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Bmp activity establishes a gradient of positional information throughout the entire neural plate

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SUMMARY

Bone morphogenetic proteins (Bmps) are key regulators of dorsoventral (DV) patterning. Within the ectoderm, Bmp activity has been shown to inhibit neural development, promote epidermal differentiation and influence the specification of dorsal neurons and neural crest. In this study, we examine the patterning of neural tissue in mutant zebrafish embryos with compromised Bmp signalling activity. We find that although Bmp activity does not influence anteroposterior (AP) patterning, it does affect DV patterning at all AP levels of the neural plate. Thus, we show that Bmp activity is required for specification of cell fates around the margin of the entire neural plate, including forebrain regions that do not form neural crest.

Surprisingly, we find that Bmp activity is also required for patterning neurons at all DV levels of the CNS. In *swirl/bmp2b*$^{-}$ (*swr*$^{-}$) embryos, laterally positioned sensory neurons are absent whereas more medial interneuron populations are hugely expanded. However, in *somitabun*$^{-}$ (*sbn*$^{-}$) embryos, which probably retain higher residual Bmp activity, it is the sensory neurons and not the interneurons that are expanded. Conversely, in severely Bmp depleted embryos, both interneurons and sensory neurons are absent and it is the most medial neurons that are expanded. These results are consistent with there being a gradient of Bmp-dependent positional information extending throughout the entire neural and non-neural ectoderm.

Key words: Bone morphogenic protein, Dorsoventral patterning, Neural plate, *swirl*, *somitabun*, zebrafish

INTRODUCTION

Members of the Bmp family of signalling proteins are involved in embryonic DV patterning in both vertebrates and invertebrates (Bier, 1997). Overexpression studies in fish and frogs have demonstrated that both Bmp2 and Bmp4 are potent ventralising agents that inhibit the development of dorsal structures (Dale and Jones, 1999). Within the developing mesoderm, it has been proposed that Bmp4 is a morphogen, specifying at least four cell types at different concentrations (Dosch et al., 1997). However, it seems unlikely that either Bmp2 or Bmp4 can diffuse over significant distances in the embryo and any gradient in Bmp activity is therefore unlikely to arise from Bmp proteins diffusing far from their site of secretion (Jones et al., 1996; Nikaido et al., 1999). Instead, the level of Bmp signalling may be modulated by a diverse group of proteins that emanate from the dorsal side of the embryo and inhibit Bmp activity. Chordin, Noggin, Cerberus and Follistatin can all bind directly to Bmps and overexpression studies have demonstrated that these proteins indeed dorsalise embryos through the inhibition of Bmp activity (Dale and Wardle, 1999; Piccolo et al., 1999). Furthermore, mutations in the *chordin* gene, *chordino*, in fish (Schulte-Merker et al., 1997) and its *Drosophila* ortholog, *sog* (Zusman et al., 1988), lead to disrupted DV patterning. Unlike Bmp2 and Bmp4, Noggin and Chordin may diffuse within the embryo and so a gradient of Bmp activity could be established through widespread production of Bmp proteins whose signalling activity is modulated by the activity of Bmp antagonists diffusing from the dorsal side of the embryo (Jones and Smith, 1998).

Bmp signalling also regulates DV patterning of the ectoderm, possibly independently of the mesoderm, by promoting epidermal fate and inhibiting neural development (Wilson and Hemmati-Brivanlou, 1995). A variety of studies also suggest a role for Bmp signalling in the development of neural crest, the roof plate and dorsal spinal cord neurons (Lee and Jessell, 1999). Neural crest cells are only specified in regions caudal to the diencephalon and in chick it has been suggested that their absence from more rostral regions may in part be due to an absence of early Bmp activity at the margin of the rostral neural plate (Muhr et al., 1997; and see Shimamura and Rubenstein, 1997). Instead, Bmp activity in the rostral CNS has been proposed to direct ventral forebrain

cells towards a hypothalamic rather than a floorplate fate (Dale et al., 1997), and regulate late developmental events in the dorsal forebrain (Furuta et al., 1997; Solloway and Robertson, 1999).

The evidence that Bmps have direct concentration-dependent effects upon cell fate in the ectoderm initially came from in vitro experiments in frogs in which cells exposed to different concentrations of Bmps, or Bmp antagonists, can develop as either epidermis, cement gland or neural tissue (Knecht and Harland, 1997; Wilson et al., 1997). These in vitro studies have been supported by analysis of zebrafish Bmp pathway mutants in which different non-neural ectodermal fates appear to be specified by different levels of Bmp activity (Nguyen et al., 1998; and see Neave et al., 1997). It therefore seems likely that ectodermal cells are just as capable of responding to alterations in Bmp activity as are mesodermal cells.

In this study, we address the role of Bmp activity in early neural patterning in zebrafish, primarily through phenotypic analysis of *swr*$^{-}$ embryos which carry a null mutation in the *bmp2b* gene (Kishimoto et al., 1997; Nguyen et al., 1998). Although there is a second *bmp2* gene in zebrafish, *bmp2a* (Martinez-Barbera, 1997), its onset of expression after the end of gastrulation suggests that it is unlikely to play a role in the early patterning described in this study. Supporting previous observations (Nguyen et al., 1998), we find that neural tissue is expanded throughout the ventral ectoderm in *swirl/bmp2b*$^{-}$ (*swr*$^{-}$) embryos. Within the neural plate, sensory neurons are absent whereas interneurons, medial neurons and even floorplate are expanded, suggesting that a gradient of Bmp activity is present throughout the neural ectoderm, from the laterally positioned sensory neurons to the medially positioned floorplate. In contrast to in vitro results in chicks (Muhr et al., 1997), we show that early Bmp signalling in zebrafish does play a crucial early role in patterning the margin of the neural plate in regions rostral to the neural crest. Early markers of dorsal diencephalic and telencephalic fate are spatially regulated by Bmp activity and therefore, at all AP levels, the medial and lateral extent of ectodermal gene expression domains may be determined by thresholds of sensitivity to Bmp signalling.

MATERIALS AND METHODS

Zebrafish lines

swirl: swr^{ta72} and *somitabun: sbn*^{tc24b} fish were maintained on a 14-hour light/10-hour dark cycle in fish facilities at UCL and the MPI in Tübingen.

Experimental protocols

Standard procedures were followed for in situ hybridisation analysis. All cDNAs used for in situ hybridisation are published with the exception of *nk2.1* (K. B. R., unpublished) and *nhg1* (neuronal-HLH-gene-1) which was kindly provided by Martin Gering. To generate dorsalised embryos we injected RNA encoding *Xenopus* Noggin. Injections were performed as previously described for *shh* (Barth and Wilson, 1995). Embryos to be sectioned were dehydrated in ethanol, embedded in Araldite and sectioned using standard procedures.

RESULTS

swirl mutant embryos (*swr*$^{-}$) harbour a mutation in the zebrafish *bmp2b* gene and exhibit a dorsalised phenotype (Kishimoto et al., 1997; Nguyen et al., 1998). To elucidate the role of Swirl in patterning the ectoderm, we compared the expression of a variety of genes expressed at specific DV and AP positions in the ectoderm of wild-type and *swr*$^{-}$ embryos.

