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Dorsal and intermediate neuronal cell types of the spinal cord are established by a BMP signaling pathway

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SUMMARY

We have studied the role of Bmp signaling in patterning neural tissue through the use of mutants in the zebrafish that disrupt three different components of a Bmp signaling pathway: *swirl/bmp2b, snailhouse/bmp7* and *somitabun/smad5*. We demonstrate that Bmp signaling is essential for the establishment of the prospective neural crest and dorsal sensory Rohon-Beard neurons of the spinal cord. Moreover, Bmp signaling is necessary to limit the number of intermediate-positioned *lim1+* interneurons of the spinal cord, as observed by the dramatic expansion of these prospective interneurons in many mutant embryos. Our analysis also suggests a positive role for Bmp signaling in the specification of these interneurons, which is independent of Bmp2b/Swirl activity. We found that a presumptive ventral signal, Hh signaling, acts to restrict the amount of dorsal sensory neurons and trunk neural crest. This restriction appears to occur very early in neural tissue development, likely prior to notochord or floor plate formation. A similar early role for Bmp signaling is suggested in the specification of dorsal neural cell types, since the *bmp2b/swirl* and *bmp7/snailhouse* genes are only coexpressed during gastrulation and within the tail bud, and are not found in the dorsal neural tube or overlying epidermal ectoderm. Thus, a gastrula Bmp2b/Swirl and Bmp7/Snailhouse-dependent activity gradient may not only act in the specification of the embryonic dorsoventral axis, but may also function in establishing dorsal and intermediate neuronal cell types of the spinal cord.

Key words: Neural patterning, Spinal cord, Neural crest, Interneuron, Dorsal sensory neuron, Dorsoventral, Bmp2b, Bmp7, Smad5.

INTRODUCTION

The establishment of different neuronal cell types along the dorsoventral axis of the spinal cord is thought to be mediated by two signaling centers: Bmp signaling from the dorsal midline and Sonic hedgehog (Shh) signaling emanating from the ventral midline (reviewed in Tanabe and Jessell, 1996). Ectopic expression, explant and grafting experiments, together with analysis of *shh* mutant mouse embryos reveals a requirement for Shh signaling from the floor plate, notochord and/or node to regionalize the ventral neural tube (reviewed in Tanabe and Jessell, 1996; Chiang et al., 1996; Ericson et al., 1996, 1997). Shh can restrict the expression of some genes to dorsal neural tube regions and inhibit development of a dorsally located interneuron subclass (Ericson et al., 1997). In zebrafish, neural plate ventralization is likely mediated by three *hedgehog*-(*hh*) related genes expressed in midline tissues (Currie and Ingham, 1996; Ekker et al., 1995; Krauss et al., 1993; Schauerte et al., 1998), and a *nodal*-related TGFβ signal, *cyclops* (Hatta et al., 1991; Rebagliati et al., 1998; Sampath et al., 1998).

In addition to a repressive role of Shh signaling from ventral regions, the establishment of dorsal spinal cord cell fates is thought to involve Bmp signaling from the adjacent epidermal ectoderm and the dorsal neural tube. Explants or grafts of chick neural plate tissue placed in contact with epidermal ectoderm results in dorsal neural marker expression in the neural tissue (Dickinson et al., 1995; Liem et al., 1995, 1997). Bmp4 or Bmp7 protein can mimic this effect of the epidermal ectoderm, while the Bmp antagonists Noggin and Follistatin act in an inhibitory manner (Knecht and Harland, 1997; Liem et al., 1997). Several Bmp and TGFβ genes are expressed in the epidermal ectoderm and/or the dorsal neural tube in the mouse and chick and could be the in vivo mediators of dorsalization of the neural tissue (reviewed in Hogan, 1996; Lee et al., 1998; Liem et al., 1997). However, loss-of-function studies in the mouse have provided little corroborating evidence for the endogenous functions of Bmps in the specification of dorsal neural tube cell fates, which may reflect redundant functions of Bmps expressed in overlapping domains (Dudley and Robertson, 1997; Furuta et al., 1997). Loss of one Bmp-related molecule expressed in the dorsal neural tube, Gdf7, causes a differentiation defect in a specific dorsal interneuron (Lee et al., 1998), consistent with a role for Bmps in the generation of dorsal neural cell types.

In the zebrafish, we have characterized mutant embryos of three genes involved in Bmp signaling: *swirl (swr)/bmp2b* (Kishimoto et al., 1997; Nguyen et al., 1998), *snailhouse* (*snh*)*/bmp7* (Schmid et al., 2000) and *somitabun* (*sbn*)*/smad5* (Hild et al., 1999). These mutant embryos display a dorsalization of the embryonic axis (Mullins et al., 1996; Nguyen et al., 1998). In all *swr/bmp2b* and many *sbn/smad5* mutants, the dorsally derived neural and paraxial mesodermal tissue extends around the circumference of the embryo, whereas the ventral ectoderm is absent or very strongly reduced. *snh/bmp7* and some *sbn/smad5* mutants exhibit a less severe dorsalization, whereby anterior neural and mesodermal tissue is expanded, but does not extend circumferentially, while ventral cell fates are greatly reduced, but not entirely absent. In more posterior regions of these two mutants, the dorsalization is more severe, including a greater expansion in neural and mesodermal tissue (Mullins et al., 1996; Nguyen et al., 1998). These dorsalized mutant phenotypes are consistent with the expected role of Bmp signaling activity in embryonic axis development.

From our previous analysis, we established that *swr/bmp2b* is essential for cranial neural crest specification (Nguyen et al., 1998). In contrast *snh/bmp7* and *sbn/smad5* mutants exhibit an expansion in cranial neural crest progenitors (Nguyen et al., 1998). The opposite effects these mutants have on neural crest development lead us to hypothesize that a low Bmp activity level is involved in neural crest specification and that different Bmp levels remained in *swr/bmp2b*, *snh/bmp7* and *sbn/smad5* mutants (Nguyen et al., 1998). Here we extend our studies to examine the endogenous role played by gastrula Bmp signaling in the development of dorsal, intermediate and ventral neural tissue. We examined spinal cord neural tissue, because of its simplicity and well-defined dorsally and ventrally located cell types.

We investigated five cell types found at different dorsoventral positions of the spinal cord. We establish that Bmp signaling is required for specification of the dorsally located trunk neural crest and Rohon-Beard sensory neurons. Moreover, Bmp signaling restricts the number of intermediate neural tube *lim1*-expressing interneurons, which are greatly expanded in many mutant embryos. Bmp signaling is also required for specification of these interneurons, since they are absent in *swr/bmp2b* mutants injected with the Bmp antagonist, *chordin*. We did not detect a change in the number of ventrally located motorneurons, while the floor plate is broadened. We examined the role of ventral signals in dorsal cell fate specification. We found a correlation in the loss of midline and tail bud *shh* expression in *no tail* mutants with a dorsalization of neural tissue, as observed by an expansion of dorsal sensory neurons and neural crest and a loss of floor plate and motorneurons. In double-mutant analysis, the expanded dorsal neurons were shown to be dependent on Bmp2b/Swirl signaling. Our analysis suggests that Bmp and Shh signaling act early in neural tissue development to specify and restrict dorsal spinal cord cell fates, respectively.

MATERIALS AND METHODS

Whole-mount in situ hybridization and mutant strains

In situ hybridizations were performed similarly to that described by Schulte-Merker et al. (1992). The following probes were used: *fkd6* (Odenthal and Nüsslein-Volhard, 1998), *krox20* (Oxtoby and Jowett, 1993), *tlx3* (P. A. and E. W., unpublished), *shh* (Krauss et al., 1993), *pax2.1* (Krauss et al., 1992; Pfeffer et al., 1998), *myoD* (Weinberg et al., 1996), *msxB* (Akimenko et al., 1995), *crestin* (Rubinstein et al., 2000), *huC* (Kim et al., 1996), *valentino* (Moens et al., 1998), *isl1* (Appel et al., 1995), *lim3* (Glasgow et al., 1997), and *lim1* (Toyama and Dawid, 1997). Triple hybridizations in Fig. 1. were performed with a fluorescein-labelled *krox20* probe and DIG-labelled *pax2.1* and *val* probes, and stained and processed as in Connors et al. (1999).

Embryos were obtained from matings between heterozygotes of the following fish: swr^{tc300a}, swr^{ta72}, swr^{tdc24}, snh^{ty68a}, sbn^{dtc24}, *no tail*^{tc41} and *floating head*^{n1}. The snh^{ty68a} mutants from our recent generations of fish used here exhibit stronger dorsalized phenotypes than we observed previously. For *sbn*, all the progeny of a cross are mutant, due to the dominant maternal-effect of this mutation (Mullins et al., 1996).

