissue, Panchision et. al. suggest distinct roles for the two BMP receptors. Their data, however, were generated partly in mouse transgenics and partly in cultured neural stem cells. There are likely to be significant differences in the cells used, timing of construct expression, and levels of construct expression between these systems and the in ovo electroporation we used, which may account for the different results.

Our data, however, are consistent with results obtained with in vitro explants of neural tissue, but expand on them in significant ways. It is clear that other signaling pathways are necessary for proper development of the neural tube; neural

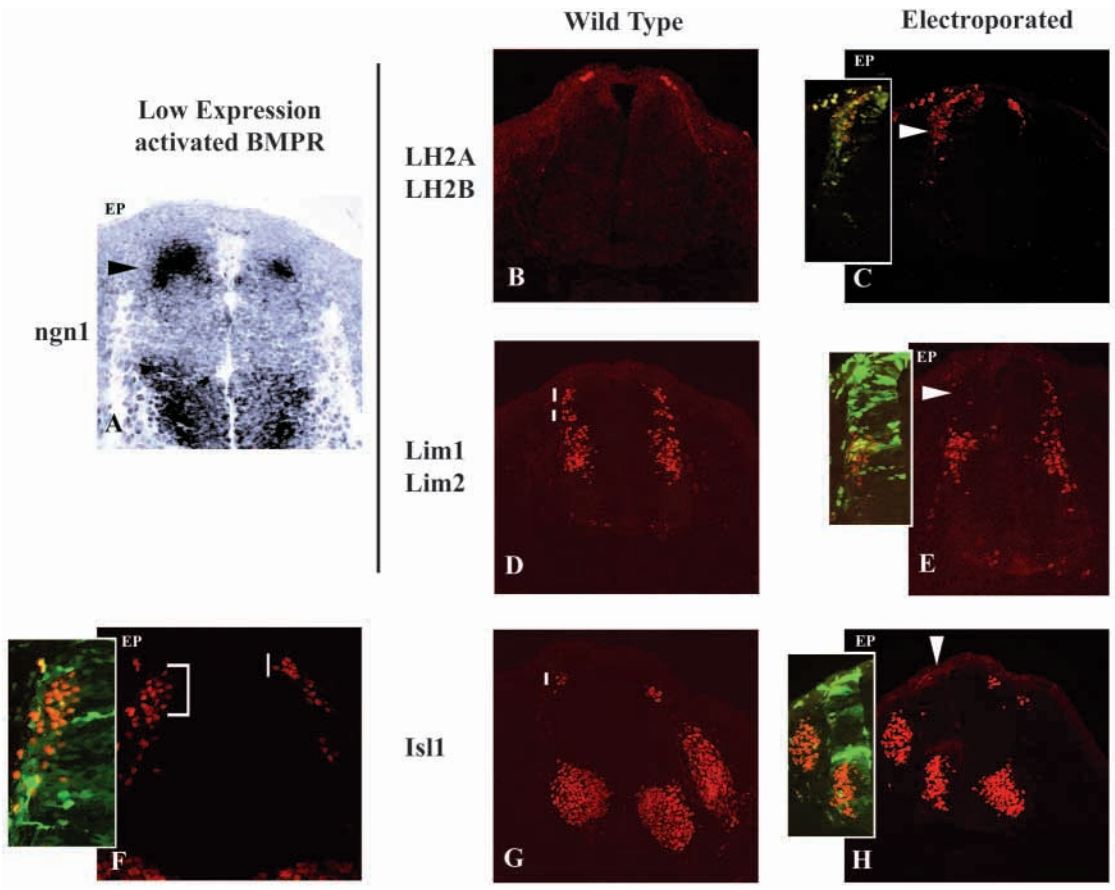

Fig. 5. BMP signaling regulates the generation of terminally differentiated dorsal interneurons. (A) Ngn1 RNA expression is activated at a threshold of BMP signaling. Ngn1 is activated by low levels of BMP signaling. A construct that weakly expresses activated BMPR-Ib (EGFP vector) results in an increase in Ngn1-expressing cells, apparently by activating it in cells ventral to its normal domain (arrowhead, stage 24). (B,C) LH2A/B-expressing cells are generated throughout the dorsal neural tube in response to high levels of BMP signaling. (B) In stage 23 wild-type samples, LH2A/B-expressing cells are generated by those cells that express Cath1 and migrate ventrally. (C) In response to high level BMP signaling, LH2A/B$^+$ cells are generated throughout the dorsal neural tube (arrowhead, stage 24). Inset shows co-localization of GFP and LH2A/B expression on the transfected side of the neural tube. (D,E) High levels of BMP signaling block the development of dorsal Lim1/2$^+$ cells. (D) In wild-type samples, Lim1/2$^+$ cells are produced in two locations within the dorsal neural tube (bars; stage 23). (E) Expression of activated BMPR reduces or eliminates the generation of the two dorsal populations of Lim1/2-expressing cells (arrowhead denotes normal region of their generation, stage 23). Inset shows GFP expression, indicating transfected cells. Note that suppression of Lim1/2 expression is specific to dorsal cells, as intermediate expression continues in transfected cells. (F-H) Isl1-expressing cells are formed within a narrow range of BMP signaling activity. (F) Samples with slight increases in BMP signaling (generated with the same vector as in A, EGFP-activated BMPR-Ib) generate Isl1-expressing cells over a broader region of the dorsal neural tube (bracketed; bar indicates endogenous domain, late stage 24). Inset shows GFP label of transfected cells. Note that Isl1 expansion is restricted to the region near its normal expression domain, despite extensive transfection. (G) In stage 24 wild-type samples, Isl1 expression marks a small population of dorsal interneurons (bar) as well as the DRG and motoneurons.(H) High levels of BMP signaling, however, abolish the generation of Isl1$^+$ interneurons (arrowhead, stage24). Inset shows that the absence of Isl1 is correlated with the presence of transfected cells marked by GFP.

explants are likely to have altered expression of the components of pathways such as Wnt and Shh (Chiang et al., 1996; Dickinson et al., 1994; Ericson et al., 1995). Additional evidence has suggested that signals arising from non-neural tissues, such as the dorsal ectoderm and somites, are also necessary for neural patterning (Bertrand et al., 2000; Liem et al., 2000; Pierani et al., 1999). Moreover, examination of the effects of BMPs in intact tissue has allowed us to identify regulatory interactions (i.e. Msx1/Dbx2) and regional-specific responses to BMP signaling (i.e. dorsal-specific changes in bHLH expression). Thus, these experiments present a clearer view of the role of BMP signaling in its proper context.

BMPs act in conjunction with *Shh* to set Pax gene expression borders

One of the earliest signs of distinct regional identity in spinal neural tissue is the expression of Pax genes, specifically Pax6 and Pax3/7 (Liem et al., 1995; Pituello et al., 1995). Our data indicate that BMP signaling plays a role in setting the expression boundaries of these Pax genes. We have found that BMP signaling promotes the expression of Pax7 in ventral tissue, both shifting its expression boundary ventrally and causing ectopic Pax7 expression. BMP signaling also appears to regulate Pax6 in a level-dependent manner. High levels of BMP signaling eliminate Pax6 expression; this repression may indicate a role for BMPs in eliminating Pax6 expression in the roofplate.

