Combinatorial Fgf and Bmp signalling patterns the gastrula ectoderm into prospective neural and epidermal domains

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Summary

Studies in fish and amphibia have shown that graded Bmp signalling activity regulates dorsal-to-ventral (DV) patterning of the gastrula embryo. In the ectoderm, it is thought that high levels of Bmp activity promote epidermal development ventrally, whereas secreted Bmp antagonists emanating from the organiser induce neural tissue dorsally. However, in zebrafish embryos, the domain of cells destined to contribute to the spinal cord extends all the way to the ventral side of the gastrula, a long way from the organiser. We show that in vegetal (trunk and tail) regions of the zebrafish gastrula, neural specification is initiated at all DV positions of the ectoderm in a manner that is unaffected by levels of Bmp activity and independent of organiser-derived signals. Instead, we find that Fgf activity is required to induce vegetal prospective neural markers and can do so without suppressing Bmp activity. We further show that Bmp signalling does occur within the vegetal prospective neural domain and that Bmp activity promotes the adoption of caudal fate by this tissue.

Key words: Zebrafish, Default model, Neural induction

Introduction

In vertebrates, the organiser and its axial derivatives have the ability to induce and pattern neural tissue when transplanted elsewhere within the embryo (Munoz-Sanjuan and Hemmati-Brivanlou, 2002; Stern, 2001). The identification of secreted inhibitors of Bmp proteins expressed in these organiser cells led to proposition of the 'neural default model', which suggests that Bmp signals induce epidermal fate whereas Bmp antagonists induce neural fate (Munoz-Sanjuan and Hemmati-Brivanlou, 2002). However, genetic analyses in zebrafish have revealed a more complex role for the Bmp pathway in early ectodermal patterning (Hammerschmidt and Mullins, 2002). These and related studies in other species have shown that high levels of Bmp signalling promote epidermal identity, whereas neural crest, lateral neural and more medial neural identities are established at progressively lower levels of Bmp activity (Aybar and Mayor, 2002; Barth et al., 1999; Nguyen et al., 2000). In all vertebrate model organisms that have been examined, most neural tissue originates close to the organiser (on what is termed the dorsal side of the gastrula in fish and frogs), supporting the notion that neural induction is dependent upon organiser-derived signals including Bmp antagonists. However, spinal cord neural tissue of zebrafish embryos originates distant to the organiser (Kimmel et al., 1990) and in this study, we show that the initial steps in its specification appear to occur independent of organiser-derived signals.

Although the Bmp signalling pathway is likely to play a role in neural and epidermal development in all vertebrates, a variety of studies have challenged the idea that antagonism of Bmp activity is sufficient to induce neural tissue (Stern, 2001; Stern, 2002; Wilson and Edlund, 2001). Indeed, several other signals may have roles in neural induction (Bally-Cuif and Hammerschmidt, 2003) and among these, Fgfs are perhaps the most extensively studied candidates (Akai and Storey, 2003). Fgfs can induce neural (or prospective neural) identity both in stem cells (e.g. Ying et al., 2003) and when ectopically expressed in vivo (e.g. Bertrand et al., 2003; Furthauer et al., 1997; Kengaku and Okamoto, 1995; Koshida et al., 2002; Lamb and Harland, 1995; Sheng et al., 2003; Streit et al., 2000; Wilson et al., 2000); in addition, abrogation of Fgf activity can in some circumstances disrupt neural induction (e.g. Bertrand et al., 2003; Hongo et al., 1999; Streit et al., 2000; Wilson et al., 2000).

In this study, we attempt to resolve the relative contributions of organiser-derived Bmp antagonists and Fgf signals to the initial steps of neural induction in zebrafish embryos. We show that Fgf activity, rather than Bmp antagonism, initiates development of prospective vegetal neural tissue that contributes to trunk and tail CNS. In vegetal prospective neural tissue, Bmp activity does not antagonise the induction of prospective neural markers, rather it promotes the ability of cells to contribute to caudal neural ectoderm. Therefore, at gastrula stages, the role of Bmp activity in the animal and vegetal ectoderm is different. In animal ectoderm, high levels

of Bmp activity push cells towards a non-neural rather than neural fate whereas in vegetal ectoderm, differential levels of Bmp activity influence the regional (rostral to caudal) character of the neural tissue.

Materials and methods

RNA probe synthesis and in situ hybridisation

RNA probes were synthesised, and in situ hybridisation procedures were performed as previously described (Itoh et al., 2002; Kudoh et al., 2002).

Fate mapping

Zebrafish embryos were injected with caged fluorescein (Molecular Probes) at the one-cell stage. Photoactivation of fluorescent dye was performed as previously described (Rohr and Concha, 2000) at around the 70-80% epiboly stage. Following photoactivation, DIC and fluorescent images were acquired using Openlab software (Improvision) with a cooled CCD camera (Hammamatsu) attached to an Axioplan microscope (Zeiss).

mRNA synthesis, injection of mRNA or morpholinos and cell transplantations

Capped RNA was synthesised by mMessage mMachine SP6 kit (Ambion) according to the manufacturer's instructions. RNA concentrations used for injection were: *bmp2b* (50 pg); *fgf3* (50 pg); XFD (300 pg); *chordin* (50 pg); and eGFP (100 pg). mRNAs were injected into cells at one- to two-cell stage in all blastomeres while the morpholino for the *chordin* gene (500 pg) (Nasevicius and Ekker, 2000) was injected at the one- to two-cell stage in the yolk. More than 15 embryos were examined in each injection experiment.

For transplantation, donor embryos were injected with various RNA constructs and cells removed at blastula or early gastrula stages using a microelectrode connected to a Hamilton syringe as previously described (Houart et al., 2002). Donor cells were gently aspirated into various positions of similar stage host embryos. To detect donor cells expressing GFP, embryos were labelled by peroxidase conjugated anti-GFP antibody (×1000 dilution) (TP401, Torrey Pines Biolabs) and detected by DAB substrate reaction after the in situ hybridisation protocol. In cases where two populations of cells were co-transplanted, donors were distinguished by injection with either fluorescein dextran (green) or rhodamine dextran (red).

Donor embryos injected with constructs encoding both Fgf3 and the truncated Fgf receptor XFD, did not show an Fgf gain-of-function phenotype and instead showed a phenotype similar to embryos expressing XFD alone. This suggests that cells from these embryos produce, but are compromised in their ability to respond to, Fgf signals. In induction assays, it is likely that Fgf3 from these cells acts directly on host cells as opposed to acting indirectly by inducing the expression of other signals within the donor cells.

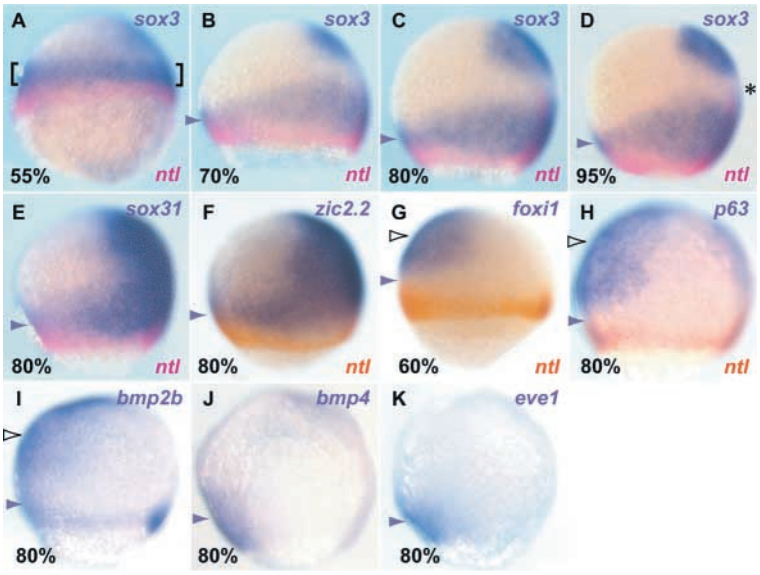

Fig. 1. Vegetal ectoderm expresses prospective neural genes at all DV positions. Lateral views of gastrulae (stage indicated in the bottom left-hand corner) with dorsal towards the right. Genes analysed are indicated on the right of the panels with typeface colour reflecting the colour of the respective expression domains. Vegetal marginal ectoderm is indicated by brackets in A and ventrovegetal ectoderm is indicated by arrowheads in other panels. *ntl* expression indicates prospective mesoderm (A-H), *foxi1* and *p63* expression shows prospective epidermis (G,H), which overlaps with the animal domain of *bmp2b* expression (white arrowheads in G-I). *foxi1* expression is initiated earlier in this domain than *p63*. *sox3*, *sox31* and *zic2.2* expression labels all prospective neural tissue (A-F) with the exception, for *sox3*, of a band of cells in the prospective midbrain and hindbrain (asterisk in D). *bmp2b*, *bmp4* and *eve1* are expressed in ventral mesoderm at the blastoderm margin in addition to ventrovegetal ectoderm (purple arrowheads in I-K).