Assay for apoptotic cells

Detection of cell death was done in fixed whole-mount embryos using a terminal transferase assay (Apoptaq Kit-peroxidase, Oncor, Inc.), with minor modifications described in Fisher et al. (1997).

mRNA injections

Synthetic *chordin* mRNA was made from chordin-S2/pCS2+ as described (Miller-Bertoglio et al., 1997). 100 pg of mRNA was injected into the yolk of 1- to 4-cell-stage embryos from crosses between homozygous adult *swr/bmp2b* fish (Nguyen et al., 1998), as described in Westerfield (1995).

Cloning of swr^{tdc24} allele

Cloning and sequencing was performed as described in Nguyen et al. (1998) and Connors et al. (1999).

Mounting and sectioning

We flattened in situ hybridized embryos by placing them in 50% glycerol/PBST and removing the yolk using watchmaker forceps. The embryos were then rinsed in PBST, methanol and benzylalcohol/benzylbenzoate, mounted in GMM on glass slides and flattened using a glass coverslip.

Sections were performed by removing the yolk (as above) and mounting the embryos in 0.5% agarose following the methanol rinse. The agarose blocks were mounted into plastic using Technovit 8100 (Energy Beam Systems), sectioned on a Leica RM 2155 microtome and counterstained with Nuclear Fast Red (Vector Laboratories) using an 8 minute incubation time.

RESULTS

Normal anteroposterior neural patterning in *swr/bmp2b*, *snh/bmp7* and *sbn/smad5* mutants

Prior to the examination of dorsoventral patterning of neural tissue in the zebrafish, we investigated the effects of loss of Bmp signaling on anteroposterior patterning of neural tissue. We examined the expression of *pax2.1* in the midbrain-hindbrain boundary, *krox20* in rhombomeres 3 and 5 of the hindbrain (Oxtoby and Jowett, 1993) and *valentino* (*val*) in rhombomeres 5 and 6 (Moens et al., 1998) in single or two-color triple hybridizations in *swr/bmp2b*, *snh/bmp7* and *sbn/smad5* whole-mount mutant embryos (Fig. 1A-D). In all mutants, an expansion in neural tissue is observed into lateral and/or ventral regions, as we previously reported for *pax2.1* (Mullins et al., 1996) and *krox20* (Nguyen et al., 1998) expression. Rhombomere 5 expression of *krox20* in magenta is

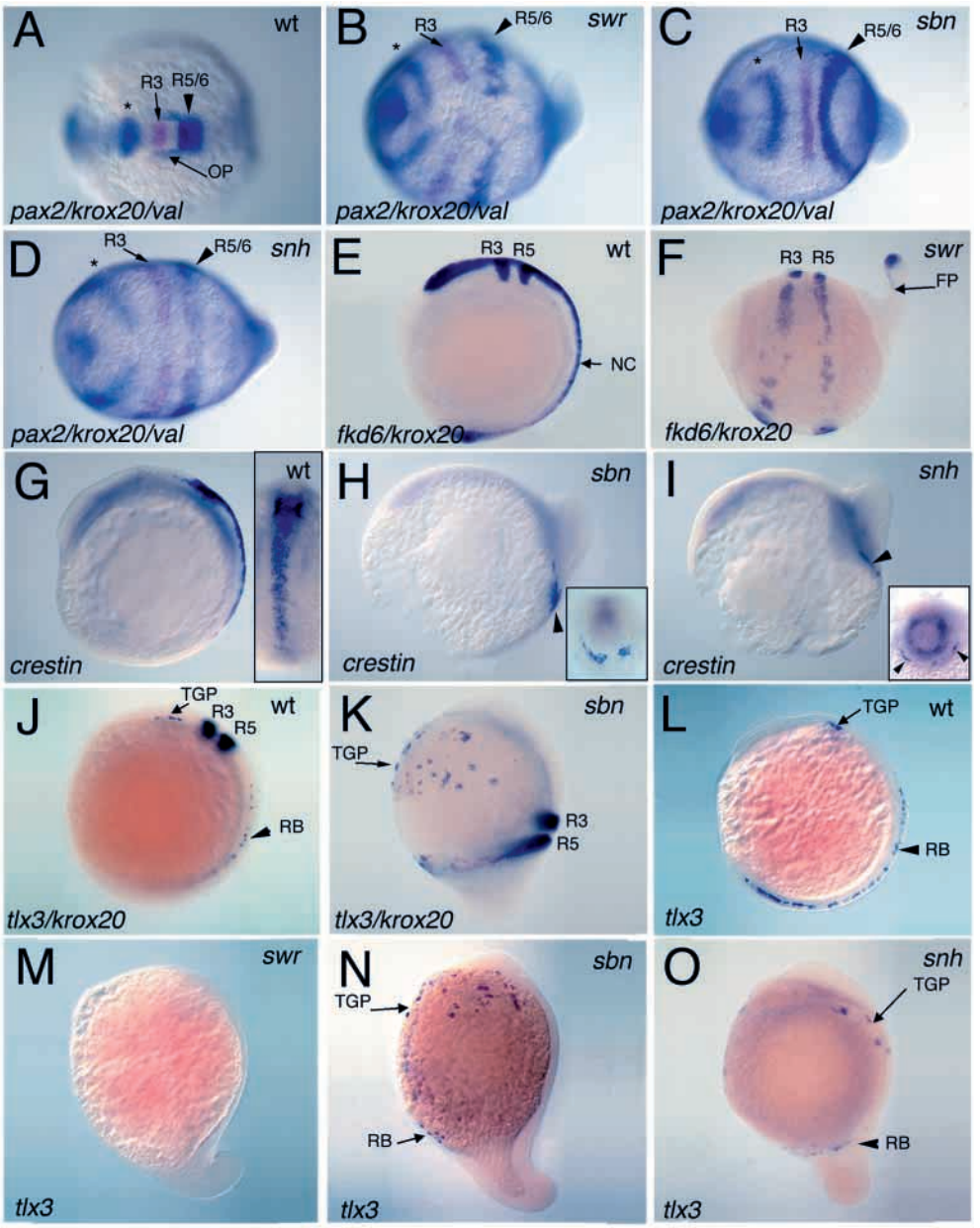


Fig. 1. Conserved anteroposterior neural patterning (A-D) and loss of trunk neural crest (E-I) and dorsal RB neurons (J-O) in dorsalized mutant embryos. (A-D) are two-color triple in situ hybridizations in 6-somite-stage wild-type (A), *swr/bmp2b* (B), *sbn/smad5* (C) and *snh/bmp7* (D) embryos (dorsal views with anterior to the left). Expression of *pax2.1* in the midbrain-hindbrain boundary (marked with an *) and *val* in rhombomeres 5 and 6 are in blue, while *krox20* expression in rhombomere 3 is in magenta. Similar results were found in 1- to 2-somite-stage embryos. Expression of *fkd6* and *krox20* in wild-type (E) and a *swr/bmp2b* mutant (F) at the 6-somite stage. *crestin* expression posterior to rhombomere 5 and within the rostral trunk of a 9-somite-stage wild-type embryo (G), and in a greatly reduced domain in *sbn/smad5* (H) and *snh/bmp7* (I) mutant embryos. Insets in G-I are dorsal or posterior views. Expression of *tlx3* and *krox20* in 7-somite-stage wild-type (J) and *sbn/smad5* (K) embryos. *tlx3* expression in 7-somite wild-type (L), *swr/bmp2b* (M), *sbn/smad5* (N) and *snh/bmp7* (O) mutant embryos. In 0/8 *swr/bmp2b*, 8/20 *sbn/smad5* and 5/8 *snh/bmp7* mutant embryos, some RB-*tlx3* expressing cells were detected, but in severely reduced numbers. Lateral views (E-O) with anterior to the top, dorsal to the right in E,G,J-O, and anterior to the left, dorsal to the top in F,H,I. R3, rhombomere 3; R5, rhombomere 5; R5/6, rhombomeres 5 and 6; OP, otic placode; NC, neural crest; FP, floor plate; TGP, trigeminal placode precursors.

not observed due to its complete overlap with *val* rhombomere 5 expression in blue. The *val* expression domain is broader (anteroposteriorly) and extends further posteriorly than *krox20* rhombomere 5 expression (data not shown), as expected. In all three mutants, as in wild type, *pax2.1* expression in the midbrain-hindbrain boundary lies most anterior, followed by a gap in expression, *krox20* rhombomere 3 expression, a second gap, *val*/*krox20* expression in rhombomere 5, and lastly *val* expression in rhombomere 6. These results indicate that anteroposterior patterning of neural tissue is relatively unaffected in these mutants. Due to the high degree of lethality of these mutants at the 14-somite stage, we were unable to investigate anteroposterior patterning of trunk neural tissue. Based on the concentric rings of *myoD* expression in the somitic mesoderm, we expect anteroposterior patterning is maintained in trunk tissues as well.