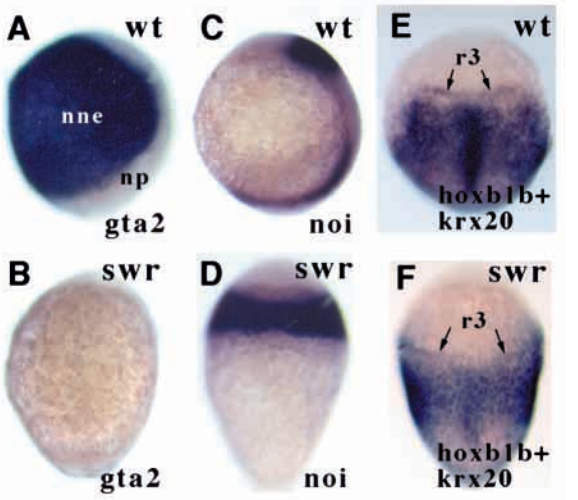

Fig. 1. The ectoderm of *swr*$^{-}$ embryos is neuralised. In lateral views, dorsal is to the right; in dorsal views, anterior is up. (A,B) Lateral views of *gta2* expression (prospective epidermis) in bud stage embryos. (C,D) Lateral views of *noi/pax2.1* expression (prospective midbrain) in 1-2 somite stage embryos. (E-F) Dorsal views of *hoxb1b* (posterior tissue) and *krx20* (r3 + r5) in 1-2 somite stage embryos. Scale in this and subsequent figures: the yolk ball of wild-type embryos is 700 μm across. nne, non-neural ectoderm; np, neural plate; r3, rhombomere 3; wt, wild-type.

AP positional values are retained throughout the neuralised ectoderm of *swr*$^{-}$ embryos

Confirming previous observations (Nguyen et al., 1998) we found that the ventral ectoderm of *swr*$^{-}$ embryos is neuralised. Thus by the end of gastrulation, markers of non-neural ectoderm such as *gta2* (Detrich et al., 1995) were absent (Fig. 1B) whereas neural markers such as *pax2.1/noi* (Krauss et al., 1991) were expanded throughout the ectoderm (Fig. 1D).

From experiments originally performed by Spemann and colleagues, it has been suggested that AP positional values within the neural plate are influenced by signals arising from the organiser and its axial derivatives (Lemaire and Kodjabachian, 1996; Harland and Gerhart, 1997). In *swr*$^{-}$ embryos, there is no duplication of organiser tissue on the ventral side of the embryo and so if AP positional values depend upon the organiser, one might expect these values to be disrupted in neural tissue specified in the ventral ectoderm, distant to the organiser. However, although the DV limits of ectodermal gene expression domains were radically altered in *swr*$^{-}$ embryos, there was no alteration in their relative AP positions. For instance, *noi/pax2.1* (Fig. 1D) was expressed in a band of cells throughout the DV extent of the ectoderm at the level of the midbrain and *hoxb1b* (Alexandre et al., 1996; renamed in Amores et al., 1998) maintained an anterior boundary of expression caudal to r3 (Fig. 1F). These and other observations (see below), indicate that AP positional values are intact throughout the entire DV extent of the ectoderm of *swr*$^{-}$

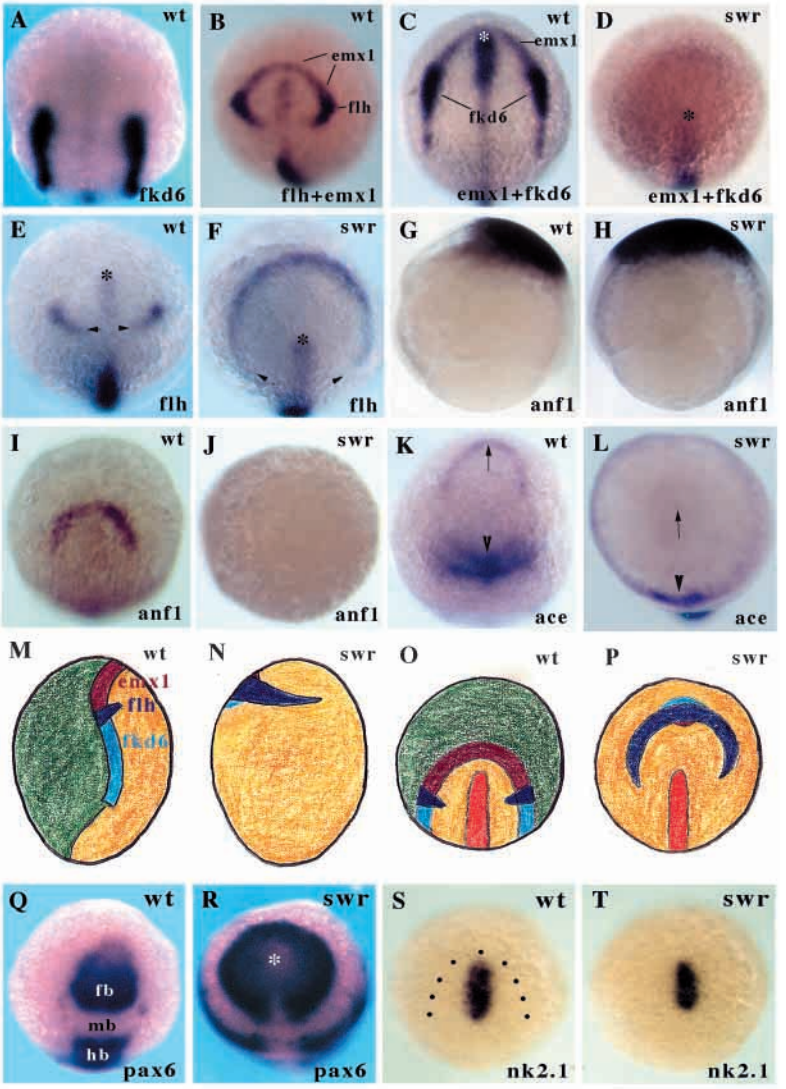

Fig. 2. Swirl/Bmp2b affects fates at the margin of the neural plate at all AP levels. Animal pole (A-F,I-L,O-T) and lateral (G,H,M,N) views of 1-2 somite stage (except G,H) embryos. (A) *fkd6* expression in prospective neural crest cells at the margins of the neural plate. (B) *flh* expression in prospective dorsal diencephalon and *emx1* in prospective telencephalic cells around the rostral margin of the neural plate. The posterior limit of *emx1* expression overlaps with *flh* expression. The posterior boundary of *flh* expression is at the anterior boundary of *fkd6* expression. (C,D) *emx1* and *fkd6* expression. In the *swr*$^{-}$ embryo, marginal expression of both genes is absent. The asterisk indicates the rostral limit of axial tissue (also marked by transient *fkd6* and *flh* expression in rostral midline) in this and other panels. (E-F) *flh* expression in prospective dorsal diencephalon. In the *swr*$^{-}$ embryo, *flh* expression is radially expanded into the ventral ectoderm and the gap between the domains of *flh* expression on the dorsal side of the embryo is expanded (arrowheads). (G-J) *anf1* expression in 80% (G-H) and 1-2 somite stage (I-J) embryos. *anf1* is initially expanded in *swr*$^{-}$ embryos but is lost by the stage that expression is restricted to prospective telencephalic cells. (K,L) *ace/fgf8* expression. Telencephalic expression (arrow) is absent in *swr*$^{-}$ embryos whereas more caudal expression domains (arrowheads) are radialised. (M-P) Schematic illustrations of marginal neural plate cell fates in wild-type (M,O) and *swr*$^{-}$ (N,P) embryos. M,N are lateral views and O,P animal pole views of bud-1 somite stage embryos. Green is non-neural tissue, yellow is neural plate. Pale blue shows *fkd6* in prospective neural crest, dark blue shows *flh* expression in dorsal diencephalic cells and maroon represents *emx1/anf/ace* expression in prospective telencephalic cells. Marginal cell fates are absent in anterior and posterior regions but at the level of the diencephalon, *flh* is expanded and some *fkd6* and *emx1* expression may be retained. (Q,R) *pax6* expression. Diencephalic and hindbrain *pax6* expression is expanded in the mutant embryo. The asterisk indicates the anterior limit of the axis. (S,T) *nk2.1* expression in the prospective hypothalamus of wild-type and *swr*$^{-}$ embryos. Dots indicate the approximate margin of the neural plate. fb, forebrain; hb, hindbrain; mb, midbrain.

embryos, at least in the head and anterior trunk regions. In tail regions however, *swr*$^{-}$ embryos exhibited severe patterning defects and AP patterning may indeed be disrupted (see Fig. 3K,P below).