Results

Prospective neural tissue is present throughout vegetal ectoderm adjacent to the germ ring

If the primary role of Bmp signalling is to antagonise neural development, then we reasoned that all neural tissue should form in regions of low Bmp activity. To determine if this is indeed the case, we used marker gene and fate mapping approaches to identify ectodermal cells destined to form CNS tissue in gastrula stage zebrafish embryos. Early markers of prospective neural tissue, such as *sox3* (Kudoh et al., 2001) (GenBank Accession Number, AY316135), *sox31* (Girard et al., 2001) and *zic2.2* (Toyama et al., 2004), are not only expressed dorsally at gastrula stages, but also in a radial band of cells along the vegetal edge of the ectoderm, adjacent to the prospective mesoderm (Fig. 1A-F). Expression of the prospective epidermal markers, *foxi1* (Solomon et al., 2003) and *p63* is complementary to that of these prospective neural genes, leaving a band of ventral vegetal ectodermal cells free of transcripts (Fig. 1G,H) (Bakkers et al., 2002). On the ventral side of the gastrula, the expression of prospective neural genes overlaps with that of *bmp2b* and *bmp4*, as well as *eve1*, a putative target of Bmp signalling (Joly et al., 1993), suggesting that Bmp activity is compatible with prospective neural gene expression (Fig. 1I-K). Altogether, these results show that ventrovegetal ectoderm expresses prospective neural rather than epidermal markers, suggesting that some prospective neural tissue is specified in a region of potentially high Bmp activity.

To determine if ventral ectoderm does indeed form neural tissue, we performed cell fate mapping of ectoderm in mid-gastrula stage embryos. At 50% epiboly, cells at all DV positions of the ectoderm can give rise to neural tissue with at least some prospective dorsal spinal cord cells located ventrally (Kimmel et al., 1990). To extend these studies, we determined the fates of ectodermal cells at mid-gastrula (70-80% epiboly), a stage by which early prospective neural genes and epidermal

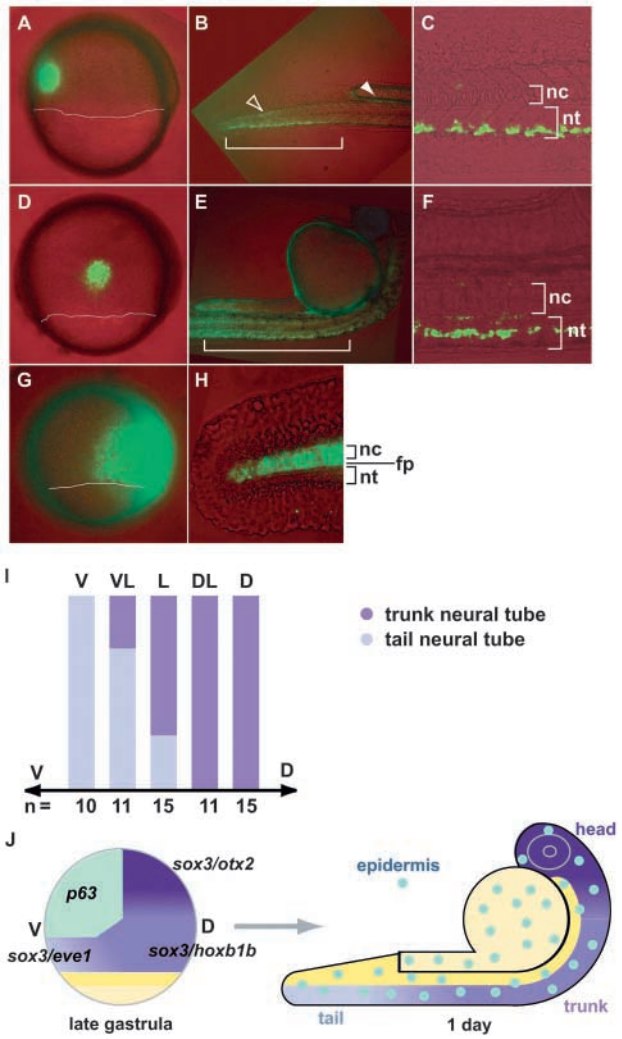

Fig. 2. Prospective caudal neural tissue is located on the ventral side of the gastrula ectoderm. Lateral views with dorsal towards the right for gastrula stage embryos and to the bottom for 1-day-old embryos. The vegetal limit of the germ ring is indicated by white lines in A, D and G. Gastrula (A,D,G) and 1-day-old (B,C,E,F,H) embryos in which fluorescein was uncaged in ventral vegetal (A) and lateral vegetal (D) ectoderm, or widely throughout dorsal and lateral ectoderm (G), then fates of fluorescent cells were examined the next day. Ventrovegetal cells (A, green fluorescent cells) give rise to tail neural tube (B, bracket; and C in high magnification), whereas laterovegetal ectodermal cells (D, green fluorescent cells) contribute to trunk neural tube (E, bracket; and F in high magnification). Besides neural tissue, some ventrally labelled cells ended up in somite muscle (B, white arrowhead) and in the pronephric duct (B, black arrowhead). In embryos with widespread dorsolateral uncaging of fluorescein (G), labelled cells in the caudal tail are restricted to floor plate, notochord and hypocord, and are absent from more dorsal neural tube (H). (I) Summary of fate-mapping data. Positions at which fluorescein was uncaged in vegetal ectodermal cells are categorised as: V, ventral; VL, ventrolateral, L, lateral; DL, dorsolateral; D, dorsal. The relative contributions of cells to the trunk (rostral to the end of the yolk extension) and tail (caudal to the end of yolk extension) neural tube are indicated by the different colours in each column. The numbers of embryos examined (*n*) for each position are indicated beneath the columns. (H) Summary of ectodermal expression and fate-mapping data. Ventral ectoderm towards the animal pole generates epidermis/non neural fates, whereas remaining ectoderm generates neural tissue with cells fated to give rise to the most caudal CNS located in ventrovegetal ectoderm and those contributing to the most rostral CNS located in dorsal animal ectoderm (see above) (Kimmel et al., 1990; Woo and Fraser, 1995). The cartoon does not indicate precursors of ventral CNS midline (such as floor plate and hypothalamus) that are positioned close to the organiser (Mathieu et al., 2002; Shih and Fraser, 1996) and distribute widely along the AP axis of the CNS. Indeed, other ventral spinal cord cell types also originate closer to the gastrula organiser than do dorsal spinal cord neurones (Kimmel et al., 1990). Some of the genes selectively expressed in different regions of the ectoderm (see Fig. 7) are indicated. The dorsal (D) and ventral (V) position of cells at gastrula stage does not necessarily correspond with the eventual DV position of the structures to which the cells contribute. For example, spinal cord of the tail (a dorsal structure in 24 hpf embryos) originates from ventrally positioned ectoderm cells of the gastrula [see also recent fate-mapping studies of mesodermal precursors in *Xenopus* (Kumano and Smith, 2002; Lane and Sheets, 2002)]. fp, floor plate; hp, hypocord; nc, notocord; nt, neural tube.