Loss of trunk neural crest and dorsal sensory neurons in Bmp mutants

We next investigated the effect of loss of Bmp signaling on spinal cord neuronal cell type specification in *swr/bmp2b*, *sbn/smad5* and *snh/bmp7* mutants. Since neural tissue is expanded in these mutants, we expect some or all neural cell types to be increased in number. The effect on particular spinal cord cell types may differ depending on the role gastrula Bmp signaling plays in neural pattern formation, since the coexpression of *swr/bmp2b* and *snh/bmp7* is restricted to gastrulation stages and the tail bud during somitogenesis stages (Nikaido et al., 1997; Schmid et al., 2000). We previously demonstrated that *swr/bmp2b* mutants exhibit a strong reduction or absence of cranial neural crest, while *sbn/smad5* and *snh/bmp7* mutants display an expansion of cranial neural crest (Nguyen et al., 1998).

To determine the effect of loss of Bmp2b/Swirl signaling on trunk neural crest development, we examined *fkd6* expression, a marker of premigratory neural crest progenitors (Odenthal and Nüsslein-Volhard, 1998), at the 6-somite stage in wild-type and *swr/bmp2b* mutant embryos. To distinguish between the cranial and trunk neural crest progenitors, we also examined *krox20* expression in the hindbrain. In wild-type embryos, *fkd6* expression is observed both anterior and posterior to the *krox20* domains in the presumptive head and trunk, respectively (Fig. 1E). In *swr/bmp2b* mutants, no trunk neural crest progenitors were detected (Fig. 1F).

In *sbn/smad5* and *snh/bmp7* mutant embryos, the expansion of cranial neural crest made it difficult to unambigously determine if the trunk neural crest is present utilizing the *fkd6* marker. Instead, we examined expression of *crestin*, a marker at the 9-somite stage specific for neural crest posterior to hindbrain rhombomere 5 including the rostral trunk (Rubinstein et al., 2000) (Fig. 1G). In *sbn/smad5* and *snh/bmp7* mutant embryos at this stage, expression was absent or strongly reduced (Fig. 1H,I, see arrowheads and insets, data not shown). No expression was observed in *swr/bmp2b* mutants, as expected (data not shown, see also Rubinstein et al., 2000). Thus, although cranial neural crest is expanded in *sbn/smad5* and *snh/bmp7* mutants, trunk neural crest is greatly reduced, consistent with a stronger dorsalization in more posterior regions of these mutants, as we previously observed with the expansion of the somitic mesoderm (Mullins et al., 1996) and hindbrain (Nguyen et al., 1998, also compare R3 to R5/6 in Fig. 1C).

We next examined a dorsal neuronal cell type of the zebrafish spinal cord, the Rohon-Beard (RB) sensory neurons. RB progenitors, as well as cells of the prospective trigeminal placode, specifically express the *tlx3* gene at the 7-somite stage (P. A. and E. W., unpublished). To distinguish between these two expression domains, we examined *tlx3* expression relative to that of *krox20* in the hindbrain. In wild-type embryos, as expected, the trigeminal placode domain is anterior to *krox20*, while the RB cells lie posterior to it (Fig. 1J). Wild-type sibling embryos possess a large number of *tlx3*-expressing RB cells at this stage, whereas no or few RB cells are detectable in *swr/bmp2b*, *sbn/smad5* and *snh/bmp7* mutant embryos (Fig. 1K-O). Similar results were found using the *isl1* gene as a marker of RB cells (Appel et al., 1995; Inoue et al., 1994; Korzh et al., 1993) (Fig. 4M,N, data not shown). The trigeminal placode expression of *tlx3* was absent or strongly reduced in most *swr/bmp2b* (10/15) and many *sbn/smad5* (17/45) mutant embryos (Fig. 1M, data not shown). In the remaining *swr/bmp2b*, *sbn/smad5* and all *snh/bmp7* mutant embryos, the trigeminal placode precursors were typically present in a dispersed lateral or ventrolateral domain (Fig. 1K,N,O). In summary, Bmp signaling is required not only for trunk neural crest specification, but also for the specification of the dorsally located RB neurons of the spinal cord.

The loss of neural crest and RB neurons could be caused by cell death rather than an effect on their specification. To address this question, we performed an assay for cell death in the dorsalized mutant embryos at the bud, 3- and 7-somite stages. Although we did detect sporadic cell death in a subset of both wild-type and mutant embryos, an increase in cell death was not observed in *swr/bmp2b*, *sbn/smad5* or *snh/bmp7* mutant embryos at any of these stages (data not shown). We previously showed that cell death is not increased in these dorsalized mutants during gastrulation stages (Nguyen et al., 1998). Thus, a lack of specification, rather than apoptosis, appears to cause the absence of neural crest and RB progenitors.

Intermediate, *lim1*-expressing interneurons are expanded in the dorsalized mutants

To investigate if an intermediate-located spinal cord cell fate depends on gastrula Bmp signaling, we examined an interneuron cell type that expresses *lim1* (Toyama and Dawid, 1997), the first known marker specifically expressed in this neuronal subtype beginning at about the 6-somite stage. The exact correspondence of these *lim1* interneurons to those in the mouse or chick is unclear. In the chick a large population of cells expresses *lim1* in the spinal cord, and additional markers have been used to subdivide Lim1/2+ cells into different neuronal cell types (Ericson et al., 1997). In wild-type zebrafish embryos at the 7-somite stage, *lim1* is expressed in a distinct population of cells at an intermediate dorsoventral position within the neural rod (the ‘neural tube’ prior to neurocoel formation, Papan and Campos-Ortega, 1994) (Fig. 2A,4E). The *lim1* gene is also expressed in a small number of cells in the brain, the presumptive pronephric system and notochord (Fig. 2A) (Toyama and Dawid, 1997). In *swr/bmp2b*, *sbn/smad5* and *snh/bmp7* mutant embryos, *lim1* expression is observed in the notochord and in dispersed cells extending into ventral and ventrolateral regions of the embryo, which may correspond to an expanded interneuron domain (Fig. 2B-E). *lim1* expression is also found in a solid, posterior domain in some *snh/bmp7* and *sbn/smad5* mutants, possibly representing a remnant of the pronephric system (Fig. 2E, data not shown).

To distinguish between the *lim1* interneuron and pronephric expression domains in these mutants, we utilized the *pax2.1* marker. *pax2.1* is expressed at the 5- to 6-somite stage in the midbrain-hindbrain boundary, the otic placode and the same pronephric cells as *lim1* (Toyama and Dawid, 1997), but is not expressed in the interneurons at this stage (Fig. 2F) (Krauss et al., 1992; Pfeffer et al., 1998). Using this marker, we examined the effects of these mutations on the pronephric system, independent of the effects on the interneurons. In all *swr/bmp2b*, most *snh/bmp7* and some *sbn/smad5* mutant embryos, we observed *pax2.1* midbrain-hindbrain expression, but did not detect the pronephric expression domain (Fig. 2G,I). In some *snh/bmp7* and *sbn/smad5* mutants, we detected a small remnant of *pax2.1* pronephric expression, very similar to that observed with *lim1* (compare Fig. 2E and H). A reduction or absence of the pronephric domain is expected, since it derives from ventral mesoderm (Kimmel et al., 1995), which is strongly reduced or absent in these mutants. We never observed dispersed expression in ventrolateral regions, as seen with *lim1* expression. These results provide strong evidence for a specific expansion of *lim1+* interneurons in the dorsalized mutant embryos.

We counted the number of *lim1*-expressing (*lim1+*) neuronal cells and estimate a range from near normal numbers in *swr/bmp2b* and *sbn/smad5* mutants to a 5- and 2.5-fold expansion in *swr/bmp2b* (n=13) and *sbn/smad5* (n=16) mutants, respectively. In *snh/bmp7* mutants (n=8) an average 5-fold expansion in interneurons was observed. The range in the number of *lim1+* interneurons and their dorsoventral

location in *swr/bmp2b* and *sbn/smad5* mutants may reflect slightly different levels of Bmp activity remaining in these mutants (see also Discussion). In conclusion, Bmp2b/Swirl and Bmp7/Snh signaling, together with Smad5/Sbn, are essential for establishing the correct number of intermediate *lim1+* interneurons in the spinal cord.