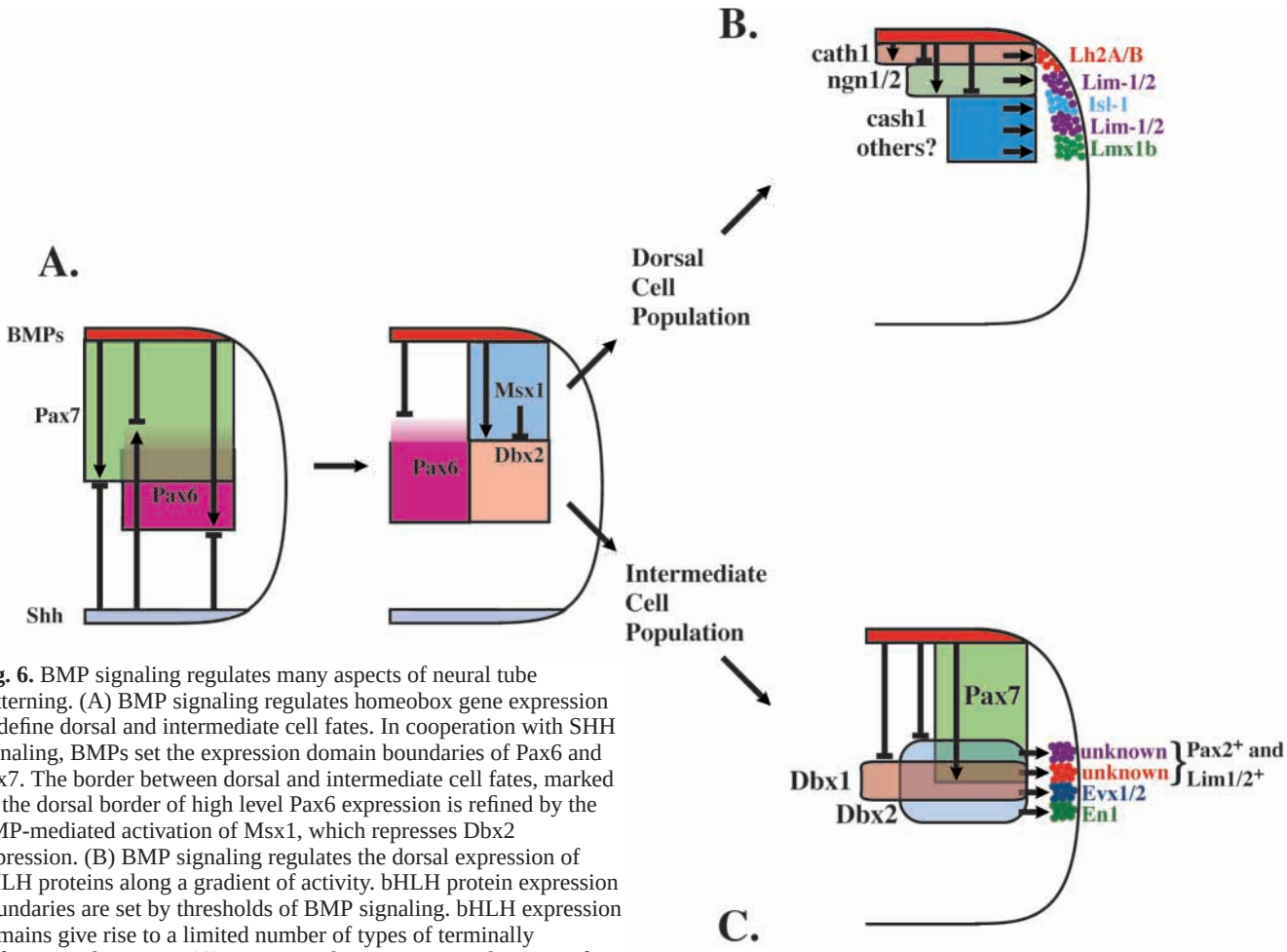

Fig. 6. BMP signaling regulates many aspects of neural tube patterning. (A) BMP signaling regulates homeobox gene expression to define dorsal and intermediate cell fates. In cooperation with SHH signaling, BMPs set the expression domain boundaries of Pax6 and Pax7. The border between dorsal and intermediate cell fates, marked by the dorsal border of high level Pax6 expression is refined by the BMP-mediated activation of Msx1, which represses Dbx2 expression. (B) BMP signaling regulates the dorsal expression of bHLH proteins along a gradient of activity. bHLH protein expression boundaries are set by thresholds of BMP signaling. bHLH expression domains give rise to a limited number of types of terminally differentiated neurons. (C) BMP signaling promotes a diversity of intermediate cell fates. BMP regulation of Pax7 sets a dorsal limit on the generation of Evx1-expressing neurons. BMP regulation of the dorsal border of Dbx1-expressing cells may help divide the $Pax2^+$, $Lim1/2^+$ cells into two distinct progenitor populations.

Moderate levels of BMP signaling shift the border of the intermediate domain of high Pax6 expression ventrally, while in samples with extensive construct expression, the high level expression domain is eliminated entirely. Finally, we have found that low levels of BMP signaling are capable of expanding the intermediate domain of Pax6 expression ventrally.

Previous data had indicated that the Pax genes were also regulated by SHH signaling. In the case of Pax7, the absence of expression in ventral tissues is caused by a SHH-mediated repression (Goulding et al., 1993; Hynes et al., 2000; Liem et al., 1995). Pax6 expression also depends on SHH; high levels of SHH repress Pax6 expression in ventral cells, while lower levels activate Pax6 in the intermediate region of the neural tube (Ericson et al., 1997b). Thus, the majority of Pax expression domain boundaries appear to be set by the combined action of the BMP and SHH signaling pathways (see Fig. 6A). The