genes are expressed in complimentary domains (Fig. 1). Cell-fate mapping was performed by uncaging fluorescein within vegetal ectodermal cells, or transplanting biotin-dextran-labelled cells into the vegetal ectoderm, and determining the fate of the labelled cells on the following day. These experiments showed that ventrally located vegetal ectoderm gives rise to caudal spinal cord, whereas progressively more dorsal vegetal ectoderm contributes to progressively more rostral spinal cord and hindbrain (Fig. 2A-F). Next, to determine if any dorsal cells give rise to tail neural tissue, we uncaged fluorescein within the entire dorsal vegetal ectoderm (and underlying mesendoderm) (Fig. 2G), In these cases (*n*=8), fluorescent cells were observed widely in the head and trunk, whereas in the posterior part of the tail, fluorescent cells were restricted to the floor plate, notochord and hypocord, and were absent from the rest of the neural tube (Fig. 2H). Therefore, the tail floor plate is exceptional in being derived from dorsal tissue (Amacher et al., 2002; Shih and Fraser, 1995; Shih and Fraser, 1996), whereas other regions of the tail neural tube are derived from ventral, vegetal ectoderm.

These experiments allow us to conclude that vegetal ectodermal cells on the ventral side of the gastrula, which are distant from the organiser and within a region of *bmp* expression, do contribute to the tail spinal cord.

Bmp antagonists and other organiser-derived signals are not essential for induction of markers of prospective neural tissue in vegetal regions of the ectoderm

Most models suggest that neural induction is dependent upon secreted molecules, notably Bmp antagonists, emanating from the dorsal organiser (Kodjabachian et al., 1999; Munoz-Sanjuan and Hemmati-Brivanlou, 2002); but the presence of prospective neural tissue in the ventrovegetal region of the gastrula challenges this idea. We therefore performed several analyses to test if Bmp antagonists or other organiser-derived signals are necessary for the expression of markers of the vegetal, prospective neural ectodermal domain. Vegetal expression of the prospective neural markers *sox3* and *zic2.2* is unaffected following overexpression of *bmp2b* (*n*=55), in mutant embryos lacking the activity of the Bmp antagonist,

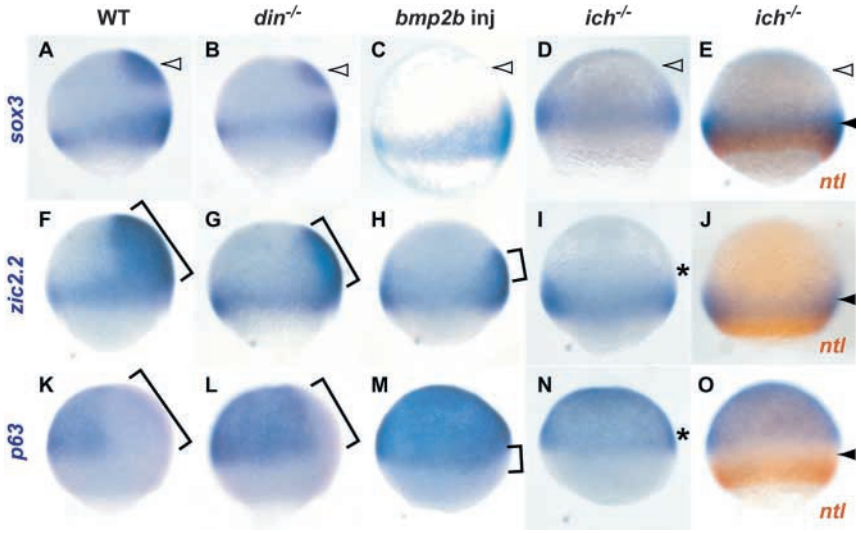

Fig. 3. Prospective neural fate in the vegetal ectoderm is initiated independent of the organiser and antagonism of Bmp activity. Lateral views of gastrula stage wild-type (A,F,K), *chordin* mutant (*din*$^{-/-}$) (B,G,L), *bmp2b*-injected (C,H,M) and *ichabod* mutant (*ich*$^{-/-}$) (D,E,I,J,N,O) embryos with dorsal towards the right (when discernable). Genes analysed by in situ hybridisation are indicated to the left of each row. In E, J and O, embryos were double stained with a probe for *ntl*, which marks marginal mesoderm cells. The gap (black arrowhead) between *ntl* and *p63* expression domains complementarily expresses *sox3* and *zic2.2* in *ich*$^{-/-}$ embryos. The white arrowheads in A-D indicate the position at which *sox3* is expressed in prospective forebrain neural tissue in wild-type embryos. Brackets in F-H,K-M indicate the reduction in prospective anterior neural plate size in *din*$^{-/-}$, *bmp2b*-injected and *ich*$^{-/-}$ embryos.

Chordin (*din*) (*n*=9), and in *ichabod*$^{-/-}$ embryos (*n*=20) that are defective in β-catenin signalling and entirely lack the organiser (Hammerschmidt and Mullins, 2002; Kelly et al., 2000) (Fig. 3A-J). In all these situations, rostral neural tissue is reduced or absent, whereas expression of the epidermal marker *p63* extends further dorsally consistent with previous observations (Fig. 3K-O) (Hammerschmidt and Mullins, 2002; Kelly et al., 2000). By contrast, irrespective of the severity of the ventralised phenotype, *p63* expression does not expand vegetally, always retaining a gap between its expression domain and that of *ntl* in the nascent mesoderm (Fig. 3O). These results indicate that expression of markers of prospective vegetal neural tissue is not dependent upon the organiser. They also suggest that induction of these markers is either unaffected by high levels of Bmp signalling or that Bmp activity in vegetal regions of the gastrula can be antagonised by mechanisms independent of the organiser.

Fgf receptor activity is required for induction of expression of markers of vegetal prospective neural tissue

As vegetal ectodermal markers are expressed around the entire circumference of the gastrula, signals from the germ ring (the blastoderm margin) are likely to contribute to their induction. Fgf genes are good candidates for encoding these signals as several are expressed in the germ ring (Draper et al., 2003; Furthauer et al., 1997; Koshida et al., 2002) and previous studies have implicated this pathway in neural induction (Akai and Storey, 2003; Stern, 2001; Wilson and Edlund, 2001). We therefore assessed the requirement for Fgf signalling in the induction of vegetal *sox3* expression.

Expression of vegetal ectodermal markers was examined in embryos that had been treated from the 1024-cell stage with SU5402, an inhibitor for Fgf receptor activity (Mohammadi et al., 1997) that should abrogate activity of zygotically encoded Fgf signals emanating from the germ ring. In embryos treated with 60 μM SU5402, vegetal *sox3* expression is variably decreased, whereas the animal (anterior) domain of *sox3* expression expands vegetally on the dorsal side of the embryo (Fig. 4A-D). With a higher dose of SU5402 (90 μM), more than 90% of embryos showed a severe phenotype equivalent to that shown in Fig. 4D (*n*>25). In these embryos, the vegetal ectodermal markers *sox31* and *zic2.2* behaved similarly to *sox3*, showing loss of ventrovegetal expression and retention of dorsal expression (Fig. 4E-H). Supporting the notion that the remaining prospective neural tissue on the dorsal side of these embryos has anterior character, expression of *otx2*, which is restricted to the prospective anterior neural tissue of gastrula stage wild-type embryos, is expanded vegetally on the dorsal side of the embryo (Fig. 4I,J) (see also Koshida et al., 1998; Kudoh et al.,