Swirl activity is required for patterning the prospective neural crest, dorsal diencephalon and telencephalon

Bmp signalling is important for specification of neural crest fates (Lee and Jessell, 1999). We therefore analysed the regulation of *fkd6*, an early marker of neural crest (Odenthal and Nüsslein-Volhard, 1998) that is expressed in bilateral bands of cells at the margin of the neural plate (Fig. 2A). In most *swr*$^{-}$ embryos, *fkd6* expression is absent (Fig. 2D; and see Ngyuyen et al., 1998). However, on the ventral side of the embryo, at the level of the midbrain, some *swr*$^{-}$ embryos showed a small patch of *fkd6* expression (Fig. 2N,P) suggesting that in rostral regions, early steps in neural crest specification can occur.

The role of Bmp activity in the specification of marginal neural plate fates rostral to the prospective neural crest is unclear (Muhr et al., 1997; Shimamura and Rubenstein, 1997; Golden et al., 1999) and we therefore examined whether marginal cell fates at diencephalic and telencephalic levels are regulated by Swirl activity.

floating head (*flh*) is expressed in the prospective dorsal diencephalon (Fig. 2E; Masai et al., 1997), just rostral to neural crest markers (Fig. 2M,O). In *swr*$^{-}$ embryos, *flh* expression expands throughout the ventral ectoderm (Fig. 2F). In addition to the ventral expansion in expression, the distance between the left and right domains of *flh* expression on the dorsal side of the embryo was increased (compare Fig. 2E,F), suggesting that cell fates medial to the domain of *flh* expression are expanded (see below). Finally, in wild-type embryos, *flh* is expressed in a DV gradient with highest levels of transcripts laterally and expression tailing off medially in the neural plate (Fig. 2E). This graded expression was retained, although expanded, in *swr*$^{-}$ embryos, with highest levels of expression at the ventral midline of the embryo (Fig. 2F).

Analysis of expression of *fgf8*, *emx1* and *anf1* all indicate that telencephalic fates are also dependent upon Swirl function. Genes characteristic of the telencephalon are expressed in a horseshoe-shaped band of cells around the rostral margin of the neural plate (Houart et al., 1998). *anf1* is initially expressed

throughout the anterior neural plate (Fig. 2G; Kazanskaya et al., 1997) but by the 1-2 somite stage, expression becomes restricted to the rostral margin of the neural plate (Fig. 2I). In *swr*⁻ embryos, *anf* expression is initially expanded throughout the anterior ectoderm (Fig. 2H). However, this expanded expression is not maintained, and by the stage at which *anf* expression is normally restricted to marginal cells, it is absent in *swr*⁻ embryos (Fig. 2J). *fgf8* is expressed in a variety of locations (Reifers et al., 1998) including a narrow band of cells at the rostral margin of the neural plate (Fig. 2K). This rostral domain of expression is lost in *swr*⁻ embryos (Fig. 2L). Finally, *emx1* is expressed in cells around the margin of the rostral neural plate fated to become telencephalon (Morita et al., 1995; Z. Varga, personal communication). At the 1-2 somite stage, the posterior boundary of *emx1* expression reaches the anterior boundary of *fkd6* expression (Fig. 2C). In *swr*⁻ embryos, *emx1* expression was either absent (Fig. 2D) or severely reduced with weak posterior radialised expression in the region of co-localised expression with *flh* (Fig. 2N,P).

These results indicate that marginal neural plate fates at telencephalic and midbrain/hindbrain levels are absent in *swr*⁻ embryos, whereas marginal cell fates at the level of the diencephalon are expanded (Fig. 2N,P). We show below that this expansion may be due to higher levels of residual Bmp activity being retained in diencephalic regions of *swr*⁻ embryos.

Medial and ventral forebrain is present in *swr*⁻ embryos

Although telencephalic gene expression is absent in *swr*⁻ embryos, other forebrain territories are present. *pax6* (Krauss et al., 1991) is normally expressed throughout the eye fields and much of the forebrain (Fig. 2Q). In *swr*⁻ embryos, expression is radialised and as with other markers, the relative positions of boundaries of expression along the AP axis are maintained (Fig. 2R).

The most rostral territory of the ventral forebrain is the hypothalamus. In vitro, Bmp7 in combination with Shh induces markers of hypothalamic tissue at the expense of floorplate tissue (Dale et al., 1997), raising the possibility that in vivo, Bmp activity may regulate the specification of the hypothalamus. *nk2.1* is expressed in hypothalamic tissue (K. B. R., unpublished observations) of wild-type embryos (Fig. 2S) and this expression is relatively normal in *swr*⁻ embryos (Fig. 2T). These results indicate that in contrast to more dorsal forebrain territories, absence of Swirl has little effect upon the specification of the hypothalamus.

Swirl determines the DV position at which specific classes of neurons are specified

Bmp family genes (including *swirl*) are expressed in non-neural ectoderm adjacent to the neural plate and it has been