regulation of Shh-responsive genes by BMPs shown here does not reflect BMP regulation of Shh expression, as Shh transcript remained unaltered in virally infected samples (data not shown). It is possible be that these signaling pathways interact downstream of ligand expression, as it has been found that BMP activity can influence the reception of *Shh* signaling (Liem et al., 2000).

BMPs coordinate the expression of two homeobox proteins and help to generate distinct dorsal and intermediate cell populations

The dorsal border of Pax6 expression is not a sharp boundary such as those that frequently separate distinct cell types, suggesting that other factors may contribute to the delineation of dorsal and intermediate cells. We have found that a sharp border between dorsal and intermediate cell types is generated at this location by the BMP-regulated expression of two homeobox proteins (Msx1 and Dbx2) (Fig. 6A): the dorsal border of Pax6 expression corresponds to both the ventral border of Msx1 and the dorsal border of Dbx2. We have shown that the BMP-mediated activation of Msx1 expression sets a dorsal boundary on Dbx2 expression. As the expression of Dbx2 appears to be necessary for the proper development of several intermediate cell types (Pierani et al., 1999), BMP regulation of Msx1 limits the population of cells that can serve as progenitors for intermediate neurons. In contrast to Dbx2, expression of Msx1 is not sufficient to generate dorsal cell types (compare Fig. 2 with Fig. 4). Thus, Msx1 may only be one of several competence factors that promote dorsal identity. A requirement for multiple factors to generate dorsal competence may explain why misexpression and loss-of-

function mutations in many of the genes expressed in restricted patterns in the neural tube have limited phenotypes in this tissue (Mansouri and Gruss, 1998; Tremblay et al., 1996). Nevertheless, the different responses of bHLH proteins in dorsal and ventral neural tissue suggest that these tissues have been assigned distinct developmental potentials.

