epidermis and the suppression of neural development. *In situ* hybridization shows that RNA for Bmp-4 is present in the entire animal cap at the start of gastrulation, as well as in the ventral and lateral marginal zone[8,9]. At later stages, the transcript disappears from the portion of the ectoderm that becomes the neural plate[9]. Although our experiments cannot establish definitively that Bmp-4 is the endogenous epidermal inducer, the demonstration that epidermis can be induced at all is significant, because this has not previously been reported in any vertebrate embryo.

If Bmp-4 or a related substance acts within the gastrula animal cap to impose an epidermal fate, how does neural tissue form? Dispersion experiments imply that a block of epidermal induction may be sufficient. We have shown that the neuralizing effect of the truncated activin receptor can be attributed to interference with Bmp-4 signalling. However, the activin-binding protein follistatin, which also neuralizes *Xenopus* ectoderm when supplied as RNA[7], does not block Bmp-4 in our assay. These observations present a paradox, to which we can envisage two possible resolutions. It is possible that the endogenous epidermal inducer is not Bmp-4 but a related molecule, which can be bound by follistatin. Alternatively, injection of follistatin RNA may prevent epidermal induction indirectly, by interfering with an early signalling process. Several molecules in addition to follistatin have recently been shown to neuralize *Xenopus* ectoderm[17-20]. It will be interesting to see whether these factors, and the endogenous signals from Spemann's organizer, act by antagonizing Bmp-4 in some way.

Our proposal that Bmp-4 acts within the ectoderm to induce epidermis and suppress neural development suggests a close relationship to mesodermal patterning in the marginal zone, and perhaps to early patterning in the fly embryo as well. Experiments involving overexpression of Bmp-4 or of a dominant-negative BMP receptor[15,21] argue that this factor acts within the marginal zone to ventralize the mesoderm and to suppress dorsal specification[8] (reviewed in ref. 22). Thus it seems that Bmp-4 may play a similar role in the marginal zone and the animal cap. The secreted protein noggin, expressed in Spemann's organizer, has been shown to neuralize ectoderm and also to dorsalize ventral mesoderm[18,23]: perhaps a capacity to antagonize Bmp-4 in some way could underlie both activities. The notion that dorsal-ventral patterning has a common basis in ectoderm and mesoderm was proposed years ago by Yamada[24]. In the *Drosophila* embryo, the boundary between the neurogenic and dorsal epidermis-forming regions of the blastoderm is thought to be established by the product of the *dpp* gene, a Bmp-4 homologue. In particular, *dpp* can induce epidermis in regions of the ectoderm that would be neurogenic in its absence[25,26].

We have shown that the secreted growth factor Bmp-4 can induce epidermis in dispersed gastrula ectoderm, suppressing neural fate. This is the first report of an epidermal inducer in early vertebrate development. Bmp-4 is expressed in the gastrula ectoderm, at the time that fate is decided, leading us to propose that Bmp-4 acts endogenously to specify epidermis, and that neural induction may involve a BMP antagonist. □

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# Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in *Xenopus*

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**In *Drosophila* the amount of neurogenic ectoderm, from which the central nervous system (CNS) derives, is regulated by a dorsal–ventral system of positional information in which two secreted molecules of antagonistic functions, decapentaplegic (dpp) and short-gastrulation (sog), play fundamental roles[1–4]. The vertebrate homologue of *dpp* is either *bmp-4* or *bmp-2* (ref. 5), and the homologue of *sog* is *chd*[4,6,7] (*s-chordin*). In *Xenopus* the CNS is induced by signals emanating from the organizer[8], and two proteins secreted by the organizer, noggin[9] and follistatin[10], have been shown to induce neural tissue in animal-cap assays. Here we report that Chd, another organizer-specific secreted factor[6], has neuralizing activity and that this activity can be antagonized by Bmp-4. Inhibition of the function of the endogenous Bmp-4 present in the animal cap[11] also leads to neural differentiation. We suggest that conserved molecular mechanisms involving *chd/sog* and *bmp-4/dpp* gene products pattern the ectoderm in *Xenopus* and in *Drosophila*.**