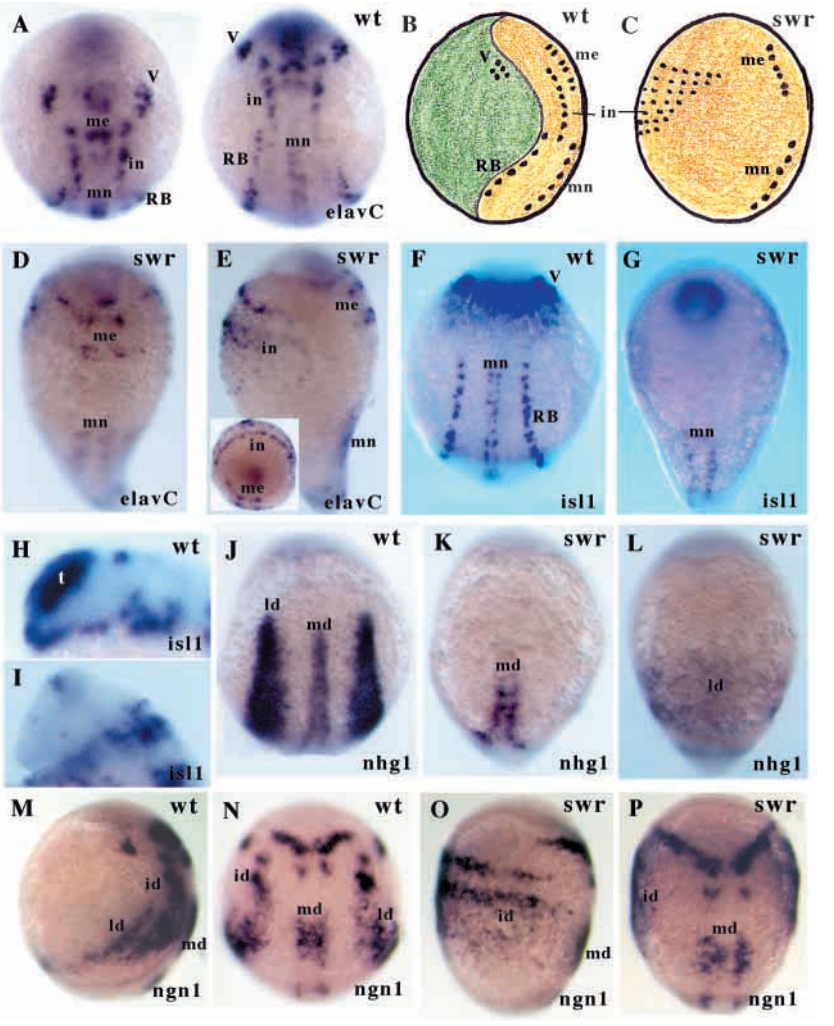

Fig. 3. Swirl/Bmp2b affects neurogenesis throughout the neural plate. All embryos are 1-2 somite stage (except H,I which are 14 somite stage). (A) Animal pole (left) and dorsal (right) views of *elavC* expression in neurons in the neural plate of wild-type embryos. (B,C) Schematic illustrations of lateral views of neuronal patterning in wild-type and *swr*⁻ embryos. Green is non-neural ectoderm, yellow is the neural plate and the dots represent neurons. In *swr*⁻ embryos, trigeminal and Rohon-Beard neurons are absent while interneurons are massively radially expanded on the ventral side of the embryo. (D) Dorsal and (E) lateral views of *elavC* expression in *swr*⁻ embryos. The interneuron population is hugely expanded. The inset shows an animal pole view of the same embryo. (F,G) Dorsal views of *isl1* expression in wild-type and *swr*⁻ embryos. The Rohon-Beard and trigeminal neurons are absent in the *swr*⁻ mutant. (H,I) *isl1* expression in wild-type and *swr*⁻ embryos. Neurogenesis is considerably reduced in the anteriormost CNS of the *swr*⁻ embryo as compared to wild type. (J,K) Dorsal and (L) ventral views of *nhg1* expression in the ectoderm of wild-type and *swr*⁻ embryos. The lateral stripes of *nhg1* expression are either absent (K) or are expanded and fused on the ventral side in *swr*⁻ embryos (L). (M,O) Lateral and (N,P) dorsal views of *ngn1* in wild-type and *swr*⁻ embryos. In the *swr*⁻ embryo, lateral domains of expression are lost, intermediate domains hugely expanded and expression in the vicinity of motorneurons is also slightly broader. in, interneurons; id, intermediate domain; ld, lateral domain; me, medial neurons; md, medial domain; mn, motor neurons; RB, Rohon-Beard neurons; V, trigeminal neurons.

proposed that Bmp signalling may be required for the specification of neurons near the neural/non-neural ectodermal interface. We therefore assessed whether these and other neurons are affected in *swr*⁻ embryos. To distinguish different classes of neurons (Fig. 3B) we used three primary criteria: expression or co-expression of markers (for instance, rostral interneurons express *elavC* but not *isl1* whereas trigeminal neurons express both genes; Fig. 3A,F); AP position with respect to other neurons and other neuroepithelial markers (for instance, trigeminal neurons, when present, were always just rostral to interneurons at the level of the midbrain, Fig. 3A); DV position with respect to other neurons in similar AP locations (for instance, although Rohon-Beard sensory neurons and motor neurons expressed *isl1* and *elavC*, motor neurons were always medial to sensory neurons; Fig. 3F).

Analysis of neuronal markers indicated that sensory neurons are absent in *swr*⁻ embryos. *elavC* is expressed in most neurons shortly after their birth (Kim et al., 1996) and labels many discrete populations of neurons in the neural plate (Fig. 3A), some of which also express *isl1* (Korzh et al., 1993; Fig. 3F). Both genes label laterally positioned Rohon-Beard sensory neurons and trigeminal ganglia (Fig. 3A,F) and in *swr*⁻ embryos, both groups of neurons are absent (Fig. 3C,E,G).

The prevalent model of ectodermal patterning suggests that Bmp signalling in the ventral ectoderm promotes non-neural fates, absence of Bmp signalling in dorsal ectoderm allows neural development and local activity of Bmp proteins at the neural/non-neural interface may influence neuronal identity. However, analysis of *elavC*, *isl1* and *ngn1* expression indicated that loss of Swirl function affects neurogenesis at all DV levels of the ectoderm. In the hindbrain, *elavC* is expressed in several clusters of interneurons and in medially positioned neurons close to the floor plate (Fig. 3A). In *swr*⁻ embryos, the distance between the bilateral clusters of medially positioned neurons was slightly increased but the size of the clusters was not significantly affected (Fig. 3D). In contrast, interneurons were vastly increased in number and distributed over the entire ventral ectoderm (Fig. 3E). Very strikingly, each DV string of neurons retained its relative AP position with respect to other clusters of neurons (Fig. 3E) suggesting that neurons at specific AP positions are serially reiterated around the DV axis. In addition to the expansion of interneurons, the number of cells between the interneurons and the medially positioned neurons was also expanded in *swr*⁻ embryos (Fig. 3D,E).

In the trunk, cell fates in intermediate regions of the neural plate were also affected dramatically in *swr*⁻ embryos. *nhg1* is a novel bHLH gene expressed in broad lateral and narrow medial domains of cells in the neural plate (M. Gering, personal communication; and Fig. 3J). The position of the lateral domains is just posterior and just medial to the bands of *fkd6* expression described above (not shown). The lateral bands of *nhg1* expression were either absent in *swr*⁻ embryos (Fig. 3K) or were occasionally fused and expanded on the ventral side of the embryo (Fig. 3L). In the trunk, *ngn1* marks medial, intermediate and lateral neurogenic domains that probably give rise to motor neurons, interneurons and sensory neurons, respectively (Blader et al., 1997). As expected, the lateral domain of *ngn1* expression was absent and the intermediate domain hugely expanded in *swr*⁻ embryos (Fig. 3O). Furthermore, the medial domain of *ngn1* expression was broader in *swr*⁻ embryos (Fig. 3P) as compared to wild type (Fig. 3N). This was mirrored by a slight broadening of the rows of *elavC*- and *isl1*-expressing motor neurons (Fig. 3D,G; and data not shown). The rows of motorneurons were also shorter in *swr*⁻ embryos than in wild type. This appeared to be due to posterior truncation as the rostral boundary of the motor neurons was not significantly altered with respect to other ectodermal markers (*nhg1+ krox20*, *elavC + krox20*, data not shown).

The majority of *swr*⁻ embryos exhibit abnormal morphogenesis during somite stages and so detailed interpretation of neuronal patterning at late stages was impractical. However, correlating with the loss of telencephalic gene expression, there was a striking loss of *isl1*-expressing neurons in the anterior brain of some surviving *swr*⁻ embryos (Fig. 3I).

In summary, neurons at all DV levels of the neural plate are affected in their position and/or number in *swr*⁻ embryos. This observation is not consistent with Swirl simply having a local role (at the neural/non-neural interface) in specifying prospective dorsal neurons.

Floorplate tissue is expanded in *swr*⁻ embryos

Signals from the organiser and its axial derivatives are required for the induction of floorplate tissue in the midline of the neural plate, and in vitro, Bmps can antagonise the floorplate inducing properties of the secreted signalling protein, Sonic hedgehog (Shh; Liem et al., 1995). We therefore examined floorplate markers in *swr*⁻ embryos to determine if loss of Swirl activity affects the midline of the neural plate. In *swr*⁻ embryos, expression of both *shh* (Krauss et al., 1993) and *twh* (Ekker et al., 1995) is expanded compared to wild type (Fig. 4B,D). *fkd4* is expressed in the floorplate and hypochord (Odenthal and

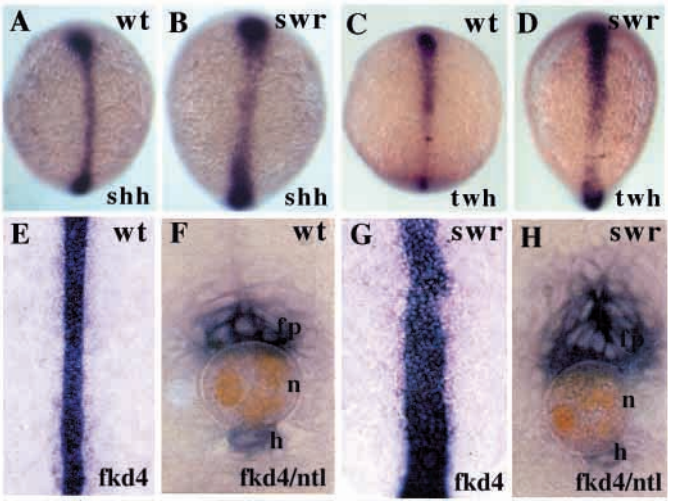

Fig. 4. Floorplate tissue is expanded in *swr*⁻ embryos. (A-D) Dorsal views of *shh* (A,B, 1-2 somite stage) and *twh* (C,D, 3 somite stage) expression in axial tissue. *shh* is expressed in ectodermal and mesodermal tissue whereas by the 3 somite stage, *twh* is limited to ectodermal tissue (Ekker et al., 1995). The expression of both genes is slightly expanded in *swr*⁻ embryos. (E-H) Whole-mount views and transverse sections of *fkd4* expression in floorplate and hypochord and Ntl (Schulte-Merker et al., 1994) expression in the notochord in 15 somite stage embryos. Floorplate *fkd4* expression is expanded in the *swr*⁻ mutants. fp, floorplate; h, hypochord; n, notochord.