BMPs contribute to the generation of a diversity of intermediate neural cell types

In addition to limiting the population of cells that are competent to generate intermediate cell types, BMPs regulate the types of interneurons generated by this Dbx2$^+$ population. Within this cell population, specific cell types are generated by populations defined by overlapping expression of homeobox proteins (Briscoe et al., 2000; Mansouri and Gruss, 1998; Pierani et al., 1999). Cells expressing both Dbx2 and Pax7 give rise to interneurons expressing Pax2 and Lim1/2 (Burrill et al., 1997). Pax7 has also been shown to help set a dorsal limit on the generation of Evx1-expressing interneurons (Mansouri and Gruss, 1998; Pierani et al., 1999). Thus, by regulating the ventral border of Pax7 expression, BMPs promote the generation of the dorsal cells at the expense of more ventral intermediate cell types, such as Evx1- and En1-expressing interneurons. Accordingly, we have seen a reduction in the generation of these ventral cell fates in embryos where BMP signaling is activated in intermediate cells.

As shown in Fig. 6C, Dbx2$^+$ cells ventral to the Pax7 expression border give rise to two cell populations, determined by the presence or absence of the homeobox protein Dbx1. As Dbx1 also has an expression border within the Pax7 expression domain, it is possible that Dbx1 performs a similar function in generating two distinct interneuron populations within the Pax2-expressing population. If this were the case, BMP-mediated regulation of Dbx1 (Pierani et al., 1999) would also be necessary for the proper patterning of other intermediate cell types. We are unaware of the expression of any protein that marks distinct populations within these cells, however, so we have been unable to test this.

BMP ligands control the generation of dorsal interneurons by regulating gene expression at distinct thresholds of activity

In addition to their role in promoting the formation of an Msx1-expressing dorsal progenitor pool, BMPs also act to subdivide this domain into discrete cell populations (see Fig. 6D). Several of the cell types generated by this region depend upon the expression of bHLH proteins (Gowan et al., 2001) that we have been found to be regulated by BMPs. We have found that high levels of BMP signaling activity promote the expression of the most dorsal bHLH protein, Cath1. The expanded domain of Cath1 expression, however, remains restricted to the dorsal neural tube. The broad expression of Cath1 correlates with the repression of other regulators expressed in this region, such as the neurogenins and Cash1. As with Cath1, this regulation is specific to dorsal neural cells; neurogenin expression in ventral cells appears to be unaltered. The restriction of these responses to dorsal cells is consistent with this region having been previously assigned a distinct developmental identity and potential. It is also consistent with findings that the dorsal and ventral regulatory sequences of the neurogenins are physically separated (Simmons et al., 2001).

Our data, when viewed in conjunction with previous data from others, indicate that thresholds of BMP signaling set the expression boundaries of dorsal regulators. We have found that high levels of BMP signaling repress Cash1 in its most dorsal domain, although expression is retained in more ventral cells of this domain. In experiments where the roofplate was genetically ablated and expression of its BMPs abolished, Cash1 expression was expanded dorsally (Lee et al., 2000; Millonig et al., 2000). Thus, it appears that BMP signaling sets the dorsal border of Cash1 expression at a precise level of activity, while expression in more ventral cells is independent of BMP activity. By contrast, dorsal Ngn1 expression is absent both in embryos with high levels of BMP activity and those in which the roofplate is absent and BMPs are not expressed (Lee et al., 2000). This suggests that the dorsal cells that express Ngn1 are formed within a limited range of BMP activity. We have confirmed this suggestion by showing that Ngn1 expression is broadly activated in the dorsal neural tube by low levels of BMP signaling. These results indicate that the BMPs collectively act to regulate genes along a gradient of activity, possibly behaving as morphogens to pattern cells within the dorsal neural tube (Fig. 6D). This function would be consistent with other descriptions of BMPs and other TGFβ superfamily members acting as morphogens during embryonic development (Dale and Jones, 1999; McDowell and Gurdon, 1999; Podos and Ferguson, 1999). The recent finding that the bHLH genes expressed in this region inter-regulate (Gowan et al., 2001) indicates the BMP activity may only have to set the boundary of a limited number of gene(s) in order to generate several distinct cell populations.