The induction of neural differentiation was assayed by the expression of N-CAM RNA, a pan-neural marker (neurons and glia), or of NF-M (neurofilament-M) RNA, a pan-neuronal marker (neurons only), in injected animal caps explanted at early gastrula stage and cultured until siblings reached the tailbud stage (stage 27). Control injections of β-galactosidase (β-gal) messenger RNA or DNA construct did not change the differentiation of the animal caps (Fig. 1*a*, lanes 2 and 3), which form atypical epidermis[9,10]. Injection of *chd* mRNA induced the expression of N-CAM and NF-M to levels comparable to those found in intact embryos of the same stage (Fig. 1*a*, lane 4). To test whether Chd would also be active at gastrulation[12], when neural induction normally occurs, we injected cytomegalovirus (CMV)-promoter-driven DNA, which was as active in neurogenesis as *chd* mRNA (Fig. 1*a*, lane 5). To test whether this property might be conserved in its *Drosophila* homologue[4], *sog* mRNA was injected and similar results obtained (Fig. 1*a*, lane

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6). Neural differentiation by Chd took place in the absence of mesoderm induction, as demonstrated by the lack of expression of α-actin RNA at the tail-bud stage (Fig. 1*a*) and of the pan-mesodermal marker Xbra and the dorsal mesoderm markers gsc, noggin and follistatin at the gastrula stage (Fig. 1*b*). Chd induced visible cement glands, which are the most anterior ectodermal structures in *Xenopus*, as confirmed by the molecular marker CG13 (Fig. 1*c*). The neural tissue induced was of the type present in the anterior CNS, as documented by the expression of the anterior neural markers XANF-1, XIF3, Otx-2 and some En-2, and by the lack of induction of the posterior neural marker XlHbox 6 (Fig. 1*c*). Histologically (Fig. 2*h*) this neural tissue is of what embryologists called the archencephalic (forebrain) type[8]. The floor-plate marker F-spondin[13] was not induced (Fig. 1*c*). We conclude that Chd, the *Xenopus* homologue of sog, has a potent neuralizing activity. Like noggin[9] and follistatin[10], Chd induces neural tissue of the anterior type.

We were prompted to study the role of *chd* in neural induction by its pattern of expression in the mesodermal mantle at the late-gastrula stage (Fig. 2*a*, *b*), in which *chd* expression foreshadows the shape of the neural plate that will appear in the overlying ectoderm (Fig. 2*c*). In *Drosophila*, genetic studies have shown that dpp antagonizes sog *in vivo*[2,3,4], and this led us to test whether Bmp-4 could antagonize Chd in *Xenopus* pattern formation. Injection of *chd* mRNA resulted in dorsalized *Xenopus* embryos (Fig. 2*d*, *e*). Injection of Bmp-4 DNA (under the control of the cytoskeletal actin promoter, CSKA, whose expression does not start until early gastrulation[9]) into adjoining vegetal blastomeres resulted in the rescue of the dorsalized pattern and the formation of rather normal trunk–tail structures (Fig. 2*f*). In this experiment Bmp-4 and *chd* were injected into different cells and because a CSKA promoter construct and synthetic mRNA were used, the signals should act non-cell-autonomously and antagonize each other after the gene products were constitutively expressed. The effectiveness of Bmp-4 when introduced as a DNA construct is in keeping with the view that Bmp-4 functions as a ventralizing agent during gastrulation[14,15]. Having found that Bmp-4 can act as an antagonist of Chd in mesodermal patterning, we next tested whether it would affect its neural-inducing activity.

Histological analysis showed that the induction of cement gland and of solid masses of neural tissue by *chd* mRNA in animal caps can be eliminated by co-injection of CSKA–Bmp-4 DNA (Fig. 2*g–i*). The same antineurogenic action of Bmp-4 was observed when a marker of mature neurons, β-tubulin type II[16], was used as a molecular probe in *in situ* hybridizations (Fig. 2*j–l*). CSKA–Bmp-4 DNA was also able to inhibit the neural induction caused by expressing *chd* from a CMV promoter (Fig. 3*a*, lanes 4 and 5). We conclude that Bmp-4 expressed at the gastrula stage has a strong antineurogenic activity in gain-of-function experiments.