Nüsslein-Volhard, 1998) and about twice the number of cells express *fkd4* in the floorplate of *swr*⁻ embryos as compared to wild type (Fig. 4E-H). These results indicate that loss of Swirl function leads to expansion of the floorplate.

swr⁻ embryos probably retain residual Bmp activity

The observations that *swirl* is a null mutation in *bmp2b* and that *bmp4* transcription is dependent upon Swirl function (Kishimoto et al., 1997) have led to the suggestion that *swirl* represents a loss of Bmp signalling activity during DV patterning (Nguyen et al., 1998). However, while marginal neural plate markers are absent in rostral and caudal regions of *swr*⁻ embryos, they are expanded at diencephalic levels. This could reflect a true difference in the response properties of marginally expressed genes at different AP locations to the loss of Bmp signalling. Alternatively, it could reflect the possibility that some Bmp activity is retained in *swr*⁻ embryos and that in the diencephalon, this residual Bmp activity is sufficient to retain, and expand, marginal gene expression. To address this issue, we overexpressed Noggin, an antagonist of Bmp signalling which might lead to a more complete suppression of Bmp signalling activity than occurs in *swr*⁻ embryos.

Analysis of gene expression patterns indicated that *noggin*-injected embryos were indeed more severely dorsalised than *swr*⁻ embryos. Similar to *swr*⁻ embryos, *noggin*-injected embryos were neuralised, as indicated by radial expansion of markers such as *anf1* and *noi*/*pax2.1*(Fig. 5A). In the most medial regions of the neural plate, the phenotype of *noggin*-injected embryos was also similar to *swr*⁻ embryos. Thus prospective hypothalamic tissue (Fig. 5B) and more caudal axial tissue (Fig. 5H) was similar to, or slightly more expanded than in *swr*⁻ embryos as assayed by *nk2.1* and *shh* expression, respectively. However, other aspects of the *noggin* overexpression phenotype were quite different from *swr*⁻ phenotype. Whereas diencephalic *flh* expression is expanded in *swr*⁻ embryos (Fig. 2F), it is absent in *noggin*-injected embryos (Fig. 5C) mirroring the loss of other marginal neural plate markers *fkd6* and *emx1* (Fig. 5D). These results suggest that Bmp signalling is required for induction of *flh* and other marginal genes at all AP levels including the diencephalon.

Neurogenesis was also affected differently in *noggin*-injected and *swr*⁻ embryos. More mildly affected injected embryos resembled *swr*⁻ mutants in that Rohon-Beard neurons were absent and motor neurons formed columns slightly further apart and slightly broader than in wild type (Fig. 5E). However, most *noggin*-injected embryos exhibited expansion of more medial cell fates than observed in *swr*⁻, and interneurons, rather than being expanded, were absent (not shown), while motor neurons and other medial neurons were expanded (Fig. 5F,G).

One interpretation of the results presented above is that different levels of Bmp activity directly or indirectly specify different cell types at different DV positions within the neuroectoderm. In *swr*⁻ and *noggin*-injected embryos, Bmp activity is progressively reduced, with the consequence that genes responsive to lower thresholds of Bmp signalling have expanded expression domains.

Different populations of cells are expanded in *sbn*⁻ embryos

To further address how altered levels of Bmp activity influence DV patterning of neural tissue, we compared patterning of the ectoderm in *somitabun*⁻ (*sbn*⁻) embryos to that in *swr*⁻ embryos. *sbn*⁻ embryos carry a mutation in Smad5 (Hild et al., 1999), an intracellular transducer of Bmp signalling, and exhibit a variable dorsalised phenotype in which some embryos are similar to *swr*⁻ while most embryos are less severely dorsalised (Nguyen et al., 1998).

Analysis of ectodermal markers confirmed that *sbn*⁻ embryos exhibit a less severe dorsalisation than *swr*⁻ embryos. Marginal neural plate markers such as *fkd6* and *emx1* which are reduced or absent in *swr*⁻ (see Fig. 2D) were variably expanded in *sbn*⁻ embryos, correlating with variable retention of non-neural markers such as *gta2* (Fig. 6A-D). While the most strongly dorsalised *sbn*⁻ embryos showed radialisation of *flh* similar to *swr*⁻, other embryos exhibited more limited expansion (compare Fig. 2E,F and Fig. 6E-G), suggesting that the DV extent of *flh* expression is dependent on the varying degrees of residual Bmp signalling activity in *sbn*⁻ embryos (see Fig. 7 below). We also observed that the severity of the *sbn*⁻ phenotype increased from anterior to posterior regions of the embryo. For instance, within a single embryo *fkd6* expression could be relatively normal in anterior regions, yet radialised in the trunk (Fig. 6C).

Analysis of neurons in *sbn*⁻ embryos confirmed that different neural populations are expanded in *sbn*⁻ and *swr*⁻

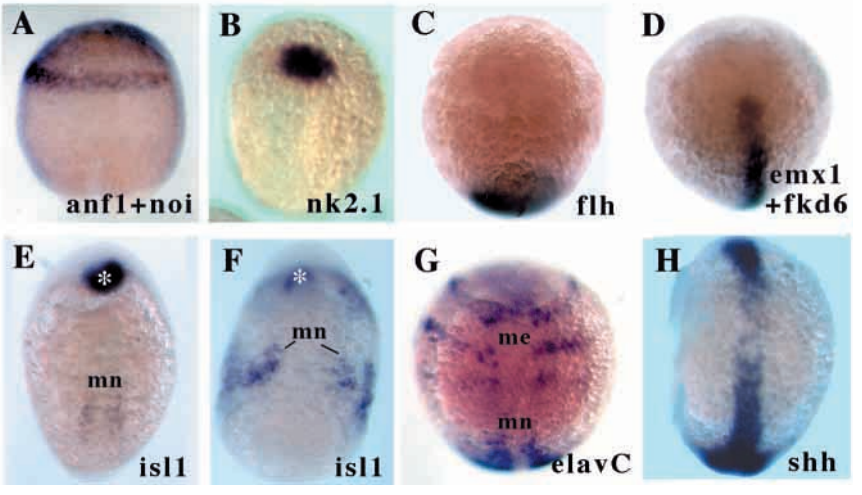

Fig. 5. Noggin-injected embryos are more severely dorsalised than *swr*⁻ embryos. Lateral (A), animal pole (B-D) and dorsal (E-H) views of of 1-2 somite stage (except A, 90% epiboly) embryos injected with *noggin* RNA. (A) *anf* and *noi* expression is radialised in *noggin*-injected embryos (similar to *swr*⁻ embryos; compare with Figs 1D and 2H). (B) *nk2.1* expression is retained in the prospective hypothalamus of the injected embryo. (C,D) Marginal neural plate expression of *flh*, *emx1* and *fkd6* is lost in *noggin*-injected embryos (compare with Fig. 2D,F). (E) Mild and (F) massive expansion of *isl1* expressing motor neurons in *noggin*-injected embryos. The asteriks indicates the pillow. (G) Radial expansion of *elavC* expressing medial neurons in a severely dorsalised *noggin*-injected embryo. (H) *shh* expression in axial tissue in a *noggin*-injected embryo. me, medial neurons; mn, motor neurons.