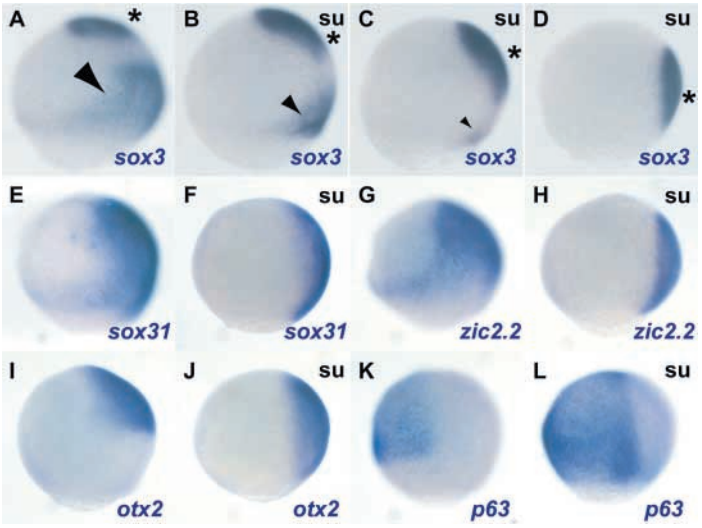

Fig. 4. Inhibition of Fgf activity prevents formation of vegetal prospective neural tissue. Lateral views with dorsal right of gastrula stage embryos (80-90% epiboly). Wild-type embryos (A,E,G,I,K) and embryos treated with SU5402, a Fgf receptor inhibitor (B,C,D, with 60 μM SU5402; F,H,J,L, with 90 μM) labelled to show *sox3* (A-D), *sox31* (E,F) and *zix2.2* (G,H) expression, the anterior neural marker gene, *otx2* (I,J), and the non neural marker gene, *p63* (K,L). The arrowheads in A-D indicate the variable decrease in vegetal *sox3* expression and the asterisks show that the animal (anterior) domain of *sox3* expression is largely unaffected (although repositioned closer to the vegetal side of the embryo).

2002). Complementing the changes in prospective neural genes, expression of the prospective epidermal marker, *p63*, was expanded ventrovegetally into the territory from which prospective neural marker gene expression is lost (Fig. 4K,L). Similar results (Fig. 5A-C), were obtained through overexpression of a truncated, dominant-negative Fgf receptor, XFD (Amaya et al., 1991; Griffin et al., 1995). These data support the idea that induction of vegetal prospective neural tissue is dependent on Fgf activity and that the residual neural tissue that remains after Fgf receptor blockade has anterior character.

Many previous studies have shown that enhanced Bmp activity during gastrulation can suppress development of rostral neural tissue (Fig. 3) (reviewed by Hammerschmidt and Mullins, 2002), and the experiments described above show that suppression of zygotic Fgf activity can suppress caudal neural development. Consistent with these observations, embryos in which Fgf activity was suppressed, while Bmp activity was enhanced showed complete absence of *sox3* expression (Fig. 5D).

Exogenous Fgf activity is able to promote expression of prospective neural markers even when the Bmp signalling pathway is active

Complementing the Fgf loss-of-function studies, increasing Fgf3 activity leads to induction of ubiquitous ectodermal expression of *sox3*, *sox31* and *zic2.2* (Fig. 5E,H,K) (Koshida et al., 2002; Furthauer et al., 1997) and this induction is unaffected by overexpression of *bmp2b* (Fig. 5F,I,L) (>90%, *n*>15). This suggests either that Fgf can induce expression of prospective neural genes in the presence of high levels of Bmp activity or that Fgf activity can block Bmp signalling.

Previous studies have shown that one mechanism by which Fgf signalling can indirectly induce neural tissue is by antagonising Bmp activity (Furthauer et al., 1997; Koshida et al., 2002; Pera et al., 2003; Wilson et al., 2000). We consequently asked if abrogation of Bmp signalling by Fgf activity is necessary for Fgf activity to induce *sox3* expression. Overexpression of *fgf3* leads to widespread induction of *chd* expression, which contributes to suppression of *bmp4* expression [and presumably lowered Bmp activity (Koshida et al., 2002); Fig. 5N,Q; >90%; *n*>30]. In this situation, the expanded expression of prospective neural markers (Fig. 5E,H,K) could, therefore, be due to suppression of Bmp activity. However, co-expression of *bmp2b* in embryos overexpressing *fgf3* suppresses *chd* expression and expands *bmp4* expression (Fig. 5O,R) (>80%, *n*>30), and yet despite this, the ectoderm still ubiquitously expresses prospective neural markers (Fig. 5F,I,L). Therefore, the Bmp pathway can be activated in the presence of Fgf signalling without affecting induction of prospective neural gene expression. These data indicate that although Fgf activity can suppress Bmp signalling by promoting expression of Bmp antagonists [and by other means (e.g. Pera et al., 2003)], this is not necessary for Fgf signalling to be able to induce markers of prospective neural identity.

Cells unable to receive Fgf signals fail to contribute to caudal neural tissue

To further explore the epistatic relationships between the Fgf and Bmp pathways, we examined the consequences of locally activating or suppressing Fgf signalling. *fgf3*-expressing ectodermal cells transplanted into animal pole regions of host embryos induce *sox3* non-autonomously in surrounding host cells (Fig. 6A; >80%; *n*>20). This induction still occurs if the donor cells are from embryos co-expressing a truncated Fgf receptor (Fig. 6B), suggesting that the Fgf signal from the donor cells acts directly on the host (100%; *n*=19). When both donor and host cells are overexpressing *bmp2b*, *fgf3*-expressing cells still induce *sox3* and suppress expression of the epidermal marker gene, *foxi1* (Fig. 6C,D; 100%; *n*=22), again suggesting that exogenous Bmp activity does not block induction of prospective neural marker genes by Fgf.

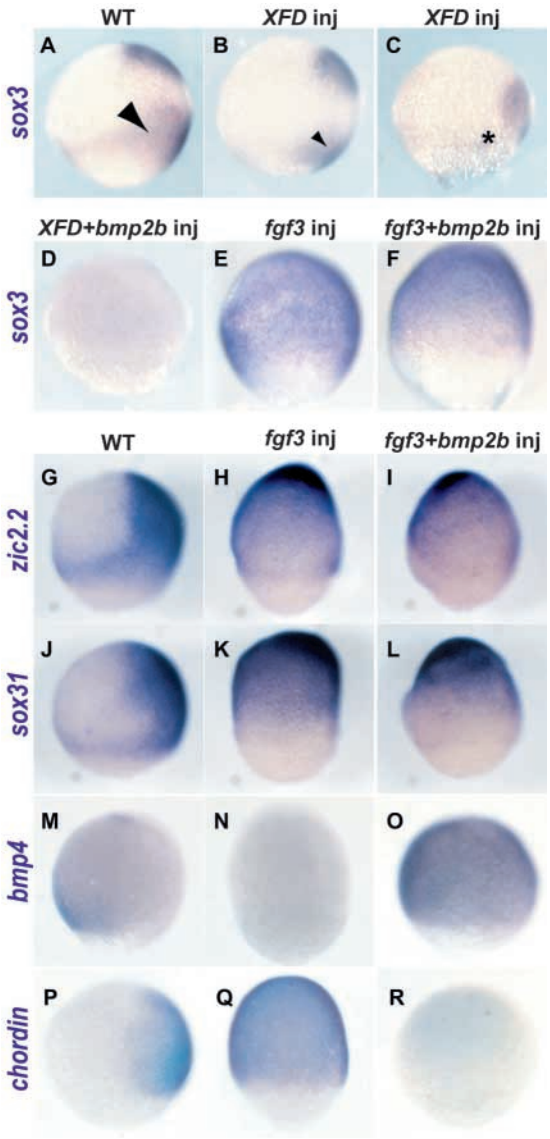

Fig. 5. Fgf can induce prospective neural markers independent of antagonism of Bmp activity. Lateral views with dorsal towards the right (where dorsal is distinguishable) of late gastrula stage wild-type embryos and embryos overexpressing the RNAs indicated above the columns. Genes analysed by in situ hybridisation are indicated to the left of each row. The phenotypes of XFD-expressing embryos are similar to those of SU5402-treated embryos (Fig. 4) with vegetal *sox3* expression reduced (A,B, arrowhead; C, asterisk) and animal expression shifted vegetally on the dorsal side of the embryo.