The role of Bmp signaling in the expansion of *lim1+* interneurons

There are at least two mechanisms by which Bmp signaling could regulate *lim1+* interneuron cell fate specification. One possibility is that Bmp signaling acts in a strictly repressive manner to limit the number of *lim1+* cells, i.e. only those cells that escape a particular level of Bmp signaling can differentiate into *lim1+* interneurons. According to this model, Bmp activity in many mutants is reduced below this repressive level, leading to an expansion of the interneurons. In a second mechanism, a particular Bmp signaling level may act positively to specify interneuron cell fates, and this activity level may be present in an expanded domain in some of the dorsalized mutant embryos.

To distinguish between these two mechanisms, we examined the effect of injecting mRNA encoding the Bmp antagonist, Chordin (Miller-Bertoglio et al., 1997; Sasai et al., 1994), into homozygous *swr/bmp2b* mutant embryos. If Bmp signaling acts only to repress *lim1+* interneurons, then reducing any remaining Bmp signaling in *swr/bmp2b* mutants would increase or have no effect on the number of *lim1+* cells. In contrast, if a particular level of Bmp signaling remains in *swr/bmp2b* mutant embryos and causes the expansion of *lim1+* cells, then lowering Bmp signaling further would result in a reduction in the number of *lim1+* cells. Following overexpression of *chordin* mRNA in *swr/bmp2b* mutant embryos (derived from crosses between homozygous *swr/bmp2b* mutant fish, Nguyen et al., 1998), we found that the number of *lim1+* interneurons was markedly reduced compared to the uninjected control mutant embryos (Fig. 3A-D). While all uninjected *swr/bmp2b* mutants exhibited *lim1+* neuronal cells and 2/3 displayed a clear expansion (Fig. 3A,B), 86% of the *chordin*-injected *swr/bmp2b* mutants displayed no or almost no *lim1+* cells (n=94, Fig. 3C).

The loss of *lim1+* cells in *chordin*-injected *swr/bmp2b* mutants was not simply due to a loss in general gene expression, since the circumferential expression of *krox20* and *myoD* was unaffected in the injected embryos (Fig. 3E). Moreover, *chordin* overexpression did not merely cause a reduction of trunk tissue, since *myoD* is still expressed in the trunk somitic mesoderm. To address whether trunk neural tissue is specifically affected, i.e. are all trunk spinal neurons absent, we examined the expression of *huC*, a marker of multiple neuronal cell types of the brain and spinal cord (Kim et al., 1996). In trunk regions, *huC* is specifically expressed in a medial and lateral row of neural cells (Fig. 3F) (Appel and Eisen, 1998). In *swr/bmp2b* mutants, the lateral neural cells are expanded (Fig. 3G, arrows), similar to the *lim1+* interneurons. In *chordin*-injected *swr/bmp2b* mutants, these lateral cells are strongly reduced or absent, like the *lim1+* interneurons, whereas the medial neural cells are still present (Fig. 3H, arrowheads). Hence, medial trunk neural cells do not appear reduced, while more lateral cells appear specifically affected. Thus, these results favor a mechanism whereby Bmp signaling acts in the specification of *lim1+* interneurons in a positive manner, and that some Bmp signaling activity is present in *swr/bmp2b* mutant embryos.

The Bmp signaling activity in *swr/bmp2b* mutants could be the activity of other Bmp protein(s) or partial Swirl/Bmp2b activity due to hypomorphic rather than null *swr/bmp2b* mutations. Both *swr/bmp2b* alleles that we examined, swr^{tc300a} and swr^{ta72}, are point mutations within the *bmp2b* ligand domain that could retain some Bmp2b activity. swr^{tc300a} corresponds to a missense mutation in a highly conserved cysteine residue and swr^{ta72} alters the stop codon, extending the open reading frame by six amino acids. The nature of these mutations leaves open the possibility that they are not null mutations. We cloned and sequenced a third *swr/bmp2b* allele, swr^{tdc24}, which was isolated in a screen for dominant mutations (van Eeden et al., 1999). The swr^{tdc24} cDNA sequence reveals a nonsense mutation in the N-terminal pro-domain at codon position 64. Translation of the swr^{tdc24} mRNA is expected to yield a truncated peptide of 63 amino acids, which does not include the ligand domain. Thus, it is likely to be a null allele of *swr/bmp2b*. We examined *lim1* expression in swr^{tdc24} mutant embryos and found a very similar expansion of *lim1+* cells (Fig. 3I) as in the other *swr/bmp2b* mutant allele embryos. Therefore, the Bmp activity remaining in *swr/bmp2b* mutant embryos appears not to be residual Bmp2b activity.

Ventral neural tissue in Bmp mutant embryos

We investigated whether loss of Bmp2b/Bmp7 signaling affects the specification of ventral neural cell fates: the floor plate and primary motorneurons. We examined *shh* expression at the 14-somite stage, since all *swr/bmp2b* and strong *sbn/smad5* and *snh/bmp7* mutant embryos die shortly after this point (Mullins et al., 1996; Schmid et al., 2000). The dorsalized mutants at this stage exhibit an abnormal morphology, due to a presumptive reduction in dorsal convergence and a constriction of the yolk caused by the expanded somites around the circumference of the embryo (Mullins et al., 1996). Typically the axis of the embryo undulates, with more posterior aspects swirled upon itself.

At the 14-somite stage, *shh* is expressed in both the notochord and floor plate of the anterior trunk (Krauss et al., 1993). The expression of *shh* appears relatively normal in the dorsalized mutants, except for the abnormal shape of the axis (Fig. 4A-D). As observed with the prospective notochord (Mullins et al., 1996), the length of the anteroposterior axis is shortened (Fig. 4A,B, insets). In cross-sections, we found the floor plate *shh* expression domain to be broadened from the normal 1 cell in wild-type embryos to 2 to 5 cells in *swr/bmp2b*, *sbn/smad5* and *snh/bmp7* mutants (Fig. 4F-J). This broadening of the floor plate may be due to a reduced amount of dorsal convergence and/or an increase in the number of floor plate cells, as discussed below.

The morphology of the sectioned neural tissue indicates that the normal medial convergence of neural tissue to form the neural tube (Papan and Campos-Ortega, 1994; Schmitz et al., 1993; Woo and Fraser, 1995) does not occur in these mutants and the neural plate remains open. We previously showed that, at the end of gastrulation, the prospective notochord is broader mediolaterally and shorter in its anteroposterior length in *swr/bmp2b*, *sbn/smad5* and *snh/bmp7* mutants compared to wild-type embryos (Mullins et al., 1996). However, we found

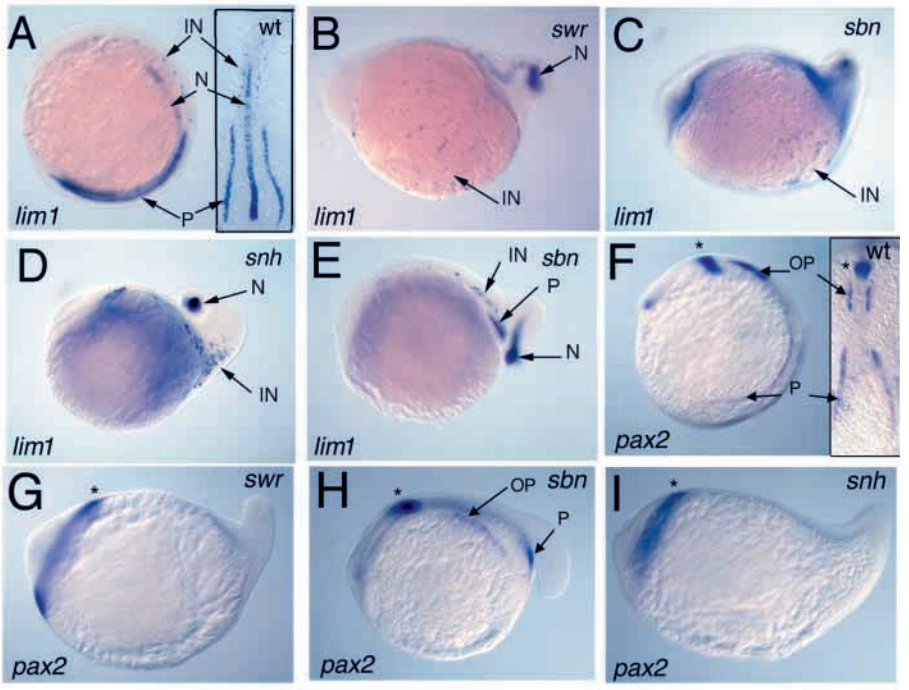
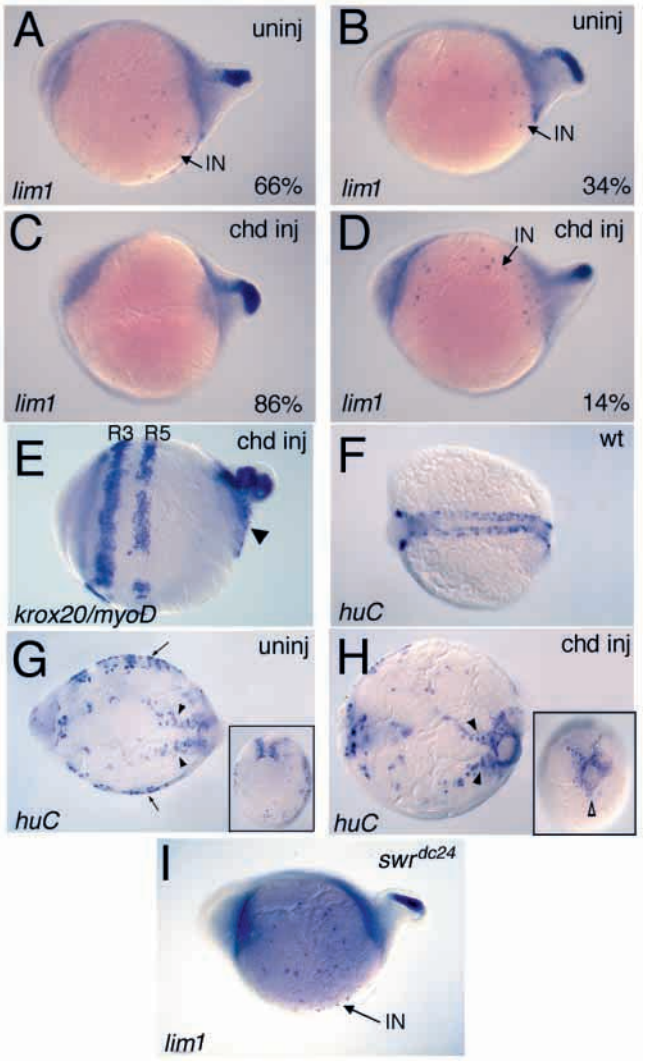