The animal cap (as well as the ventrolateral marginal zone) has significant amounts of zygotic Bmp-4 transcripts at the early gastrula stage[11]. To test whether these endogenous transcripts might prevent neural differentiation in the animal cap, directing the tissue towards a more ventral epidermal fate, we pursued a loss-of-function approach. Injection of mRNA encoding a dominant-negative Bmp-2/4 receptor[17,18] induced the neural markers N-CAM and NF-M and, as expected in case of a block in the Bmp-4 signalling pathway, this could not be reversed by co-injection of Bmp-4 DNA (Fig. 3*b*, lanes 6 and 7). The dominant-negative receptor interacts not only with Bmp-4, but also with Bmp-2 (refs 17, 18) and perhaps with other related factors[19]. To determine whether Bmp-4 itself was responsible for the anti-neurogenic activity in the animal cap, we used antisense RNA. It has recently been shown that antisense Bmp-4 can dorsalize *Xenopus* ventral mesoderm and that the antisense approach works in *Xenopus*, at least for genes expressed early after mid-blastula (H.S., A. Fainsod, C. Niehrs, Y.S. and E.M.D.R., manuscript submitted). When full-length antisense Bmp-4 RNA was injected, neural markers were induced, and this effect, unlike that of the dominant-negative receptor, could be reversed by co-