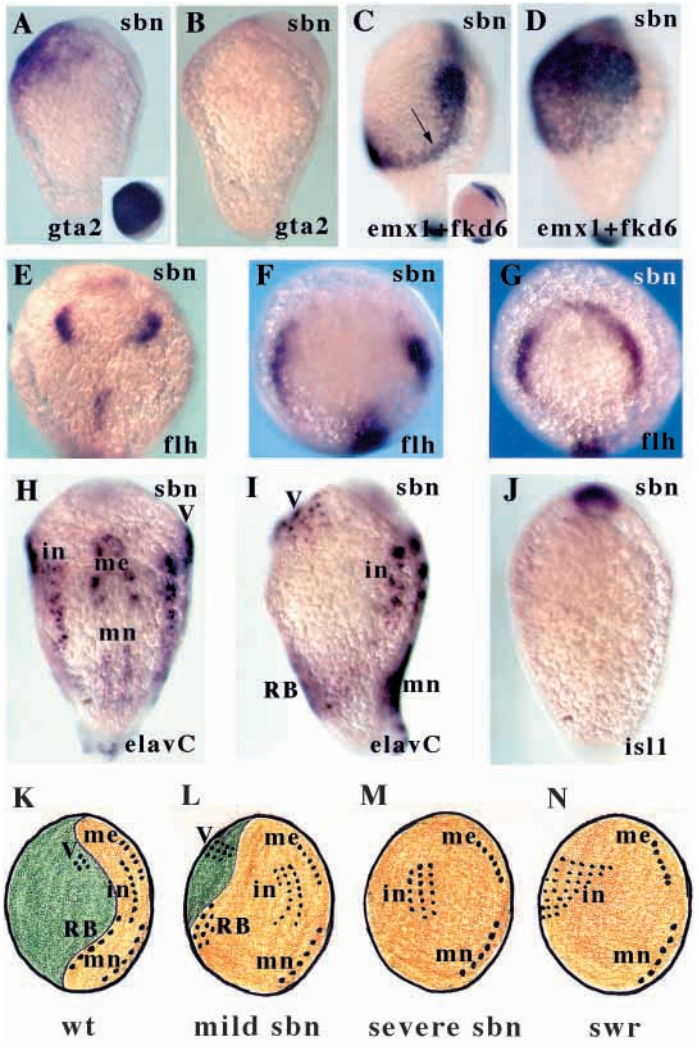

Fig. 6. Sbn affects marginal cells fates and patterns of neurogenesis. All panels show 1-2 somite stage embryos. (A,B) Lateral views of *gta2* expression (prospective epidermis) in mild (A) and severe (B) *sbn*$^{-}$ embryos. The inset panel shows expression in a wild-type embryo. Expression is retained in the anterior ventral region of the mildly affected embryo. (C,D) Lateral views of *emx1* (prospective telencephalon) and *fkd6* (prospective neural crest) expression in mild (C) and severe (D) *sbn*$^{-}$ embryos. The inset panel shows expression in a wild-type embryo. In the mildly affected embryo, expression is relatively normal in rostral regions, consistent with retention of *gta2* expression (see A) in ventral ectoderm. However, more caudally, *fkd6* expression is radialised (arrow). In the severely affected embryo, *emx1* and *fkd6* are expanded throughout the ventral ectoderm consistent with loss of *gta2* in this region (see B). (E-G) Animal pole views of *flh* expression (prospective epiphysis) in progressively more severe *sbn*$^{-}$ embryos. *flh* expression is variably expanded in the *sbn*$^{-}$ embryos (see Fig. 2E,F for wild-type and *swr*$^{-}$ expression). (H) Dorsal and (I) lateral views of *elavC* expression in a *sbn*$^{-}$ embryo. Wild-type pattern of expression is shown in Fig. 3A,B. In these mildly affected *sbn*$^{-}$ embryos, interneurons are slightly expanded and dorsal sensory neurons are broadly expanded on the ventral side of the embryo. (J) Ventral view of *isl1* expression in a severely affected *sbn*$^{-}$ embryo. Trigeminal and Rohon-Beard neurons are absent. (K-N) Summary schematics of patterns of neurogenesis in wild-type, *sbn*$^{-}$ and *swr*$^{-}$ embryos. In the mildly affected *sbn*$^{-}$ embryo, some non-neural ectoderm is retained and trigeminal and RB neurons are expanded on the ventral side of the embryo. In the severe *sbn*$^{-}$ embryo, trigeminal and RB neurons are lost and in the *swr*$^{-}$ embryo, interneurons are expanded throughout the ventral ectoderm. in, interneurons; me, medial neurons; mn, motor neurons; RB, Rohon-Beard neurons; V, trigeminal neurons.

embryos. Similar to *swr*$^{-}$, medial clusters of neurons were relatively normal in *sbn*$^{-}$ (Fig. 6H); however, interneurons were specified closer to the dorsal midline than in *swr*$^{-}$ and were not present throughout the ventral ectoderm (compare Fig. 3A,D and Fig. 6H). Instead, there was variable expansion or loss of the laterally positioned trigeminal neurons and Rohon-Beard cells (Fig. 6I,J). Together these results indicate that the radialised neural tissue in *noggin*-injected embryos has a more dorsal (medial) character than neural tissue in *swr*$^{-}$ embryos which in turn has a more dorsal character than neural tissue in *sbn*$^{-}$ embryos.

DISCUSSION

A Bmp-dependent gradient of positional identity is present across the entire gastrula ectoderm

Cells at all DV positions of the ectoderm are affected by the level of Bmp activity within the zebrafish embryo suggesting that discrete ectodermal domains are established between specific thresholds on a Bmp-dependent gradient of positional information. For example, *flh* is normally induced in cells at the margin of the neural plate that will later contribute to the epiphysis in the dorsal diencephalon (Masai et al., 1997). In wild-type embryos, we suggest that *flh* is not expressed in ventral ectoderm because Bmp activity is too high and it is not expressed in dorsal ectoderm because Bmp activity is too low (Fig. 7B). When Bmps are overexpressed, all cells are exposed to a high level of Bmp signalling, *flh* is not induced (data not shown) and cells form more ventral ectodermal derivatives (Fig. 7A). Conversely, if the Bmp gradient is progressively lowered, as in *sbn*$^{-}$ and *swr*$^{-}$ embryos, then *flh* is excluded from a larger domain of the dorsal ectoderm but is expressed in a progressively greater proportion of the ventral ectodermal cells, reflecting the larger number of cells that fall between the thresholds for induction of *flh* expression (Fig. 7C,D). If Bmp signalling is reduced even further through overexpression of the Bmp antagonist Noggin, then no ectodermal cells are exposed to sufficient Bmp activity to induce *flh* (Fig. 7E). Thus while *swr*$^{-}$ embryos and *noggin*-injected embryos are both completely neuralised, the DV identity of the neural tissue in such embryos is very different.