The regulation of the dorsally expressed genes by BMP signaling has significant consequences for the generation of differentiated dorsal interneurons. The expanded expression of Cath1 drives the generation of LH2A/B$^+$ cells throughout the dorsal third of the neural tube. The expansion of Lh2A/B$^+$ interneurons appears to come at the expense of other cell fates, as the generation of other classes of dorsal interneurons, marked by Isl1 or Lim1/2, is reduced or eliminated. Thus, the presence of high levels of BMP signaling throughout the dorsal third of the neural tube appears to generate a uniform pool of progenitors and a single type of differentiated neuron in this region. Small increases in BMP signaling in dorsal cells, however, can expand the generation of other cell types in this region (Ngn1 and Isl1, see Fig. 5A,F). This reinforces the suggestion that a gradient of BMP signaling activity is essential for the generation of a diversity of dorsal interneurons (Fig. 6D).

BMP signaling and transcription factor codes

Previous work has shown that homeobox containing proteins play a significant role in the generation of differentiated neural cell types (Briscoe et al., 2000). A homeobox 'code', generated by overlapping and exclusive domains of expression, provides the positional information necessary for generating many classes of neurons at precise locations in the ventral neural tube. We have shown that BMPs contribute to the generation of the homeobox code. BMP regulation of Pax6 expression sets a dorsal limit on the population of cells that give rise to motoneurons. By regulating the ventral boundary of Pax7 expression, BMPs act to limit the ability of cells to adopt ventral fates. BMPs also act to set a dorsal boundary to Dbx2-derived intermediate cell fates via activation of the homeobox

protein Msx1. We also expect that the BMP-regulated dorsal border of Dbx1 expression will play a similar role in subdividing the intermediate neural tube as its ventral border performs (Pierani et al., 1999).

It remains to be determined whether Msx1 plays a role in directly providing positional information to differentiating neurons. It is clear, however, that several types of differentiated neurons arise from the Msx1 expression domain. The division of this domain into distinct progenitor populations depends on the expression of bHLH proteins (Gowan et al., 2001; Lee et al., 1998). Consistent with this, we have found that alterations in the differentiated cells generated by cells within the Msx1 expression domain correlate with changes in the expression of bHLH proteins (Cash1, Ngn1, Ngn2 and Cath1) expressed by undifferentiated cells in this region.

Restricted expression of bHLH proteins, however, does not appear to be the only mechanism for patterning the dorsal neural tube. The cells that express Cash1 appear to give rise to at least three types of differentiated neurons ($Isl1^+$, a second $Lim1/2^+$ population and $Lmx1b^+$), but no currently identified bHLH protein appears to further subdivide the Cash1 expression domain. In addition, any direct relationship between bHLH protein expression and cell fate is restricted to dorsal cells, as most of these bHLH proteins are also broadly expressed in ventral cells. Thus, the activity of any bHLH code may ultimately depend on the homeobox proteins that define the dorsal cell population in which they function.

Modulation of BMP responses

It is clear from these data that the effectors of BMP signaling must interact with other factors to generate the responses seen here. The activation of Msx protein expression by BMP signaling appears to be a general downstream response, as it is seen in a variety of tissues (Pizette and Niswander, 1999; Streit and Stern, 1999; Wang et al., 1999; Yamamoto et al., 2000). Within the neural tube, however, the responses of other genes are more finely regulated. The response of all other markers examined here was restricted to neural cells, even when viral infection spread to surrounding tissues. Within the developing neural tube, responses to BMP signaling were frequently restricted to specific regions (e.g. Cath1 activation in the dorsal neural tube). Thus, at the transcriptional level, the effectors of BMP signaling must be interacting with other regulators of transcription or other signaling pathways.

Previous data have suggested that BMPs may cooperate with the activin members of the TGFβ superfamily, which are also expressed by the roofplate. Experiments using neural explants had previously indicated that several of the effects seen here might also be mediated by activin signaling (Liem et al., 1997; Pituello et al., 1995). We have found, however, that expression of an activated activin receptor (Chang et al., 1997) in vivo is incapable of generating the alterations of gene expression seen here. Although activin signaling expanded the expression of Isl1, the expression of many of the BMP-responsive genes examined here (including Msx, *Pax6*, *Cash1* and *Lh2a*/*b*) was unaltered in embryos expressing this construct (J. T. and L. N., unpublished). Thus, these responses appear to be specific to BMP-mediated signaling.

In addition to their regulation of patterning within the neural tube, it is worthwhile noting that BMPs have also been implicated in earlier stages of neurogenesis, including the generation of neural progenitors from the neurectoderm and the formation of the roofplate and neural crest (Baker and Bronner-Fraser, 1997; Liem et al., 1997; Liem et al., 1995), as well as later events, such as the directing the migration of differentiated dorsal interneurons (Augsburger et al., 1999). Many of these processes overlap temporally (e.g. the generation of the roofplate and neural crest occur while the generation of patterning information is in progress), and thus should be viewed as different responses to the continual presence of BMP signaling. How the response to BMP signaling is redirected to different processes or used for several processes simultaneously also provides an interesting avenue for future study.