Next, we locally suppressed Fgf signalling by transplanting XFD-expressing donor cells to various positions in the prospective neural ectoderm of host embryos. *sox3* expression was suppressed in XFD-expressing cells transplanted to dorsal, lateral or ventral vegetal ectoderm (Fig. 6E-J) (>80% *n*=30), and *foxi1* was ectopically induced in transplants targeted to ventral-vegetal ectoderm (Fig. 6K,L) (>70%; *n*=27). These results suggest that ventral vegetal ectoderm needs to receive Fgf to express *sox3*, otherwise it expresses the prospective epidermal marker, *foxi1*.

To directly assess if Fgf signals are essential for vegetal ectoderm to form neural tissue, we traced the eventual fate of XFD-expressing donor cells transplanted to wild-type hosts. In these experiments, labelled wild-type cells were co-transplanted with XFD-expressing cells to the same locations in the vegetal ectoderm of unlabelled host embryos at the end of blastula stage. When the donor cells were transplanted to dorsal side, wild-type cells primarily contributed to the hindbrain, whereas the XFD-expressing cells localised more anteriorly, mainly in the midbrain (*n*=14). However, when transplanted to ventral vegetal ectoderm, wild-type donor cells contributed to spinal cord and muscle (see also Kimmel et al., 1990) whereas XFD-expressing cells were excluded from the CNS and found in tissues such as the epidermis and fin (*n*=8). These results suggest that Fgf signalling is required for vegetal ectoderm to contribute to caudal neural tissue. They also suggest that the consequences of suppression of Fgf signalling in cells in dorsal and ventral domains of the vegetal ectoderm are different: dorsally, cells with compromised Fgf signalling frequently move into anterior neural tissue; ventrally, cells move into the prospective epidermis and are excluded from neural tissue. These results are consistent with analyses of embryos in which XFD is expressed ubiquitously (e.g. Griffin et al., 1995) (Fig. 3) and which show loss of posterior neural structures and anteriorisation of remaining CNS tissue on the dorsal side of the embryo.

Bmp and Fgf activities in ventrovegetal regions of the ectoderm cooperatively promote prospective caudal neural fate

If Bmp signalling can still occur in vegetal ectoderm without suppressing *sox3* expression, then this raises the issue of what Bmp activity is doing in this domain. Within animal pole ectoderm, Bmp activity promotes expression of markers of ventral, non-neural, ectodermal fates (such as *p63* and *foxi1*) at the expense of expression of dorsal neural markers [such as *otx2* (Hammerschmidt and Mullins, 2002); Fig. 7B, parts i,iv; C, parts i,iv]. In vegetal ectoderm, DV organisation does not correspond to neural versus non-neural fates, but rather is predictive of the anterior-to-posterior (AP) position that neural cells will occupy within the caudal CNS (Fig. 2). This raises the possibility that in vegetal ectoderm, Bmp activity promotes regional specification within the prospective neural ectoderm rather than neural/non-neural fate specification. Supporting a global role for Bmp signalling in promoting ventral fates in the gastrula, irrespective of their neural or non-neural destiny, expression of the ventral vegetal marker *eve1* is induced by overactivation of Bmp signalling, whereas the dorsal vegetal marker *hoxb1b* is suppressed [Fig. 7B, parts ii,iii; C, part ii,iii; similar results have been observed in mutants or embryos with other modulations affecting Bmp signalling (e.g. Bakkers et al., 2002; Hammerschmidt et al., 1996; Mullins et al., 1996)]. As cells within the domain of *eve1* expression of wild-type embryos give rise to more posterior neural fates than cells within the ectodermal domain of *hoxb1b* expression, then this suggests that graded Bmp activity in vegetal ectoderm

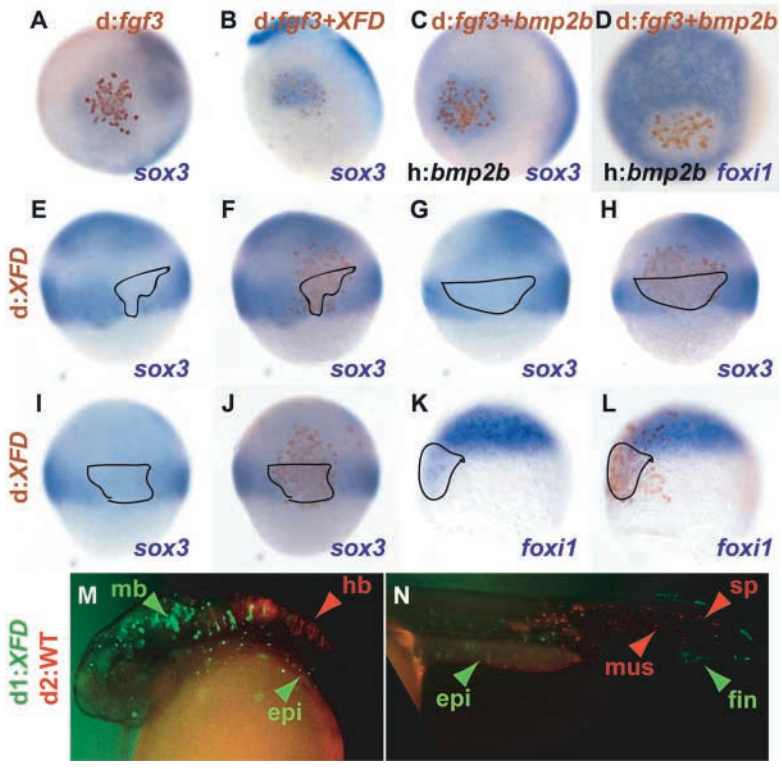

Fig. 6. Fgf signalling can locally induce *sox3* expression and is required for cells to contribute to caudal CNS. (A-L) Views of late gastrula stage wild-type embryos in which donor (d) cells (brown) overexpressing the genes/constructs indicated top right or left side of panels were transplanted at late blastula stage. In C and D, the host (h) embryos are overexpressing the RNAs indicated in the bottom left-hand corner. Genes analysed are indicated in the bottom right-hand corner. Orientation varies depending on the location of the donor cells: (A) laterodorsal, (B) lateral, (C,D) animal, (E,F) dorsal, (G,H) lateral and (I-L) ventral views. In E-L, in situ stained (purple) embryos were photographed before (E,G,I,K) and after (F,H,J,L) staining to reveal donor cells (brown). The domains where *sox3* expression was suppressed (E-J) or *foxi1* expression was ectopically induced (K,L) are outlined. (M,N) Lateral views of the head (M) and tail (N) of 1-day-old embryos in which XFD-expressing donor cells (d1, green) were mixed and co-transplanted with wild-type donor cells (d2, red) to the same positions in dorsal (M) or ventral (N) vegetal ectoderm at around 50% epiboly stage. In the dorsal transplant, wild-type cells localise to the hindbrain (hb), whereas XFD cells are distributed in the midbrain (mb) and on the surface of the 1-day-old embryo, possibly in the epidermis (epi) (M). In the ventral transplant, wild-type cells localise to tail spinal cord (sp) and somite muscle (mus), whereas XFD-expressing cells are excluded from the spinal cord and localise predominantly to the fin and epidermis (N).