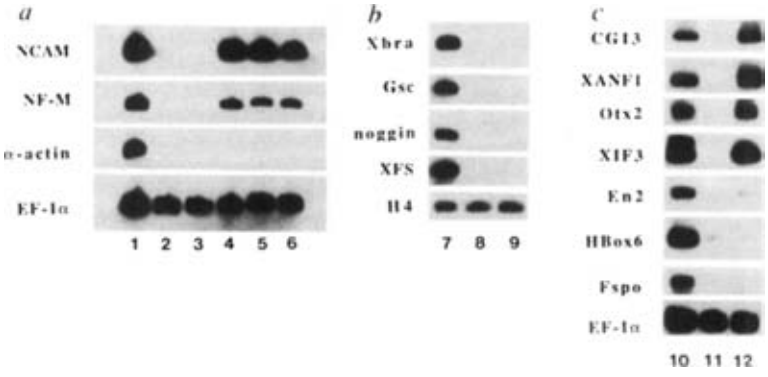


FIG. 1 Chd can induce anterior neural markers in the absence of mesoderm induction. Animal caps were excised from injected embryos at stage 10, cultured until stage 27 (*a*, *c*) or stage 11 (*b*), and assayed for the expression of marker mRNAs. Lanes contained: 1, RNA extracted from whole embryos at stage 27; 2, embryos injected with β-gal mRNA[4]; 3, with pCMV–β-gal DNA[12]; 4, *chd* mRNA[6]; 5, pCMV–chd (constructed for this study); 6, *sog* mRNA[4]; 7, activin-treated stage 11 animal caps[6]; 8, control β-gal mRNA; 9, *chd* mRNA; 10, whole-embryo RNA at stage 27; 11, control β-gal mRNA; 12, *chd* mRNA. Note that *chd* mRNA, *chd* DNA and *sog* mRNA induce the neural markers N-CAM and NF-M[26] without increasing the mesodermal marker α-actin. Xbra, gsc, noggin and follistatin (XFS) are not induced by *chd* mRNA injection. *chd* mRNA, but not control β-gal mRNA, induces cement gland (CF13 (ref. 27)) and anterior neural markers (XANF-1 (ref. 28), Otx-2, XIF3 (ref. 26) and some En-2) but not the posterior marker XlHbox 6 (Hox-b9). The floorplate marker F-spondin (Fspo) is not induced. Elongation factor 1α (EF-1α) and histone H4 were used as RNA-loading controls. METHODS. For all RNAs 0.3 ng, and for the CMV plasmids 0.1 ng, were injected per blastomere. Embryos were injected into each animal blastomere at the 8-cell stage. Animal caps were explanted at the gastrula stage and cultured in 0.3× modified Barth's solution until siblings reached the desired stage[6]. Only healthy animal caps in which cells continued to divide were used for further analysis. Total RNA was isolated by using the RNA-STAT kit[6], using two extractions to reduce contamination by genomic DNA. For each set of primers the number of cycles required for the linear range in the reverse transcription polymerase chain reaction (RT–PCR) was determined empirically. Many of the primer sets, conditions for PCR and the number of cycles were as described (H.S., A. Fainsod, C. Niehrs, Y.S. and E.M.D.R., submitted, and refs. 10, 11, 28), but the following were new. *CG13*, (forward primer (F), 5′-AGTTGTTGATTTTGTGAAAACC; reverse primer (R), 5′-CTTCTTCTAAATCAACAACAGG; 28 cycles); *Otx-2* (accession no. U19813) (F, 5′-GGATGGATTTGTTGCACCAGTC; R, 5′-CACTCTCCGAGCTCACTTCTC; 30 cycles); NF-M (accession no. M25696) (F, 5′-GCGGGTACCTTCTAATAGTCAC; R, 5′-GGCTTGGCTGTGGTTCTGAAGG; 28 cycles); XIF3 (F, 5′-ATCCTCCAGGCTATCCACCTCC; R, 5′-TAGCGGACCTTCTCTATGAAGC; 28 cycles); F-spondin (F, 5′-TCTGGCAGTATGTGGCAACGTC; R, 5′-GTACAATGCTCGCCTTGAGTCTC; 30 cycles); noggin (F, 5′-ATGATCATTCCCAGTGCCTTGTGAC; R, 5′-AGATTAGTCCAAGAGTCTCAGCATGAGC; 30 cycles); follistatin (F, 5′-ATGGTAAATGAAAGGATCCAGCCGGGCATG; R, 5′-ATTCACTTACAGTTGCAAGATCCACTGTG; 30 cycles). The DNA expression construct pCMV–*chd* was constructed by subcloning a *Hin*dIII–*Xba*I fragment of pSP35–*chd* (ref. 6) into pCDM8 (Invitrogen). Previous studies[12] have shown that a similar construct (pCMV–βgal) accumulates a significant amount of the protein product only after gastrulation starts. mRNAs were synthesized with SP6 polymerase by using the 'Message Machine' kit (Ambion); all synthetic mRNAs used in this study were engineered so that they contained 5′ leader and 3′ trailer sequences derived from the *Xenopus* β-globin mRNA[4,6].

injection of CSKA-Bmp-4 DNA (Fig. 3*b*, lanes 8 and 9). As specificity controls, we used ΔBmp-4 antisense RNA lacking the mature carboxy-terminal region that is conserved among TGF-β growth factors, which behaved as its full-length antisense counterpart (lane 10); ΔBmp-2 antisense RNA, which had no neural inducing activity (lane 11); and, as a negative control, *gsc* antisense RNA, which is not expressed in animal caps and, as expected, had no effect (lane 12). We conclude from these loss-of-function experiments that Bmp-4, but not Bmp-2, is required in the animal cap to prevent neural differentiation.

The genes *chd*, *noggin* and *follistatin* are expressed in the organizer, induce neural tissue of the archencephalic type and can dorsalize mesoderm. A dorsalizing function for follistatin has not been noted before, but as shown here *follistatin* mRNA, like *noggin* and *chd*, is able to induce dorsalization, partial secondary axes and the formation of dorsal tissues in ventral marginal zone (VMZ) explants in microinjected *Xenopus* embryos (Fig. 2*m–o*). This supports the view[20] that follistatin might not bind exclusively activin, and indicates that follistatin can counteract ventral signals. Because of these common features, we tested whether the antineurogenic activity of Bmp-