Summary

A model for the action of BMP signaling in patterning the neural tube is depicted in Fig. 6. Initially, BMPs act in conjunction with Shh signaling to set expression boundaries for the Pax genes. The Pax6 expression boundary that marks the border between dorsal and intermediate cells is refined by the BMP-mediated activation of Msx1 in dorsal cells, which in turn represses the intermediate marker, Dbx2. This generates two pools of progenitor cells (intermediate and dorsal) with distinct developmental potentials. In intermediate cells, BMPs contribute to the generation of overlapping patterns of homeobox protein expression; their regulation of Dbx1, Dbx2 and Pax7 generates at least three distinct populations of progenitor cells. In dorsal cells, our data suggest that patterning information is provided in part by mutually exclusive expression of members of the bHLH family of proteins. bHLH gene expression is set at specific thresholds of BMP signaling activity, which ultimately results in populations of mature neurons differentiating along a gradient of BMP activity.

The authors acknowledge T. Jessell, J. Johnson, C. Abate-Shen, J. Eggenschwiler and members of their laboratory for advice, reagents and critical reading of this manuscript; and Louise Howe, Cathy Chesnutt and Joan Seoane for assistance with these experiments. Confocal images were obtained with the help of Katia Manova and the staff of the Molecular Cytology facility at the Sloan-Kettering Institute. This work was supported by an NRSA fellowship (C. W.) an award from the Irma T. Hirschl Trust (L. N.) and by the MSKCC Cancer Center support grant (CA-08748). L. N. is an assistant investigator of the Howard Hughes Medical Institute.

REFERENCES

Augsburger, A., Schuchardt, A., Hoskins, S., Dodd, J. and Butler, S. (1999). BMPs as mediators of roof plate repulsion of commissural neurons. *Neuron* **24**, 127-141.

Baker, C. V. and Bronner-Fraser, M. (1997). The origins of the neural crest. Part I: embryonic induction. *Mech. Dev.* **69**, 3-11.

Basler, K., Edlund, T., Jessell, T. M. and Yamada, T. (1993). Control of cell pattern in the neural tube: regulation of cell differentiation by dorsalin-1, a novel TGF beta family member. *Cell* **73**, 687-702.

Bei, M. and Maas, R. (1998). FGFs and BMP4 induce both Msx1-independent and Msx1-dependent signaling pathways in early tooth development. *Development* **125**, 4325-4333.

Bendall, A. J., Ding, J., Hu, G., Shen, M. M. and Abate-Shen, C. (1999). Msx1 antagonizes the myogenic activity of Pax3 in migrating limb muscle precursors. *Development* **126**, 4965-4976.

Beppu, H., Kawabata, M., Hamamoto, T., Chytil, A., Minowa, O., Noda, T. and Miyazono, K. (2000). BMP type II receptor is required for

gastrulation and early development of mouse embryos. *Dev. Biol.* **221**, 249-258.

Bertrand, N., Medevielle, F. and Pituello, F. (2000). FGF signalling controls the timing of Pax6 activation in the neural tube. *Development* **127**, 4837-4843.

Briscoe, J., Pierani, A., Jessell, T. M. and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* **101**, 435-445.

Burrill, J. D., Moran, L., Goulding, M. D. and Saueressig, H. (1997). PAX2 is expressed in multiple spinal cord interneurons, including a population of EN1+ interneurons that require PAX6 for their development. *Development* **124**, 4493-4503.

Chalepakis, G., Stoykova, A., Wijnholds, J., Tremblay, P. and Gruss, P. (1993). Pax: gene regulators in the developing nervous system. *J. Neurobiol.* **24**, 1367-1384.

Chan-Thomas, P. S., Thompson, R. P., Robert, B., Yacoub, M. H. and Barton, P. J. (1993). Expression of homeobox genes Msx-1 (Hox-7) and Msx-2 (Hox-8) during cardiac development in the chick. *Dev. Dyn.* **197**, 203-216.

Chang, C., Wilson, P. A., Mathews, L. S. and Hemmati-Brivanlou, A. (1997). A Xenopus type I activin receptor mediates mesodermal but not neural specification during embryogenesis. *Development* **124**, 827-837.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-413.

Dale, L. and Jones, C. M. (1999). BMP signalling in early Xenopus development. *BioEssays* **21**, 751-760.

Dickinson, M. E., Krumlauf, R. and McMahon, A. P. (1994). Evidence for a mitogenic effect of Wnt-1 in the developing mammalian central nervous system. *Development* **120**, 1453-1471.