Fig. 2. Expansion of *lim1+* interneurons and loss of pronephric precursors in *swr/bmp2b*, *sbn/smad5* and *snh/bmp7* mutants. Lateral views with anterior to the top, dorsal to the right in A,F, and anterior to the left and dorsal to the top in B-E,G-I. *lim1* expression in 7-somite wild-type (A), *swr/bmp2b* (B), *sbn/smad5* (C, E) and *snh/bmp7* (D) mutant embryos. Note the expansion of the interneuron domain in B-D. The insets in A and F are flattened whole-mount embryos. Note the small remnant of the *lim1* pronephric domain observed in a *sbn/smad5* embryo (E). Expression of *pax2.1* in wild-type (F), *swr/bmp2b* (G), *sbn/smad5* (H) and *snh/bmp7* (I) embryos. Note the absence of the *pax2.1* pronephric domain in *swr* and *snh* mutants and the remnant of pronephric expression in the *sbn* mutant. The otic placode domain is also absent in the *swr* and *snh* mutant, but is slightly laterally positioned in the *sbn* mutant compared to wild-type. * indicates the midbrain-hindbrain boundary domain; IN, interneurons; N, notochord; OP, otic placode; P, pronephric domain.

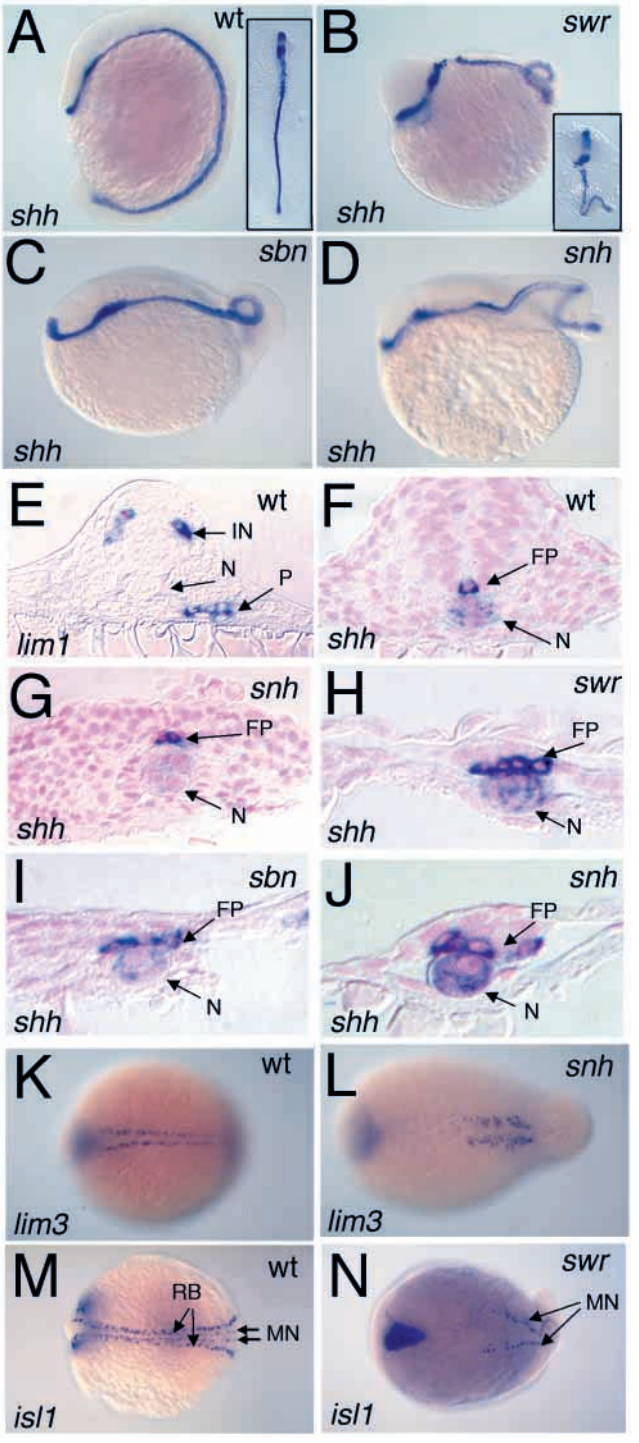


approximately normal numbers of notochord cells in both *swr/bmp2b* mutant and wild-type embryos (Mullins et al., 1996). We speculated that the abnormal morphology of the notochord was due to a reduction in dorsal convergence, which has indeed been shown for *sbn/smad5* mutant embryos (L. Solnica-Krezel, personal communication). Thus, the broader floor plate may be caused by reduced dorsal convergence and a shortening in the length of the anteroposterior axis, similar to that observed with the notochord in these mutants. However, we cannot determine if the broader floor plate is, in part, also due to an increased number of *shh*-expressing floor plate cells in these mutants caused by a loss in Bmp signaling.

We examined the expression of *lim3*, a marker of primary motorneurons (Appel et al., 1995; Glasgow et al., 1997) at the 5- to 7-somite stage, in *swr/bmp2b*, *sbn/smad5* and *snh/bmp7* mutant embryos. We did not detect an expansion in motorneurons in these mutants (Fig. 4K,L, data not shown), as might have been expected if Hh signaling is increased from the

Fig. 3. Expansion of *lim1+* interneurons depends on Bmp activity. Lateral (A-E,I) and dorsal (F-H) views, anterior is to the left. In 6- to 7-somite uninjected *swr/bmp2b* homozygotes, 66% show a large expansion in *lim1+* interneurons (A) and 34% exhibit a lesser or no expansion in interneurons (B). In the *chordin*-injected *swr/bmp2b* mutants, we see a shift to 86% with no or very few (<10) interneurons (C) and 14% that still exhibit normal or expanded numbers (D). Other head and trunk gene expression is unaffected as seen with *krox20/myoD* expression in a *chordin*-injected *swr/bmp2b* embryo (E). The arrowhead points to circular *myoD* expression in the somitic mesoderm. Expression of *huC* in 5- to 7-somite wild-type (F), uninjected (G) and *chordin*-injected (H) *swr/bmp2b* mutant embryos. (G) Arrows mark the lateral neural cells; (G,H) arrowheads indicate the medial trunk neural cells and insets are posterior views, dorsal to the top, showing a loss of lateral neural cells in ventral regions of injected mutants, while medial neural cells remain. (I) Expansion of *lim1+* interneurons in *swr/bmp2b*^{tdc24} presumptive null mutants.

broadened floor plate. Instead it appeared as though the motorneurons were reduced in number (Fig. 4K,L). However, in counting the *lim3*+ motorneurons, we found similar numbers of cells in *swr/bmp2b* (34, *n*=3) and *sbn/smad5* (36, *n*=6) mutants compared to wild-type embryos (31, *n*=3). The superficial appearance of a reduction in motorneurons is likely due to the anteroposterior shortening and medial-lateral broadening of the motorneuron domain caused by reduced dorsal convergence (as discussed above). We also examined *isl1*, which is expressed in the ventral motorneurons, the RB neurons and additional cell types within the prospective head region (Appel et al., 1995; Inoue et al., 1994; Korzh et al., 1993). The motorneuron expression of *isl1* was very similar to our observations with *lim3* (Fig. 4M,N, data not shown). Thus, a loss in Bmp2b/Swirl, Bmp7/Snh or Smad5/Sbn activity does not affect specification of the primary motorneurons. Moreover, the *shh*-expressing notochord and floor plate, which are mediolaterally broadened in these mutants, do not cause an expansion in the number of primary motorneurons.