Previous models have focussed upon the role of Bmps in the specification of ventral ectodermal derivatives such as epidermis, placodes and neural crest rather than upon their role in patterning the dorsal ectoderm. Indeed, by the time the neural plate has formed, *bmp2/4* expression is confined to non-neural ectoderm and it appears that Bmp proteins are poorly diffusible and have only local activity (Jones et al., 1996; Nikaido et al., 1999). Given that it is unlikely that Bmps can diffuse very far from the margin of the neural plate, one is faced with the problem of how cells in medial positions in the neural plate are responsive to the alterations in Bmp activity in *swr*$^{-}$

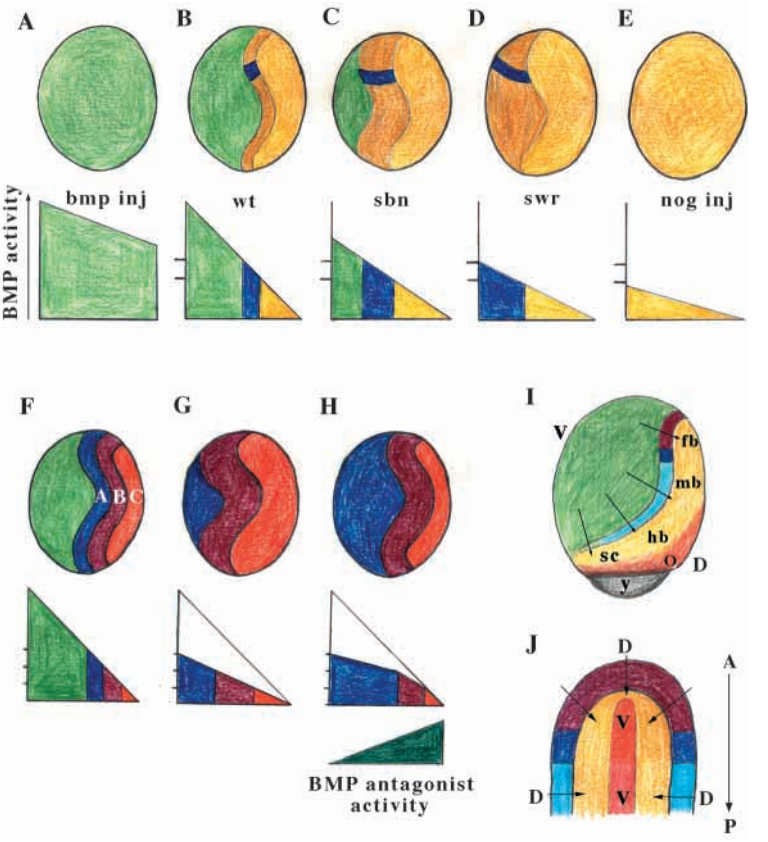

Fig. 7. Bmp activity in ectodermal patterning. Schematics of lateral views of embryos at 1-2 somite stage (except I-J). Dorsal is to the right and anterior is up. (A-E) In the wild-type situation (B), *flh* expression (dark blue) is shown at the margin between the neural plate (yellow) and the non-neural ectoderm (green). *flh* may be induced between specific thresholds on a gradient of Bmp-dependent positional information present across the entire ectoderm (B, graph). When Bmp proteins are overexpressed (A) there is no induction of *flh* as all cells are exposed to too high a level of Bmp activity. Conversely, progressive lowering of Bmp activity in *sbn*$^{-}$ and *swr*$^{-}$ embryos (C,D) results in progressively more ectodermal cells expressing *flh* on the ventral side of the embryo. However, if *noggin* is overexpressed (E), then *flh* is again not induced as the overall level of Bmp activity is too low. (F-H) A, B and C represent three DV domains in the neural plate. If the gradient of Bmp-dependent positional information was equally affected at all DV positions in *sbn*$^{-}$ and *swr*$^{-}$ mutants, then one would expect to observe an equivalent expansion of all domains that are retained in the mutants (G). Instead, we observe that more ventral ectodermal domains are expanded more than dorsal ectodermal domains (H). This implies that the gradient of Bmp-dependent positional information is more severely affected ventrally than dorsally. An explanation for this is that on the dorsal side of the embryo, this gradient is primarily defined by the activity of Bmp antagonists, whereas on the ventral side of the embryo it is defined by activators of Bmp signalling (H, graphs). (I,J) Lateral view (I) of an embryo at 90% epiboly. The arrows indicate the putative orientation of the DV axis at different AP positions in the prospective neural plate. Prospective axial tissue and mesendoderm is shown in orange. By the time that the neural plate has formed (J), the orientation of the DV axis at the anterior tip of the neural plate is the same as the prospective AP axis and is perpendicular to the DV axis at the lateral margins of the neural plate. In J, axial midline tissue is shown in red/orange. A, anterior; D, dorsal; fb, forebrain, fp; hb, hindbrain; mb, midbrain; o, organiser; P, posterior; sc, spinal cord; V, ventral; y, yolk.

and *sbn*$^{-}$ embryos. One possibility is that only mesodermal cells are responsive to graded Bmp activity and they transmit vertical signals to specify all DV positions in the overlying ectoderm. While we cannot discount this possibility completely, it is unlikely given in vitro results that ectodermal cells are responsive to graded Bmp activity in the absence of mesoderm (Knecht et al., 1995) and the observation that embryos entirely lacking mesendodermal derivatives, exhibit DV patterning of the ectoderm (Feldman et al., 1998; Gritsman et al., 1999; Thisse and Thisse, 1999).

One hypothesis to explain how Bmp proteins establish a gradient across the entire ectoderm is that cells are responding to Bmp activity from very early stages and for extended periods of time. Thus at early blastula stages *bmp2b* and *bmp4* are expressed throughout most of the ectoderm and over time, expression regresses from the organiser until it is restricted to non-neural ectoderm (Nikaido et al., 1997). Concommitantly, antagonists of Bmp activity progressively spread from the organiser inhibiting Bmp function (Miller-Bertoglio et al., 1997). A consequence of these two events is that over time, cells near the organiser will have been exposed to a low amount of Bmp signals for a short period of time, whereas cells distant from the organiser will have been exposed to higher levels of Bmp activity for longer periods of time. If ectodermal cells can integrate the temporal duration and concentration of Bmp signals, then this provides a means by which a Bmp-dependent gradient of positional information may be established throughout the ectoderm. A similar scenario has been proposed to explain the activity of Activin as a morphogen, where relatively small differences in receptor occupancy are integrated by cells over time to promote different cell fates (Dyson and Gurdon, 1998; Gurdon et al., 1998).

If this model is correct, it raises a few interesting problems. For instance, it challenges the simple concept of neural induction in which there is a binary choice between non-neural fate and neural fate dependent upon presence or absence of Bmp activity. Our model suggests that even at the time of neural induction, cells positioned close to the organiser are likely to have encountered less Bmp activity for a shorter period of time and consequently have a more “dorsal” identity than more laterally positioned neural cells. To our knowledge, there are no experiments that have addressed whether newly induced neural tissue does exhibit any intrinsic differences in DV positional identity. However, the earliest genes to be expressed in the neural plate are generally expressed throughout this tissue, whereas genes characteristic of specific DV positions are expressed only at later stages. This observation does suggest a biphasic response to Bmp activity in which the earliest neural genes are activated when Bmp activity is simply below a certain level whereas later genes may have more refined responses to thresholds along the gradient of Bmp-dependent positional information (Fig. 7F).

Alterations in Bmp activity affect the gradient of positional information in the ectoderm in a non-linear fashion

Examination of the consequences of the lowered Bmp activity in *swr*$^{-}$ and *sbn*$^{-}$ embryos suggests that cells distant to the organiser are more responsive to altered Bmp levels than are cells close to the organiser. If one represents the gradient of Bmp-dependent positional information across the ectoderm as a straight line (Fig. 7F) then if *swr* or *sbn* mutations affected this gradient equally at all DV locations, one would expect an equivalent expansion of all cell domains that are retained in the mutants (Fig. 7G). This is not what is observed. Instead, cell domains at ventral positions are expanded more than dorsal ectodermal domains (Fig. 7H). Bmp activity in the dorsal ectoderm may be primarily regulated by the activity of inhibitors of Bmp function, such as Chordin and Noggin, emanating from the organiser. The buffering activity of such proteins may explain the lower impact of mutations such as *swr*$^{-}$ and *sbn*$^{-}$ on the dorsal side of the embryo. Thus the overall gradient of Bmp-dependent positional information in the ectoderm may depend primarily upon the local action of activating components of the Bmp pathway in the ventral ectoderm and primarily upon inhibitors of Bmp function in dorsal ectoderm (Fig. 7H).