Dudley, A. T. and Robertson, E. J. (1997). Overlapping expression domains of bone morphogenetic protein family members potentially account for limited tissue defects in BMP7 deficient embryos. *Dev. Dyn.* **208**, 349-362.

Dunn, N. R., Winnier, G. E., Hargett, L. K., Schrick, J. J., Fogo, A. B. and Hogan, B. L. (1997). Haploinsufficient phenotypes in Bmp4 heterozygous null mice and modification by mutations in Gli3 and Alx4. *Dev. Biol.* **188**, 235-247.

Epstein, D. J., Marti, E., Scott, M. P. and McMahon, A. P. (1996). Antagonizing cAMP-dependent protein kinase A in the dorsal CNS activates a conserved Sonic hedgehog signaling pathway. *Development* **122**, 2885-2894.

Ericson, J., Thor, S., Edlund, T., Jessell, T. M. and Yamada, T. (1992). Early stages of motor neuron differentiation revealed by expression of homeobox gene Islet-1. *Science* **256**, 1555-1560.

Ericson, J., Muhr, J., Jessell, T. M. and Edlund, T. (1995). Sonic hedgehog: a common signal for ventral patterning along the rostrocaudal axis of the neural tube. *Int. J. Dev. Biol.* **39**, 809-816.

Ericson, J., Morton, S., Kawakami, A., Roelink, H. and Jessell, T. M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. *Cell* **87**, 661-673.

Ericson, J., Briscoe, J., Rashbass, P., van Heyningen, V. and Jessell, T. M. (1997a). Graded sonic hedgehog signaling and the specification of cell fate in the ventral neural tube. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 451-466.

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

Fekete, D. M. and Cepko, C. L. (1993). Replication-competent retroviral vectors encoding alkaline phosphatase reveal spatial restriction of viral gene expression/transduction in the chick embryo. *Mol. Cell. Biol.* **13**, 2604-2613.

Goulding, M. D., Lumsden, A. and Gruss, P. (1993). Signals from the notochord and floor plate regulate the region-specific expression of two Pax genes in the developing spinal cord. *Development* **117**, 1001-1016.

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.

Graham, A., Francis-West, P., Brickell, P. and Lumsden, A. (1994). The signalling molecule BMP4 mediates apoptosis in the rhombencephalic neural crest. *Nature* **372**, 684-686.

Guillemot, F. and Joyner, A. L. (1993). Dynamic expression of the murine Achaete-Scute homologue Mash-1 in the developing nervous system. *Mech. Dev.* **42**, 171-185.

Hamburger, V. and Hamilton, H. L. (1992). A series of normal stages in the development of the chick embryo. 1951. *Dev. Dyn.* **195**, 231-272.

Helms, A. W. and Johnson, J. E. (1998). Progenitors of dorsal commissural interneurons are defined by MATH1 expression. *Development* **125**, 919-928.

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

Holmes, G. and Niswander, L. (2001). Expression of slit-2 and slit-3 during chick development. *Dev. Dyn.* **222**, 301-307.

Horton, S., Meredith, A., Richardson, J. A. and Johnson, J. E. (1999). Correct coordination of neuronal differentiation events in ventral forebrain requires the bHLH factor MASH1. *Mol. Cell. Neurosci.* **14**, 355-369.

Hynes, M., Ye, W., Wang, K., Stone, D., Murone, M., Sauvage, F. and Rosenthal, A. (2000). The seven-transmembrane receptor smoothened cell-autonomously induces multiple ventral cell types. *Nat. Neurosci.* **3**, 41-46.

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, 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.

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.

Liem, K. F., Jessell, T. M. and Briscoe, J. (2000). Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites. *Development* **127**, 4855-4866.

Luo, G., Hofmann, C., Bronckers, A. L., Sohocki, M., Bradley, A. and Karsenty, G. (1995). BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. *Genes Dev.* **9**, 2808-2820.

Ma, Q., Kintner, C. and Anderson, D. J. (1996). Identification of neurogenin, a vertebrate neuronal determination gene. *Cell* **87**, 43-52.

Maeda, R., Kobayashi, A., Sekine, R., Lin, J. J., Kung, H. and Maeno, M. (1997). Xmsx-1 modifies mesodermal tissue pattern along dorsoventral axis in Xenopus laevis embryo. *Development* **124**, 2553-2560.

Mansouri, A. and Gruss, P. (1998). Pax3 and Pax7 are expressed in commissural neurons and restrict ventral neuronal identity in the spinal cord. *Mech. Dev.* **78**, 171-178.

Marti, E., Bumcrot, D. A., Takada, R. and McMahon, A. P. (1995). Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. *Nature* **375**, 322-325.