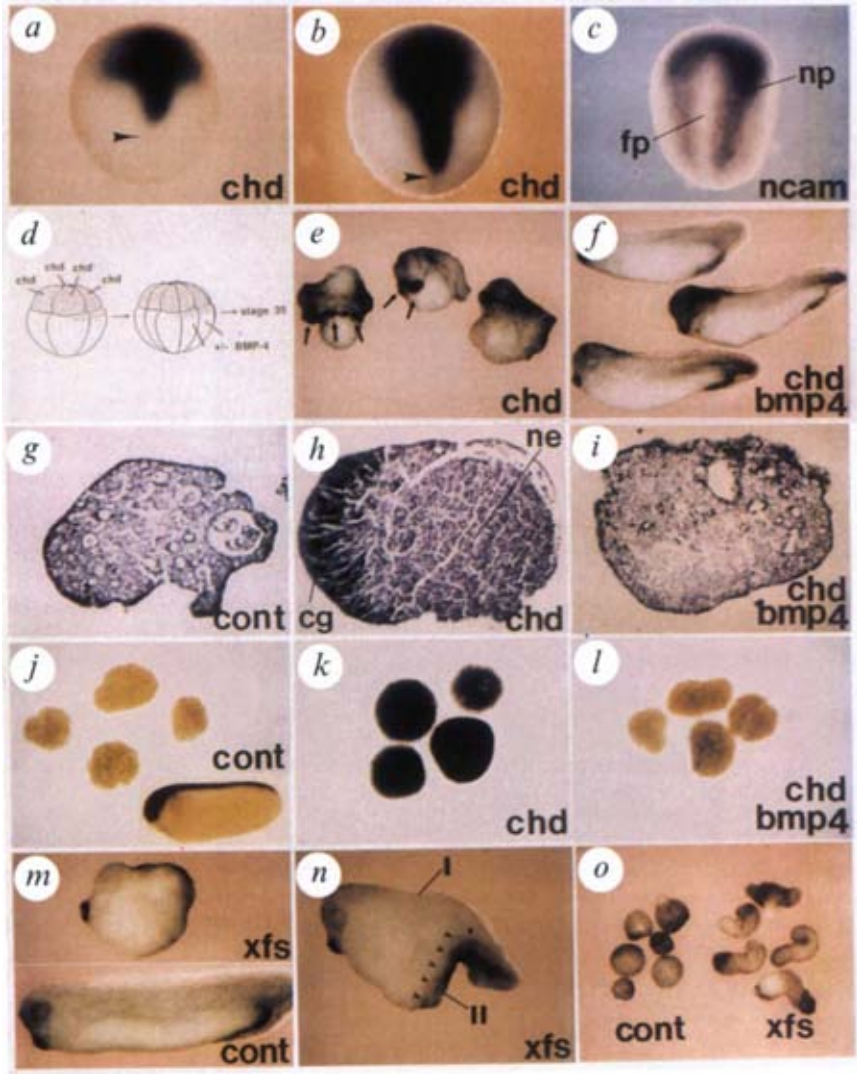


FIG. 2 *chd* mRNA is expressed at the right time and place to act as a neural inducer, and its effects can be antagonized by injected *Bmp-4* DNA. *a*, *In situ* hybridization of *chd* mRNA at late gastrula (stage 12); expression in the mesoderm foreshadows the shape of the future neural plate, which will appear later in the ectoderm. *b*, At early neurula (stage 13) *chd* expression in the mesodermal layer extends more posteriorly, reaching the closing blastopore (arrowhead) and starts converging towards the midline. *c*, N-CAM hybridization *in situ* demarcates the neural plate (np) in the ectoderm of a stage-14 *Xenopus* neurula; the prospective floorplate does not express N-CAM (fp). The shape of the neural plate resembles that of *chd* expression in the mesoderm. *d*, Experimental design used to test the antagonism between Chd and Bmp-4 in patterning the marginal zone. *chd* mRNA was injected into the four animal blastomeres at the 8-cell stage, and at the 16-cell stage two adjoining vegetal blastomeres were injected with a control plasmid or with Bmp-4 DNA. *e*, Embryos injected with *chd* mRNA and control pCSKA–β-gal plasmid; note that the embryos are dorsalized[6] (dorsoanterior index (DAI) 7.3, $n=15$) and have expanded cement glands (arrows) and short trunk–tail structures. *f*, Embryos injected radially with *chd* as in *e*, but also pCSKA–Bmp-4 DNA; the dorsalized phenotype has been rescued[11] and that the resulting embryos have normal trunk–tail structures (DAI 5.7, $n=18$; data from two independent experiments). *g*, Histological appearance of animal caps injected with control β-gal mRNA; this morphology corresponds to atypical epidermis. *h*, After injection with *chd* mRNA together with control pCSKA–β-gal plasmid, a large mass of neural tissue (small basophilic cells with prominent nuclei, ne) as well as cement gland tissue (large ectodermal vacuolated cells containing melanin pigment, cg). *i*, Co-injection of pCSKA–Bmp-4 DNA together with *chd* mRNA eliminates neural and cement gland differentiation. *j*, Control animal caps injected with β-gal mRNA and hybridized *in situ* with the neuronal marker β-tubulin II. No signal is seen in the animal caps; inset shows expression of the marker[16] in tailbud embryos. *k*, Expression of β-tubulin indicates differentiation of neurons after injection of *chd* mRNA