A ventral signal restricts the number of dorsal neuronal cell types

A possible increase in Shh signaling from the floor plate of the dorsalized mutants may cause, in part, the loss of the trunk neural crest and RB cells, and/or expansion in *lim1*+ interneuron cells. To investigate the role of floor plate Shh signaling in the specification of dorsal and intermediate neural cell types, we examined *no tail* (*ntl*) and *floating head* (*flh*) mutant embryos, which both lack a notochord. Floor plate *shh* expression is expanded in *ntl* mutants from 1 to 3-5 cells throughout most of the trunk, which may cause the expansion of motorneurons also observed in these mutants (Glasgow et al., 1997; Halpern et al., 1997; Odenthal et al., 1996). In the caudalmost five trunk segments of *ntl* mutants (which lack a tail), however, the floor plate is absent (Halpern et al., 1997, 1993). *flh* mutant embryos possess a very small number of *shh*-expressing floor plate cells (Beattie et al., 1997; Halpern et al., 1995; Odenthal et al., 1996), which is reflected in an overall reduction of motorneurons (Beattie et al., 1997; Glasgow et al., 1997).

In regions of *ntl* mutants where the floor plate is expanded, we did not detect a change in the neural crest or dorsal RB cells, indicating that additional floor plate Shh signaling does not affect specification of these cell types (Fig. 5A,B, data not shown). In *flh* mutant embryos where *shh* expression is reduced, the neural crest and RB cells also appeared normal in number (Fig. 5C-F). Even in regions where midline *shh* expression was absent, no changes were detectable. In contrast, in caudal regions of *ntl* mutants, where *shh* expression is absent, the RB precursors appeared expanded (Fig. 5A,A′,B,B′ (marked with a *)). A shortening of the anteroposterior axis in caudal regions could account for an apparent expansion in the RB cells in *ntl* mutants. However, measurement of the length of the axis in wild-type (*n*=3) and mutant (*n*=3) embryos at the 7-somite stage showed no alteration in *ntl* mutants. Upon

Fig. 4. Examination of floor plate and motorneurons in dorsalized mutant embryos. *shh* expression in wild-type (A), *swr/bmp2b* (B), *sbn/smad5* (C) and *snh/bmp7* (D) mutant embryos at the 14-somite stage. The axis of the wild-type embryo (inset A) is straight, while the axis of the *swr/bmp2b* mutant (inset B) is curved. An intermediate dorsoventral spinal cord position of *lim1*+ cells in a cross-section of a 14-somite-stage wild-type embryo (E). Cross-sections of 14-somite-stage embryos showing floor plate *shh* expression 1 cell wide in wild-type (F) and 2 to 5 cells wide in *snh/bmp7* (G,J), *swr/bmp2b* (H) and *sbn/smad5* (I). *lim3* expression in the motorneurons of 7-somite-stage wild-type (K) and *snh/bmp7* (L) mutant embryo. Expression of *isl1* in the RB neurons and motorneurons in wild-type (M) and *swr/bmp2b* (N), where the motorneurons are apparent, but RB cells are absent. Anterior is to the top, dorsal to the right in A. Anterior is to the left, dorsal to the top in B-D. In K-N, dorsal views, anterior is to the left. FP, floor plate; P, pronephric region.

counting the number of *tlx3+* cells, we estimate a 50% increase in RB cells in this posterior region in *ntl* mutants (n=5) compared to wild-type embryos (n=5). The *tlx3* expression domain is altered, forming a loop in *ntl* mutants, perhaps due to the reduced size of the tail bud (Halpern et al., 1993). The expression of *fkd6* in the neural crest progenitors and *msxB* in a region overlapping the trunk neural crest and the RB cells (Ekker et al., 1997), is also expanded in the caudalmost regions of *ntl* mutant embryos (Fig. 5G-J).

The expansion versus normal number of dorsal cell types in *ntl* and *flh* mutants, respectively, may reflect differences in the tail bud expression domain of *shh* in these two mutants. In addition to the floor plate and notochord, *shh* is also expressed within the tail bud. Both mutants display an absence of midline *shh* expression along short anteroposterior lengths of the axis. However, only in caudal regions of *ntl* mutants are all three *shh* expression domains absent (best seen in Fig. 5J′), while *flh* mutants retain weak expression within the tail bud (Fig. 5D′, arrowhead, see also Beattie et al., 1997; Odenthal et al., 1996). Hence, the tail bud expression domain of *shh* may be critical in restricting the number of dorsal neural cell fates.

We asked whether the expanded number of RB cells in caudal segments of *ntl* mutants depended on Bmp2b signaling. We addressed this question by generating *ntl*;*swr* double-mutant embryos. We found that, in crosses between double heterozygous fish, 3/16 of the embryos exhibited the *swr/bmp2b* mutant phenotype, another 3/16 displayed the *ntl* mutant phenotype, and 1/16 had a combination of the two mutant phenotypes, corresponding to the double mutants. In 7-somite-stage double-mutant embryos, the *tlx3+* RB progenitors were absent (data not shown), demonstrating that *swr/bmp2b* is epistatic to *ntl* with respect to RB cell specification and the expansion of RB cells in *ntl* mutants depends on Bmp2b signaling.

The expansion of RB and neural crest cells in *ntl* mutants may reflect a dorsalization of the neural tissue caused by a loss of *hh* expression in the notochord, floor plate and tail bud in caudal trunk regions. Since motorneurons are very sensitive to Hh signaling, we analyzed the motorneuron expression of *lim3* and *isl1* in caudal segments of *ntl* mutants. The additional expression of *isl1* in the RB cells allowed us to compare the posterior extents of the motorneuron and RB cell populations. We examined expression of these genes in *ntl* mutants from the same brood to ensure that mutants were of the exact same stage. We found that the RB neurons extended further posteriorly than the motorneurons in *ntl* mutant embryos (Fig. 6A-D). These data are consistent with caudal segments of *ntl* mutants exhibiting a dorsalization of neural tissue caused by a complete loss of Hh signaling.

Lastly, we addressed whether specification of the intermediate *lim1+* interneurons of the spinal cord is either positively or negatively affected by changes in floor plate Shh signaling levels. In anterior trunk regions of 15-somite-stage *ntl* mutants, the *lim1+* interneurons appeared normal in number (Fig. 6G,H), indicating that an increase in Shh signaling did not affect specification of these cells. In comparing the posterior extent of *lim1+* interneurons to that of the motorneurons and RB cells, we found that *lim1* expression extended beyond that of the motorneurons, but did not extend as far posteriorly as the RB cells (Fig. 6E,F; compare F to D and E). Thus, these interneuronal precursors may not be responsive to Shh signaling, while they are sensitive to Bmp signaling.

DISCUSSION

An early genetic requirement for Bmp signaling in dorsal neural tissue specification

Here we show that mutants defective in Bmp2b/Swirl, Bmp7/Snh or Smad5/Sbn activity display strongly reduced or no neural crest or dorsal sensory RB neurons of the spinal cord. An increase in apoptosis is not observed in these mutants indicating that the loss of dorsal cell types is caused by a lack of their specification. Our genetic analysis strongly supports explant and ectopic expression studies indicating that Bmp signaling plays a role in the specification of dorsal spinal cord cell fates (Liem et al., 1995, 1997; Neave et al., 1997). Our results provide the first genetic evidence for the role of a Bmp signaling pathway in dorsal spinal cord cell fate specification.

Previous studies suggested a role for Bmp signaling from the epidermal ectoderm lying adjacent to the neural plate as well as Bmp and other TGFβ signaling within the dorsal neural tube at later stages. The restricted coexpression of *bmp2b/swr* and *bmp7/snh* to the ventral gastrula and tail bud during somitogenesis stages in the zebrafish indicates a very early role for Bmp signaling in the establishment of dorsal spinal cord cell fates. Expression of *bmp2b/swr* and *bmp7/snh* is not detectable in the epidermal ectoderm, roof plate, or the dorsal neural tube. Hence, the Bmp2b/Swirl and Bmp7/Snh activity required for dorsal neural cell fate specification of the rostral spinal cord functions during and/or at the end of gastrulation. The similarity in the defects observed in *sbn/smad5* mutants to those in *bmp2b* and *bmp7* mutants suggests that Bmp2b/Bmp7 signaling is mediated through the downstream-acting transcriptional regulation of Smad5/Sbn, together perhaps with Smad1. Our experiments do not address whether Bmp2b and Bmp7 signaling acts directly or indirectly in the specification of dorsal neural cell fates; however, the early specification of these cells is consistent with a direct role.