Bmp signalling affects cell identity at all AP levels of the neural plate

For many years, there has been disagreement regarding the definition of the AP and DV axes in the forebrain. The primary issue of contention has been whether to define the telencephalon as a separate anterior subdivision of the neural tube or whether it constitutes a dorsal compartment of the rostral forebrain. Fate mapping studies show that prospective telencephalic cells are generally anterior to prospective diencephalic cells (e.g. Woo and Fraser, 1995), yet morphological considerations and gene expression analysis suggest telencephalon should be considered as dorsal tissue (Rubenstein et al., 1998). These two points of view are reconciled if prospective telencephalic tissue, through being located at the margin of the neural plate, is considered to be both anterior and dorsal. We have shown that Bmp activity is not restricted to the lateral neural plate, rather it extends all the way around the anterior margin of the neural plate. Thus the AP axis and the DV axis (as defined by signalling pathways specifying AP and DV values) have the same orientation at the anterior margin of the neural plate (Fig. 7I,J). DV patterning mutations, such as *swr*$^{-}$, that affect specification of marginal cell fates may therefore result in anterior/dorsal truncations of CNS tissue, such as loss of telencephalic fates.

Our analysis has demonstrated that Bmp signalling plays a critical role in the specification of cell types at all AP levels of the ectoderm. It has previously been suggested that in chicks, an absence of Bmp activity in rostral regions may help explain the absence of neural crest in such regions (Muhr et al., 1997). However, our in vivo observations indicate that Bmp activity in fish specifies all marginal cell fates irrespective of their AP identity. Thus, alterations in Bmp activity affect the DV extent of expression of markers of neural crest, diencephalon and telencephalon but have no effect on their relative AP positions. It therefore seems unlikely that in fish, fate choice between neural crest and more rostral marginal fates could be mediated by differences in Bmp activity at different AP positions. Our results argue the opposite; cells at different AP locations but equivalent DV positions appear to respond similarly to alterations in Bmp activity.

We suggest that the role of Bmp activity in the specification of neural crest in fish is to define a DV compartment of the ectoderm that is responsive to other instructive signals that specify neural crest identity. The region of competence to form neural crest may be defined between upper and lower thresholds of Bmp activity (as desribed for *flh* above; and see Nguyen et al., 1998). Thus at early stages, Bmp signalling alone may have no instructive role in specifying neural crest identity – instead, the concerted activity of signals that establish AP pattern in the ectoderm (and distinguish neural crest from more rostral marginal fates) together with Bmp signals may establish prospective neural crest at the correct AP and DV locations of the ectoderm.

The severity of mutations in the Bmp pathway varies along the AP axis

Although Bmp signalling is required at all AP levels of the ectoderm, the severity of the dorsalised phenotype increases in posterior regions. For instance, in *sbn*$^{-}$ mutants, marginal fates can be relatively normal in rostral regions but radialised in more posterior regions (Fig. 5C). This could either be due to the same genes being differentially sensitive to Bmp activity at different AP positions, or more likely, different degrees of Bmp activity are retained in the mutants at different AP locations. One contributing factor to this AP gradient in phenotypic severity may relate to the temporal regulation of Bmp activity. Bmp signalling autoregulates such that transcription of *swirl/bmp2b*, *bmp4* and *chordin* are all dependent upon Swirl activity. In *swr*$^{-}$ embryos, *bmp4* is initially expressed normally but over a period of a few hours, transcription is lost in the ectoderm (Kishimoto et al., 1997), while *chordin* expression is expanded (Miller-Bertoglio et al., 1997), suggesting that as embryos get older there will be less residual Bmp activity. Thus if posterior neural tissue acquires DV positional identity later than anterior tissue, it may be exposed to lower levels of Bmp signalling and show a more dorsalised phenotype.

Some aspects of the *swr*$^{-}$ phenotype phenocopy embryos in which the rostral margin of the neural plate has been ablated

Removal of a single row of cells (row1) at the rostral margin of the prospective neural plate midway through gastrulation leads to a failure of induction of telencephalic gene expression and reduced neurogenesis (Houart et al., 1998). In *swr*$^{-}$ embryos, there is also a loss of telencephalic gene expression and there appears to be a loss of neurons in the anterior brain in some embryos that survive beyond gastrulation, suggesting that Swirl activity and row1 activity may be related. One possibility is that Swirl itself is responsible for row1 activity. However, it seems unlikely that Swirl could impart AP positional information and the apparent diffusibility of row1 activity (Houart et al., 1998) also makes it less likely that Swirl is solely responsible for this activity. Nevertheless the ability of row1 cells to induce more posterior markers of marginal neural plate fates has not been tested and so it remains a possibility that a component of row1 activity is the general specification of marginal cell fates.

Perhaps more likely is that Bmp signalling within the ectoderm is required for the establishment of row1 activity. For instance, row1 itself may be induced by a specific level of Bmp signalling or by boundary interactions between two adjacent domains of ectodermal cells that are specified by thresholds of Bmp signalling activity and the loss of this boundary in *swr*$^-$ embryos may account for the loss of telencephalic induction.

Swirl activity limits the extent of the floorplate

In *swr* mutants, floorplate is expanded suggesting that Swirl activity normally limits the extent of floorplate tissue in the medial neural plate. There is currently some debate as to whether floorplate induction is initiated in the organiser or at later stages when the medial neural plate is underlain by notochord (Dodd et al., 1998). *swirl* is expressed in the organiser but not in cells of the prospective notochord once these cells have involuted suggesting that prospective floorplate cells are more likely to be influenced by Swirl activity at early stages when they are close to the organiser. How Swirl influences floorplate formation is unclear. In vitro studies suggest that it could antagonise the activity of the floorplate inducer, Shh (Liem et al., 1997). However, Nodal activity is also required for floorplate specification (Rebagliati et al., 1998; Sampath et al., 1998) and so an antagonistic interaction between the Nodal and Bmp signalling pathways is also a possibility.

Bmp signalling may define neurogenic domains in the ectoderm

One of the consequences of the altered levels of Bmp activity in *swr*$^-$ and *sbn*$^-$ embryos is that certain populations of neurons are hugely increased in number. However, these Bmp pathway mutations probably do not exhibit a true neurogenic phenotype. We observed no obvious alterations in the density of neurons in mutant embryos, rather, the neurons were distributed over larger areas of the ectoderm. This suggests that Notch/Delta signalling is probably intact in mutant embryos, and the phenotype is rather due to ectopic initiation of neurogenesis in a greater number of ectodermal cells. Thus Bmp activity is more likely to be acting upon genes that define territories of neurogenesis within the ectoderm (Masai et al., 1997; Sasai, 1998) than upon genes directly involved in neuronal production.

In *Drosophila*, the products of early acting DV patterning genes, including Dpp, the Bmp2/4 homolog, spatially regulate the domains of neurogenesis in the ectoderm (Skeath, 1999). Analogous to vertebrates, most research on Dpp has focused upon its role in the non-neural ectoderm adjacent to the territory that generates neurons (Bier, 1999). However, a number of observations suggest that Dpp may indeed have graded activity over most or all of the neurogenic regions of the ectoderm. For instance, *vnd/NK2* and *msh* are expressed in columns of cells that give rise to medial and lateral neurons respectively, and the expression domains of both of these genes expand in *dpp* mutants (Mellerick and Nirenberg, 1995; D'Alessio and Frasch, 1996).

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