McDowell, N. and Gurdon, J. B. (1999). Activin as a morphogen in Xenopus mesoderm induction. *Semin. Cell Dev. Biol.* **10**, 311-317.

McMahon, J. A., Takada, S., Zimmerman, L. B., Fan, C. M., Harland, R. M. and McMahon, A. P. (1998). Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev.* **12**, 1438-1452.

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.

Muramatsu, T., Mizutani, Y., Ohmori, Y. and Okumura, J. (1997). Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos in ovo. *Biochem. Biophys. Res. Commun.* **230**, 376-380.

Niwa, H., Yamamura, K. and Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193-199.

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.

Perez, S. E., Rebelo, S. and Anderson, D. J. (1999). Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo. *Development* **126**, 1715-1728.

Pierani, A., Brenner-Morton, S., Chiang, C. and Jessell, T. M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97**, 903-915.

Pituello, F., Yamada, G. and Gruss, P. (1995). Activin A inhibits Pax-6

expression and perturbs cell differentiation in the developing spinal cord in vitro. *Proc. Natl. Acad. Sci. USA* **92**, 6952-6956.

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

Podos, S. D. and Ferguson, E. L. (1999). Morphogen gradients: new insights from DPP. *Trends Genet.* **15**, 396-402.

Potts, W. M., Olsen, M., Boettiger, D. and Vogt, V. M. (1987). Epitope mapping of monoclonal antibodies to gag protein p19 of avian sarcoma and leukaemia viruses. *J. Gen. Virol.* **68**, 3177-3182.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A. and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81**, 445-455.

Selleck, M. A., Garcia-Castro, M. I., Artinger, K. B. and Bronner-Fraser, M. (1998). Effects of Shh and Noggin on neural crest formation demonstrate that BMP is required in the neural tube but not ectoderm. *Development* **125**, 4919-4930.

Shimeld, S. M., McKay, I. J. and Sharpe, P. T. (1996). The murine homeobox gene Msx-3 shows highly restricted expression in the developing neural tube. *Mech. Dev.* **55**, 201-210.

Simmons, A. D., Horton, S., Abney, A. L. and Johnson, J. E. (2001). Neurogenin2 expression in ventral and dorsal spinal neural tube progenitor cells is regulated by distinct enhancers. *Dev. Biol.* **229**, 327-339.

Solloway, M. J., Dudley, A. T., Bikoff, E. K., Lyons, K. M., Hogan, B. L. and Robertson, E. J. (1998). Mice lacking Bmp6 function. *Dev. Genet.* **22**, 321-339.

Sommer, L., Ma, Q. and Anderson, D. J. (1996). neurogenins, a novel family of atonal-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. *Mol. Cell. Neurosci.* **8**, 221-241.

Streit, A. and Stern, C. D. (1999). Establishment and maintenance of the border of the neural plate in the chick: involvement of FGF and BMP activity. *Mech. Dev.* **82**, 51-66.

Suzuki, A., Ueno, N. and Hemmati-Brivanlou, A. (1997). Xenopus msx1 mediates epidermal induction and neural inhibition by BMP4. *Development* **124**, 3037-3044.

Tremblay, P., Pituello, F. and Gruss, P. (1996). Inhibition of floor plate differentiation by Pax3: evidence from ectopic expression in transgenic mice. *Development* **122**, 2555-2567.

Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M. and Pfaff, S. L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* **79**, 957-970.

Walther, C. and Gruss, P. (1991). Pax-6, a murine paired box gene, is expressed in the developing CNS. *Development* **113**, 1435-1449.

Wang, W., Chen, X., Xu, H. and Lufkin, T. (1996). Msx3: a novel murine homologue of the Drosophila msh homeobox gene restricted to the dorsal embryonic central nervous system. *Mech. Dev.* **58**, 203-215.

Wang, Y. H., Rutherford, B., Upholt, W. B. and Mina, M. (1999). Effects of BMP-7 on mouse tooth mesenchyme and chick mandibular mesenchyme. *Dev. Dyn.* **216**, 320-335.

Wieser, R., Wrana, J. L. and Massague, J. (1995). GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. *EMBO J.* **14**, 2199-2208.

Williams, R., Lendahl, U. and Lardelli, M. (1995). Complementary and combinatorial patterns of Notch gene family expression during early mouse development. *Mech. Dev.* **53**, 357-368.

Yamada, T., Pfaff, S. L., Edlund, T. and Jessell, T. M. (1993). Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate. *Cell* **73**, 673-686.

Yamamoto, T. S., Takagi, C. and Ueno, N. (2000). Requirement of Xmsx-1 in the BMP-triggered ventralization of Xenopus embryos. *Mech. Dev.* **91**, 131-141.

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.

Zou, H., Wieser, R., Massague, J. and Niswander, L. (1997). Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev.* **11**, 2191-2203.