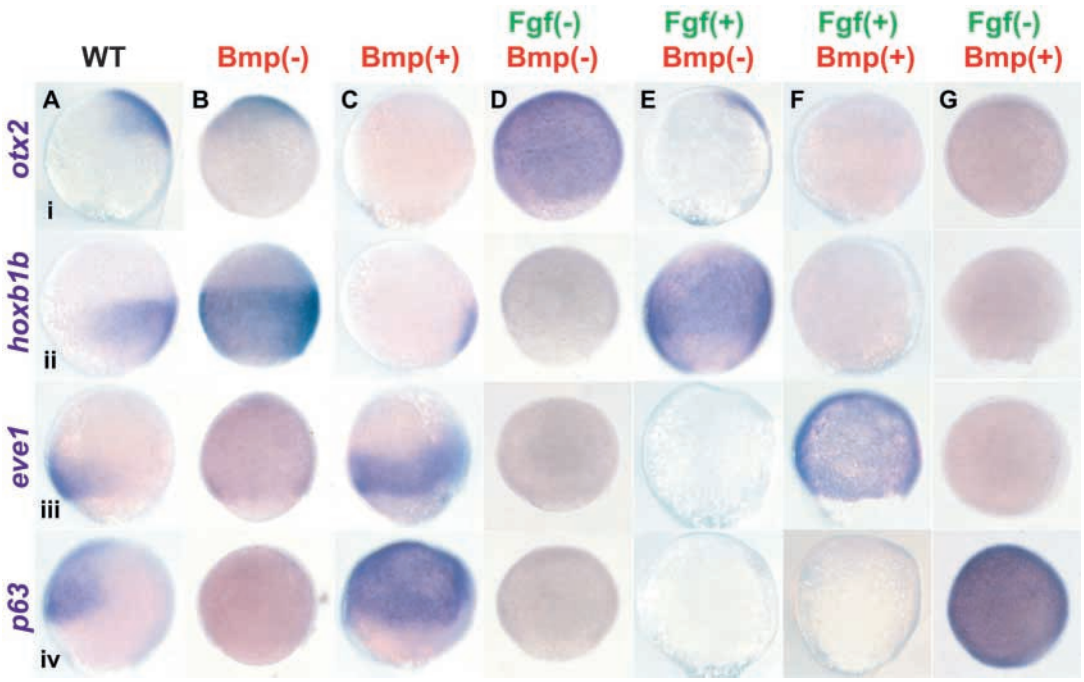

Fig. 7. Combinatorial activity of Bmp and Fgf signals promotes expression of different regional markers of the gastrula ectoderm. Lateral views of late gastrula stage embryos with dorsal towards the right. Genes analysed are indicated to the left of the rows: *otx2*, prospective head neural ectoderm; *hoxb1b*, prospective trunk neural ectoderm; *eve1*, prospective tail neural ectoderm and mesoderm; *p63*, prospective epidermis. Bmp activity was modulated by expression of exogenous *chordin* or *bmp2b*; Fgf activity was modulated by expression of a dominant-negative Fgf receptor (XFD) or *fgf3*. The presumed state of activation of the two pathways is indicated in red/green above each column. Note that overexpression of *fgf3* (E) induces *chordin* (see Fig. 2Q) and therefore suppresses Bmp activity (Koshida et al., 2002).

contributes to the allocation of fates along the AP axis of the neural tube rather than to a neural versus non-neural fate choice.

These results suggest that the consequences of graded Bmp activity are different in vegetal gastrula stage ectoderm, which is exposed to Fgf signals from the germ ring than in animal ectoderm (which we predict is unaffected by these Fgf signals). To test this hypothesis further, we manipulated the levels of Bmp activity in embryos in which Fgf signalling was either ubiquitously activated or was suppressed. When Fgf activity is suppressed, the entire ectoderm expresses either the prospective epidermal marker *p63* or the prospective forebrain marker *otx2*, depending on whether Bmp activity is enhanced or suppressed (Fig. 7D,G). These data are consistent with the role of endogenous Bmp signalling in animal pole ectoderm of wild-type embryos. Conversely, ubiquitous activation of Fgf3 signalling leads to either ubiquitous *eve1* or *hoxb1b* expression, again depending on the levels of Bmp activity [Fig. 7E,F; induction of *hoxb1b* by *fgf3* is also reported elsewhere (Koshida et al., 2002)]. These data are consistent with the role we predict for endogenous Bmp signalling within vegetal ectoderm of wild-type embryos.

Alterations to Bmp activity in embryos lacking Chordin function affects the allocation of trunk versus tail fates

To explore the possibility that in vegetal regions of the ectoderm, Bmp signalling promotes the adoption of caudal neural fate, we examined marker gene expression and fates of vegetal ectoderm in *din*$^{-/-}$ mutants/morphants in which Bmp activity is enhanced. In *din*$^{-/-}$ embryos, the caudal marker, *eve1* is expanded to lateral vegetal ectoderm (Hammerschmidt et al., 1996) (Fig. 8B), whereas vegetal *sox3* expression is not significantly altered (Fig. 3B, Fig. 8D). Furthermore, while exogenous Fgf3 induces *sox3* throughout the ectoderm both in *din*$^{-/-}$ and in wild-type embryos (Fig. 8G,H), *eve1* is suppressed in wild-type embryos (n=37), whereas expression expands throughout most of the *sox3*-expressing ectoderm in *din*$^{-/-}$ mutants (n=13) (Fig. 8E,F) with the exception of the dorsal most ectoderm which retains *hoxb1b* expression in this condition (Koshida et al., 2002). These data suggest that a key role for Chordin in the vegetal ectoderm is to suppress *eve1* and tail formation, and raise the possibility that cell fate in the lateral vegetal ectoderm may be changed from trunk neural to tail neural fate in *din*$^{-/-}$ mutants. Indeed, the size of trunk may be smaller and tail bud larger in *din*$^{-/-}$ embryos compared with wild-type embryos (Hammerschmidt et al., 1996; Myers et al., 2002).

To directly assess if the expansion of *eve1* in lateral vegetal ectoderm of embryos lacking Chordin activity correlates with a switch from prospective trunk to prospective tail identity, the fate of lateral vegetal ectoderm was assessed in *din* morphants. As expected, lateral vegetal ectodermal cells contributed primarily to the trunk spinal cord in wild-type embryos (Fig. 2D,E,F; Fig. 8I,J,K). By contrast, the majority of equivalently positioned vegetal ectoderm cells in *din* morphants contribute to the tail, including the most caudal spinal cord, a region that does not receive contribution from lateral ectodermal cells in wild-type embryos (n=11) (Fig. 8L-N). Therefore in the absence of Chordin function, equivalently positioned ectodermal cells tend to contribute to more caudal regions of the CNS than in wild type, supporting the idea that Bmp activity affects the allocation of regional AP fates in the vegetal ectoderm.