Bmp signaling specifies and restricts the number of intermediate *lim1+* interneurons

We demonstrate a role for Bmp2b and Bmp7 signaling in limiting the number of *lim1+* interneurons. In some *swr/bmp2b* and *snh/bmp7* mutant embryos, we observe a 5-fold expansion in the number of *lim1+* interneurons. Specification of these interneurons also depends on Bmp activity, since they are absent in *swr/bmp2b* mutant embryos injected with the Bmp antagonist Chordin. Specification of these interneurons is not caused by low Bmp2b activity levels remaining in *swr/bmp2b* mutant embryos, since this effect is observed in *swr/bmp2b* presumptive null mutants.

It is possible that the expansion of *lim1+* interneurons depends on Bmp and Hh signaling. One class of *lim1+* interneurons in the chick, V1 neurons, can be induced by low Shh concentrations in explant assays (Ericson et al., 1997). Since the floor plate is broader in all three dorsalized mutants, although also shorter in its length, there may be more Hh signaling emanating from the floor plate, which could be required for the *lim1+* interneuron expansion. Although the number of primary motorneurons is not increased in the

dorsalized mutants, this could reflect normal tail bud expression of *shh* (see discussion below). Our analysis of *ntl* mutants, however, suggests that expanded floor plate Shh signaling is not sufficient to expand greatly or inhibit *lim1+* interneuron specification. Moreover, the enlarged floor plate of *ntl* mutants does not affect the amount of neural crest or dorsal RB neurons. Consistent with a lack of a role for Hh signaling in *lim1+* interneuron specification, recent work from Pierani et al. (1999) demonstrates that Shh is not required in vivo for *lim1+* V1 interneuron specification in the mouse.

Involvement of tail bud Hh signaling in dorsal neural cell fate restriction

Although Hh signaling may not be involved in *lim1+* interneuron specification, our results suggest a specific role for tail bud *hh* expression in the restriction of dorsal neural cell fates. In *flh* mutants, which display no notochord cells and a progressive anterior-to-posterior loss in *shh*-expressing floor plate cells, we detected no change in the amount of neural crest or RB cells, even in regions with no *shh*-expressing floor plate cells. These results are similar to those observed in the chick, where absence of the notochord and floor plate does not cause a change in the number or location of two dorsal neuronal cell types (Liem et al., 1997). In contrast, in posterior regions of *ntl* mutants, where no *shh* expression is detected in the floor plate or notochord, an apparent dorsalization of neural tissue is observed in the expansion of the neural crest and dorsal RB cells, and a loss of the floor plate and motorneurons.

Both *flh* and *ntl* mutants exhibit short axial regions absent of *shh*-expressing floor plate and notochord, yet *ntl* mutants display a dorsalization of neural tissue, while *flh* mutants do not. The apparent paradox in the dorsalization of neural tissue in caudal segments of *ntl* mutants and the normal specification of dorsal cell types in *flh* mutants can be resolved when considering the tail bud *shh* expression domain in these mutants. As noted previously, *shh* is expressed weakly within the tail bud of *flh* mutants (Fig. 5D′) and this expression domain is postulated to specify the primary motorneurons found in *flh* mutant embryos (Beattie et al., 1997). We similarly hypothesize that *hh* tail bud expression ventralizes neural tissue at a stage prior to overt floor plate or notochord development. In posterior regions of *ntl* mutants, the tail bud, in addition to the floor plate and notochord expression domains of *shh* are absent, which could explain the dorsalization of the neural tissue in these regions. A similar explanation could be made for the lack of expansion of dorsal neurons in the chick notochord extirpation experiment (Liem et al., 1997), while removal of both the notochord and node causes an expansion in a dorsal interneuron cell type (Fedtsova and Turner, 1997). Explant assays from *Xenopus* also suggest an early specification of ventral spinal cord cell types (Saha et al., 1997). Thus, Hh signaling may act at very early stages of neuroectoderm tissue development, prior to formation of either the notochord or floor plate, to ventralize the neuroectoderm and restrict the specification of dorsal cell fates. Alternatively, the dorsalization in caudal regions of *ntl* mutants may be caused by the loss of a different, or additional, signal to Hh.

The dorsalization of neural tissue observed in *ntl* mutants requires Bmp2b signaling, as revealed from double-mutant analysis. The mechanism by which the dorsalization occurs and depends on Bmp2b activity is unclear. Perhaps a loss of Hh signaling allows a Bmp activity gradient to extend into more ventral neural regions, or a larger region of cells may be competent to respond to Bmp activity. Further investigations will be required to resolve the possible interaction of these two signaling systems.

Mechanism of Bmp action in neural patterning

Models for dorsoventral patterning of the embryonic axis hypothesize a gradient of Bmp activity that establishes different cell types along the dorsoventral axis in a concentration-dependent manner (Dosch et al., 1997; Jones and Smith, 1998; Knecht and Harland, 1997; Neave et al., 1997; Nguyen et al., 1998; Wilson et al., 1997). This same Bmp2b/Bmp7-dependent gradient acting during gastrulation may function in the specification of the neural crest, RB neurons and *lim1+* interneurons of the rostral spinal cord in the zebrafish. In such a model, two Bmp activity levels must be functional in prospective trunk neural tissue: a low level specifying the *lim1+* interneurons, while a higher level specifies the neural crest and dorsal RB neurons, which appear to be derived from a similar progenitor population (Artinger et al., 1999, R. A. Cornell and J. S. Eisen, personal communication). Bmp activity levels higher than that required for neural crest/RB specification are expected to inhibit neural tissue development (Hawley et al., 1995; Nguyen et al., 1998; Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995). Modulation of Bmp activity levels supports the action of a Bmp gradient in neural crest specification (V. H. N. and M. C. M., unpublished).

The two postulated Bmp activity levels are consistent with the distances of these progenitor cells from the Bmp-expressing non-neural ectoderm: the prospective neural crest and RB neurons lie close to the neural/non-neural ectodermal boundary, whereas intermediate neural tube progenitors lie more distant or closer to the future ventral midline (Papan and Campos-Ortega, 1994; Schmitz et al., 1993). We hypothesize that the higher Bmp activity level, required for trunk neural crest/RB neuron specification, is present in a greatly reduced domain or absent in these mutants. In contrast, the low Bmp signaling level that specifies *lim1+* cells is present in all *swr/bmp2b*, *sbn/smad5* and *snh/bmp7* embryos, and, in some of these mutants, this level is present in an expanded domain. The higher Bmp activity level may also inhibit *lim1+* interneuron specification.

If a gastrula Bmp activity functions in the establishment of *lim1+* cells, what is the source of Bmp activity in *swr/bmp2b*, *sbn/smad5* and *snh/bmp7* mutants? The *snh*^{ty68a} allele analyzed here is a hypomorphic mutation retaining some Bmp7 activity (Schmid et al., 2000), which may function in this process. In *sbn/smad5* mutants, the closely related *smad1* gene is expressed and may mediate some Bmp function in these mutants, possibly together with residual maternal Smad5 activity (Hild et al., 1999). In *swr/bmp2b* and *sbn/smad5* mutants, *bmp4* and *bmp7* expression is initiated, but is not maintained during gastrulation stages (Hild et al., 1999; Kishimoto et al., 1997; Nguyen et al., 1998; Schmid et al., 2000). Thus, this early *bmp* expression may provide the presumptive low Bmp activity remaining in these mutants. In *sbn/smad5* and *snh/bmp7* mutants, diminishing Bmp activity levels remaining as development proceeds may cause the opposite effects observed in these mutants on specification of