Discussion

Our study has revealed that combinatorial activity of the Bmp

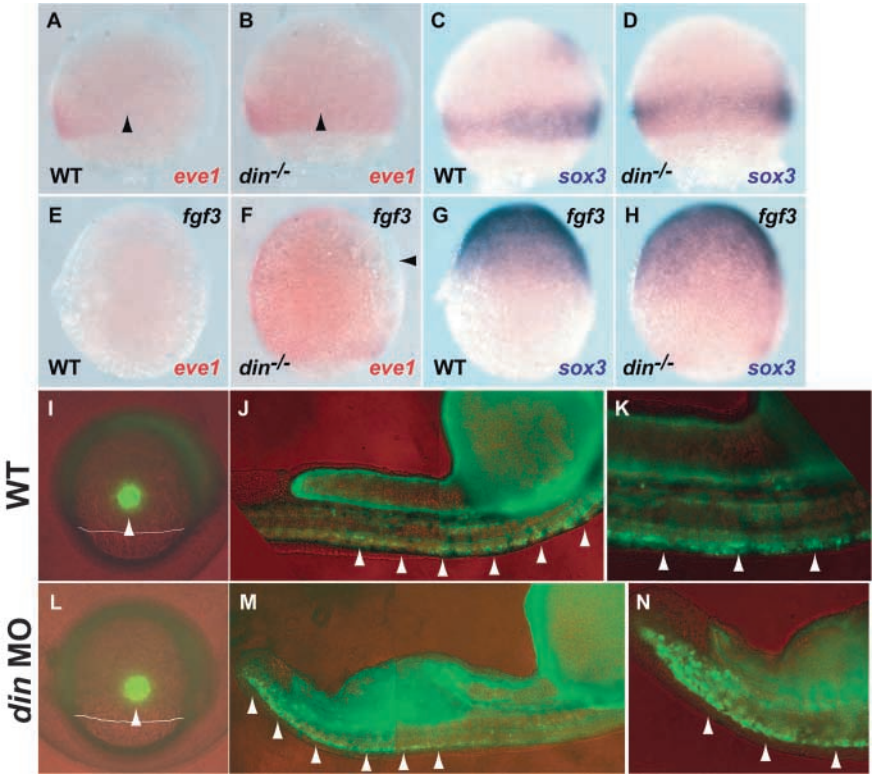

Fig. 8. Lateral vegetal ectoderm contributes to tail rather than trunk spinal cord in *chordino* mutant embryos. (A-H) Wild-type (wt), and *chordino* mutant (*din*$^{-/-}$) gastrula stage embryos, with or without injection of *fgf3*, stained initially to show *eve1* expression (red; A,B,E,F) and subsequently to show *sox3* expression (purple; C,D,G,H). Arrowheads in A and B indicate the position of vegetal lateral ectoderm, where *eve1* expression is absent in wild type (A) while expanded in *din*$^{-/-}$ (B). The arrowhead in F indicates the dorsal most ectoderm where *eve1* expression is absent. (I-N) Lateral views of gastrula (I,L) and 1-day-old (J,K,M,N) wild-type (I-K) and *chordin* morphant (din MO; L-N) in which fluorescein was uncaged in lateral vegetal ectoderm (I,L, arrowheads) and fates of fluorescent cells were traced on the next day (J,K,M,N). In the wild-type embryo, vegetal lateral ectoderm cells mostly give rise to trunk neural tube (J,K, arrowheads), whereas in the *chordin* morphant, the cells occupy the tail, including the caudal spinal cord (M,N, arrowheads). White lines in I,L indicate the vegetal limit of the germ ring.

and Fgf signalling pathways during blastula and gastrula stages contributes to the subdivision of the embryonic ectoderm into prospective neural and epidermal domains. We show that in vegetal ectoderm, initiation of neural fate specification requires Fgf activity and can occur in the presence of Bmp signalling activity (Fig. 9). Indeed, the regulation of prospective neural/non-neural marker gene expression in vegetal ectoderm appears to be independent of all dorsal organiser activity, as it still occurs in the most severely ventralised *ichabod* mutant embryos in which β-catenin-dependent development of dorsal structures fails to occur (Kelly et al., 2000). This study shows that both Fgfs and Bmp antagonists can promote neural development and may help reconcile the differing mechanisms proposed to regulate early neural development and patterning in amniotes and anamniotes.

Bmp activity promotes fates of cells on the ventral side of the gastrula

We find that Bmp activity promotes fates of cells positioned on the ventral side of the gastrula ectoderm irrespective of whether or not these cells are destined to contribute to neural or non-neural structures. In vegetal ectoderm, Bmp activity promotes tail neural fate at the expense of trunk neural cell fates whereas towards the animal pole, it promotes epidermal at the expense of neural fates (Fig. 9). Other studies have shown that Bmp activity promotes caudal and ventral mesodermal fates (Munoz-Sanjuan and Hemmati-Brivanlou, 2001; Myers et al., 2002), and so Bmp signalling may have graded activity throughout all germ layers, promoting all fates that lie on the ventral side of the gastrula. Our data helps to reconcile how high levels of Bmp activity during gastrulation can promote development of both epidermis and tail neural tissue by showing that these alternative consequences are dependent upon the level of Fgf activity. Given that the consequences of activation of the Fgf and Bmp pathways differ in different regions of the gastrula, it will be important to resolve how these two signalling pathways influence cell fate.

Bmp signalling may promote caudal neural fate by regulating cell movements

Given that Bmp activity may not directly specify cell identity (as it promotes several distinct fates for ventrally positioned cells), it is intriguing to consider how this pathway can promote the adoption of caudal neural fate. An attractive possibility is that it does so by regulating the movements of gastrulating cells. Solnica-Krezel and her colleagues (Gonzalez et al., 2000; Myers et al., 2002) have analysed movements of gastrulating cells in embryos with different levels of Bmp activity. They have shown that laterally positioned cells in the wild-type gastrula move dorsally, whereas the ventral-most cells fail to converge dorsally and consequently end up in the tail bud area. In *din*$^{-/-}$ embryos, the domain of non-convergence is expanded laterally with the likely consequence that more cells end up in the tail bud. Therefore, if Bmp-dependent regulation of dorsal convergence also occurs within the ectoderm, then this model provides an attractive explanation of why laterally positioned prospective neural cells have a higher likelihood of ending up in the tail spinal cord in *din*$^{-/-}$ embryos.

If the role of the Bmp pathway in caudal development is primarily to regulate cell movements, then other pathways may cooperate with Bmps to impose caudal cell fates. Several candidates are well documented (Munoz-Sanjuan and Hemmati-Brivanlou, 2001), and in fish, Agathon and colleagues (Agathon et al., 2003) have recently shown that entire tail structures could be induced by simultaneous activation of Bmp, Nodal and Wnt pathways. Although a role

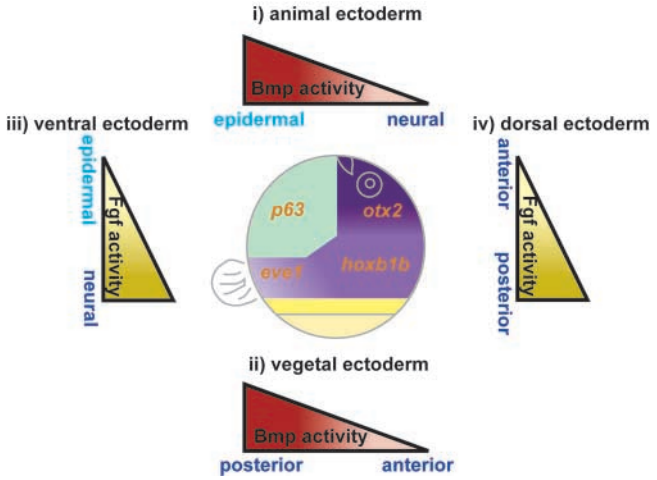

Fig. 9. Summary of results. The cartoon in the middle represents a lateral view of a gastrula stage embryo. Prospective epidermis is light green; prospective CNS is purple, with dark colour indicating prospective anterior fate and progressively lighter colour indicating progressively more posterior fate (as indicated in the legend to Fig. 2; the organisation of prospective dorsal versus ventral prospective CNS structures is not indicated). Prospective mesendoderm is yellow. Bmp activity is graded during gastrulation: high ventrally and low dorsally (i,ii). This gradient is established, at least in part, by an opposing gradient of dorsally derived Bmp antagonists such as Chordin (e.g. Hammerschmidt and Mullins, 2002). Fgf may also have graded activity during blastula and gastrula stages: high vegetally and low at the animal pole (iii,iv) (Figs 2, 3) (Furthauer et al., 2002; Roehl and Nusslein-Volhard, 2001; Tsang et al., 2002). In the animal gastrula ectoderm, high levels of Bmp activity promotes epidermal fate, while Bmp antagonists promote neural fate (i). Conversely, in vegetal ectoderm, Bmp activity promotes caudal neural fate, whereas Bmp antagonists promote the adoption of more rostral neural fate (ii). The different consequences of activation or suppression of Bmp activity in animal and vegetal ectoderm are influenced by Fgf-dependent promotion of prospective neural fate in vegetal ectoderm (iii). In addition to promoting neural specification, Fgf signalling posteriorises neural tissue in the vegetal ectoderm, most obviously on the dorsal side (iv).

for Wnt signalling in posterior development is supported by many other studies (e.g. Erter et al., 2001; Hashimoto et al., 2000; Marlow et al., 2004), a requirement for Nodal signalling to form tail tissue is less clear given that fish embryos lacking Nodal activity do still form tails that contain neural tissue (Feldman et al., 2000). Nevertheless, it is likely that cooperation between several signalling pathways (e.g. Haremaki et al., 2003) is required for ventrally positioned cells to move and adopt appropriate fates within the tail.

To our knowledge, a source of tail neural tissue distant from the dorsal organiser has not been shown for other model species and it will be of interest to determine if there are similar roles for Bmp activity in promoting caudal neural fates in these species.

Fgf signalling promotes both induction of prospective neural fate and posteriorisation of the ectoderm

We show that Fgf signalling promotes expression of prospective neural markers and is required for ectodermal cells to contribute to caudal CNS structures. This is consistent with data in other species that suggest Fgfs are important regulators of neural induction (Bertrand et al., 2003; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Streit et al., 2000; Wilson et al., 2000; Ying et al., 2003). However, there is a larger body of literature that indicates Fgf signalling promotes caudalisation of neural tissue (Bally-Cuif and Hammerschmidt, 2003; Cox and Hemmati-Brivanlou, 1995; Koshida et al., 1998; Kudoh et al., 2002) and it will be important to determine if these two activities are separable.

In this study, we show that abrogation of Fgf activity leads to loss of prospective neural tissue on the ventral side of the embryo (supporting a role for Fgf signalling in an early step of neural induction) and also anteriorisation of the residual neural tissue on the dorsal side of the embryo (supporting a role for Fgf in posteriorisation) (see also Koshida et al., 1998; Kudoh et al., 2002). Some of our data and other reports suggest that Fgf-dependent induction of prospective neural fate and posteriorisation of induced neural tissue are separable events. For example, induction of expression of the posterior neural gene, *hoxb1b* by Fgf3 depends on Chordin function (Koshida et al., 2002) and is probably mediated indirectly by retinoic acid (Kudoh et al., 2002). By contrast, induction of the prospective neural markers *sox3*, *sox31* and *zic2* is independent of Chordin activity (this study). Furthermore, Koshida et al. (Koshida et al., 2002) have shown that XFD-expressing cells transplanted to wild-type hosts are still able to express *hoxb1b*, supporting an indirect role for Fgf on *hoxb1b* expression. However, our results suggest that ventral vegetal ectodermal cells may directly need to receive Fgf signalling to specify neural fate (to induce *sox3*, to suppress *foxi1* and to contribute to caudal CNS). Therefore, at least in part, the roles of Fgf signalling in neural induction and posteriorisation by Fgf seem to be separable.

Other signals may cooperate with Fgfs during formation and differentiation of neural tissue

Although the regulation of early neural/non-neural marker gene expression in vegetal ectoderm is independent of Bmp antagonism, this does not discount the possibility that Bmp antagonists may be contributing to the maintenance and/or further differentiation of neural plate. Although there has not been any detailed analysis of mature neural tissue in *ichabod* mutant or other severely ventralised embryos, it appears that relatively few neurones are present in these fish (Gonzalez et al., 2000) (T.K., unpublished), despite the early expression of vegetal prospective neural markers. This suggests that exposing ectodermal cells to Fgf signalling alone at blastula and gastrula stage is not sufficient to maintain and promote further differentiation of the neural tube. Bmp antagonists such as Chordin and Noggin 1 may therefore contribute to later steps in the induction, maturation and/or maintenance of neural tissue. Indeed, the idea that Chordin is not necessary for the initial phase of neural induction but rather functions at a later step of neural development has been suggested from neural induction assays in chick (Stern, 2002; Streit et al., 1998).

In our studies, we noticed that although exogenous Fgf induced early prospective neural marker genes it actually appeared to inhibit the expression of markers of mature neuronal identity such as *huC* (data not shown). This is of interest in light of recent observations that, in chick, levels of

Fgf activity are believed to be high in immature neural tissue and low in mature neural tissue (Diez del Corral et al., 2003). Indeed Diez del Corral et al. propose that inhibition of Fgf activity is a necessary step in the maturation of neural tissue that allows neuronal differentiation to occur. Thus, although exogenous Fgf activity may promote early steps in neural development (induction of expression of prospective neural markers and suppression of prospective epidermal markers), it may inhibit this prospective neural tissue from progressing to a fully differentiated state. Furthermore, it has recently been shown that Sox protein activity in undifferentiated neural tissue inhibits neurogenesis, and that this activity needs to be suppressed through the activity of proneural bHLH proteins (Bylund et al., 2003). Therefore as expression of Sox genes is promoted by Fgf signalling (this study) (Streit et al., 2000), this may provide an explanation of how Fgf can promote early neural fate yet inhibit later neuronal differentiation.

The molecular mechanisms by which Fgfs and Bmp antagonists interact to regulate prospective neural/non-neural gene expression are undoubtedly complex. In our study, we have shown that Fgf3 can induce *sox3* in ectodermal cells without suppressing the ability of the Bmp pathway to induce *bmp4* and suppress *chordin* expression. This indicates that Fgf activity does not lead to a comprehensive block of Bmp signalling. However, data from other studies have shown intracellular crosstalk between Fgf and Bmp pathways by which Fgf pathway activation can antagonise Bmp pathway activity. For example, in frogs, Map kinase, a downstream effector of Fgf signalling, phosphorylates a linker domain in Smad1 thereby suppressing Bmp signalling (Pera et al., 2003). Assuming similar Fgf-dependent regulation of Smad1 activity occurs in fish, then either this is insufficient to abrogate Bmp signalling, or there may be several independently regulated intracellular responses downstream of Bmp receptor activation. In addition to intracellular crosstalk between these two pathways, Fgf signalling can profoundly affect Bmp signalling activity through the regulation of expression of secreted Bmp antagonists such as Chordin (Koshida et al., 2002; Furthauer et al., 1997) (this study).

Is Fgf signalling required for induction of anterior prospective neural tissue?

In our study and in related experiments (e.g. Griffin et al., 1995; Koshida et al., 1998), XFD injection and SU5402 treatment both suppressed posterior neural induction, whereas anterior neural fate was retained in both situations. This is consistent with some experiments in other species showing a requirement for Fgf activity only in posterior neural development (Munoz-Sanjuan and Hemmati-Brivanlou, 2001). By contrast, in chick and ascidian (and from some experiments in frogs), it has been proposed that Fgf activity is crucial for induction of neural tissue, including anterior domains (Bertrand et al., 2003; Hongo et al., 1999; Hudson and Lemaire, 2001; Streit et al., 2000; Wilson et al., 2000). One possibility is that in fish, Fgf activity may be required in prospective anterior neural tissue at lower levels or at earlier stages than in more caudal neural tissue. If so, then sufficient early Fgf activity might still be present in manipulated embryos in our experiments to allow development of rostral neural tissue. Indeed, very recent studies have shown that early activation of Fgf signalling plays an important role in the regulation of the Bmp signalling pathway in prospective anterior neural tissue (Furthauer et al., 2004) and preliminary data suggest that abrogation of Fgf activity at stages earlier than reported here leads to more severe depletion of anterior neural tissue (T.K., unpublished).

In the animal (prospective anterior) gastrula stage ectoderm, Bmp antagonists emanating from the organiser, its derivatives and other tissues are crucial for neural development, whereas in prospective caudal regions, prospective neural fate appears to be specified by Fgfs emanating radially from the germ ring (Fig. 9). Therefore, we suggest that the organiser and the germ ring constitute two distinct and at least partially independent sources of signals that promote neural development. We predict that the combined activity of germ ring and organiser signals establishes prospective neural tissue from the dorsal animal ectoderm through to the ventral vegetal ectoderm. One consequence of this view of early neural induction is that the neural to non-neural ectodermal fate choice should not only be considered as occurring between dorsal and ventral ectoderm but also, on the ventral side of the gastrula, between animal and vegetal ectoderm.

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Note added in proof

Two very recent papers examine early activity and function of the Fgf pathway in zebrafish (Furthauer et al., 2004; Tsang et al., 2004). We briefly cite the Furthauer paper in the Discussion, but it contains additional data pertinant to our study.

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