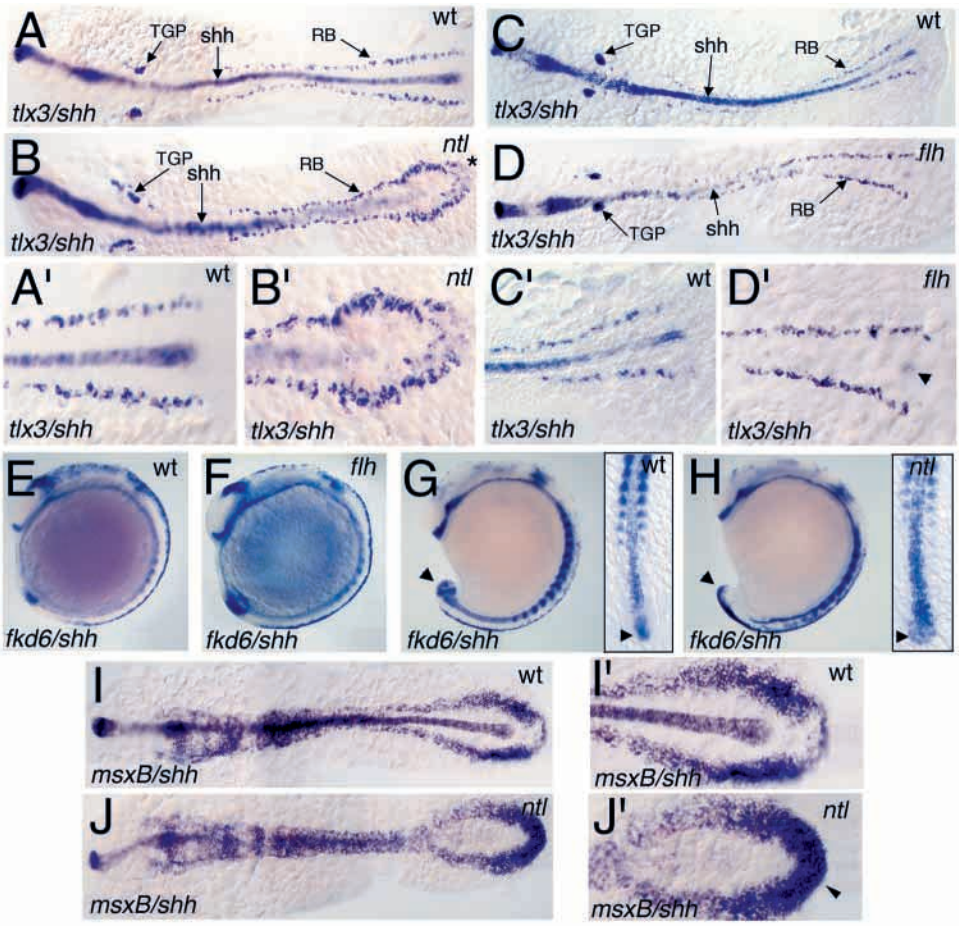


Fig. 5. Normal and expanded dorsal neural cell types in *flh* and *ntl* mutants, respectively. In 7-somite-stage embryos, rostral trunk regions of *ntl* mutants display normal numbers of *tlx3*-expressing RB precursors, while the caudalmost region, where *shh* expression is absent, exhibits an expansion in RB neurons (A,B, * marks the caudal region). (A′,B′) Enlargements of the posterior regions of A,B, showing the expanded RB cells in *ntl* mutants. In 10-somite-stage *flh* mutants, the RB cells and neural crest appear normal, even in regions where no *shh*-expressing midline cells are observed (C-F). (C′,D′) Enlargements of C,D showing weak *shh* expression in the tail bud of *flh* mutants. Caudal regions of *ntl* mutants display a mild expansion in the *fkd6* neural crest domain at the 15-somite-stage (G,H) and *msxB* expression in 7-somite-stage embryos (I,J). Arrowheads in G marks *shh/fkd6* tail bud expression in wild type. Arrowheads in H indicate the expanded *fkd6* expression domain in *ntl* mutants. (I′,J′) Enlargements of the posterior region of I,J, showing the loss of midline and tail bud *shh* expression in *ntl* mutants and expanded *msxB* expression. (A-J) Double in situ hybridizations including *shh*, except the insets in G and H where *shh* was omitted because tail bud *shh* expression obscures the *fkd6* expression in flattened embryos.

the cranial versus trunk neural crest. A similar Bmp level may specify both neural crest domains, however, the later specification of the trunk versus the cranial neural crest and diminishing Bmp activity may lead to the different effects. The range from near normal to a 5-fold expansion of *lim1+* cells and the presence of a few RB and trunk neural crest cells in some mutants may reflect slightly different Bmp activity levels remaining in mutant embryos.

The later onset of expression of *lim1* in the interneurons, relative to *tlx3*/*isl1* expression in the RB neurons, could reflect a later specification of interneuron cells via a different mechanism. The gastrula Bmp activity gradient may not directly specify *lim1+* neurons and instead a second domain of Bmp or TGFβ expression may do so. This second domain is expected to correspond to a Bmp, since *lim1+* cell specification is inhibited by *chordin* overexpression and Chordin specifically binds to the Bmp subclass of TGFβ proteins (Piccolo et al., 1996). In the mouse, chick and frog, multiple *bmp* genes are expressed in the roof plate and/or dorsally in the neural tube. Hence, a dorsal neural tube Bmp expression domain may be

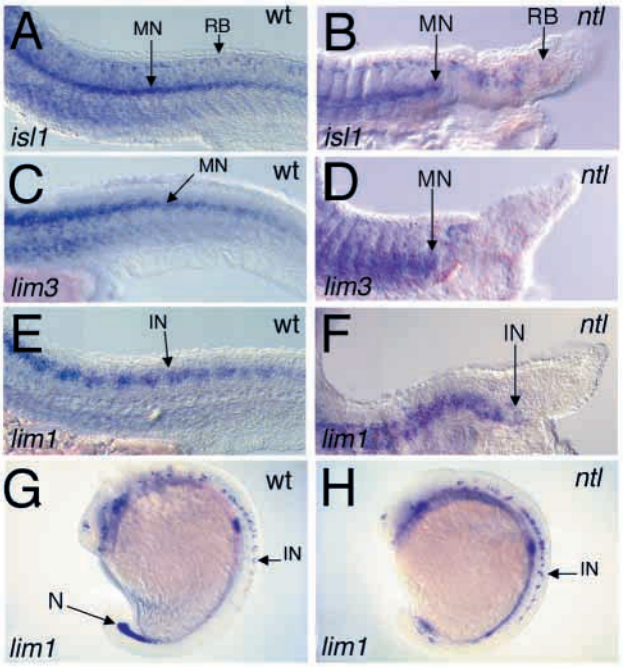


Fig. 6. Absence of ventral motorneurons and presence of dorsal RB neurons in caudal segments of *ntl* mutants. The *isl1*- and *lim3*-expressing motorneurons are absent in caudal regions of *ntl* mutants, whereas the *isl1*-expressing RB neurons are present (A-D). Arrows mark the posterior extents of the expression domains. In caudal regions of *ntl* mutants, *lim1*-expressing interneurons (E,F) extend further posteriorly than the motorneurons (B,D), but do not extend as far posteriorly as the RB neurons (B). The *lim1*-expressing interneurons appear normal in rostral *ntl* segments, where *shh* expression is expanded in the floor plate (G,H). Anterior is to the left in A-F, dorsal to the top. Anterior is to the top in G,H, dorsal to the right.

required for *lim1+* cell specification. It is possible that a low gastrula Bmp activity level induces this second Bmp domain. Alternatively, Bmp activity may not act in the specification of *lim1+* neurons, but function to inhibit *lim1+* interneuron cell proliferation, as Bmps have been implicated in restricting cell proliferation in development (reviewed in Hogan, 1996).

The Bmp activity(s) required for *lim1+* interneuron, neural crest and dorsal sensory neuron cell fate specification may not specify these cells directly. Instead it could act to generate zones of competence, within which other factors function to establish different cell fates. For example, Notch-Delta lateral inhibition is involved in the segregation of the RB neurons and trunk neural crest cells from a common population in the zebrafish neural plate (R. A. Cornell and J. S. Eisen, pers. comm.). A Bmp activity gradient may establish a progenitor population and subsequently a Notch-Delta interaction may establish the RB and neural crest cell types.

Concluding remarks

The early specification of the RB sensory neurons along with their dependence on Bmp2b/Swirl and Bmp7/Snh signaling suggests that dorsalization of neural tissue occurs at a very early stage in development, likely mediated by a Bmp activity gradient during gastrulation. Via either a direct or indirect mechanism, gastrula Bmp2b/Swirl and Bmp7/Snh activity also restricts the number of intermediate-positioned interneurons of the rostral spinal cord and, in addition, may specify these cells via a low Bmp activity level. At postgastrulation stages, the dorsalization of neural tissue is likely mediated through the posterior tail bud expression domains of *bmp2b* and *bmp7*. Ventralization of neural tissue may occur at similar stages through Shh signaling in the dorsal organizer of the gastrula and the anterior tail bud at postgastrulation stages. Further experiments will be required to establish the direct or indirect nature of these events and their precise timing in the zebrafish and other vertebrates.

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