together with control pCSKA–β-gal DNA. *l*, Differentiation of neurons is suppressed by injection of pCSKA–Bmp-4 DNA together with *chd* mRNA. *m*, Embryo with enhanced dorso–anterior structures after injection of *XFS* mRNA into the upper region of the four vegetal blastomeres at the 8-cell stage; a normal embryo (cont) is shown as well. If injected at the 2- or 4-cell stage, *XFS* mRNA interferes with gastrulation, which is probably why its dorsalizing activity has not been recognized before. *n*, Embryo with a partial secondary (II) axis (indicated by arrowheads) after injection of a single ventral-vegetal blastomere with *XFS* mRNA at the 16-cell stage; 23% of such embryos were dorsalized and 54% had secondary axes ($n=26$). Antibody markers showed no secondary notochords and histological sections showed secondaryneural tubes and somites. *o*, VMZs injected with β-gal mRNA (control, cont) or with *XFS* mRNA; the explants with follistatin elongate. METHODS. *chd* and β-gal mRNA were injected at 0.3 (*d–f*) or at 0.4 (*f–l*) ng per blastomere. pCSKA–β-gal and pCSKA–Bmp-4 DNAs were injected at 0.15 ng (*d–f*) or 0.1 ng (*g–l*) per blastomere. Whole-mount *in situ* hybridizations were according to Harland's method with minor modifications[6]. The N-CAM probe was made by PCR amplification of a complementary DNA fragment (nucleotides 260–1,790 in Genbank M25696) and subcloning into the *Xho*I and *Eco*RI sites of pBluescript KS(−); antisense N-CAM probe was made by linearizing with *Xho*I and transcribing with T7 polymerase. The antisense *chd* probe has been described[6] and the β-tubulin II probe was obtained by linearizing plasmid 24–10[26] with *Not*I and transcribing with T3 RNA polymerase. *XFS* mRNA was injected at about 0.1 ng per blastomere; two independent experiments gave similar results.

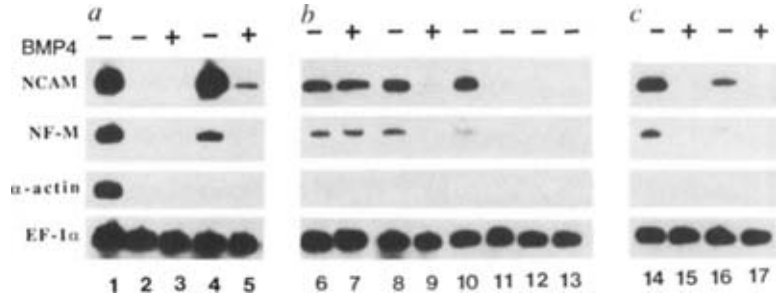


FIG. 3 Bmp-4 expression counteracts neural induction by *chd* DNA and antisense Bmp-4 RNA but not neurogenesis by dominant-negative Bmp-2/4 receptor. Lanes: 1, whole-embryo RNA at tailbud stages; 2, animal caps injected with control pCMV–β-gal DNA together with pCSKA–β-gal DNA; 3, control pCMV–β-gal DNA and pCSKA–Bmp-4 DNA; 4, pCMV–*chd* DNA co-injected with control pCSKA–β-gal DNA; 5, pCMV–*chd* co-injected with pCSKA–Bmp-4 DNA; 6, dominant-negative Bmp-2/4 receptor (DNBMPR) mRNA and pCSKA–β-gal DNA; 7, DNBMPR mRNA and pCSKA–Bmp-4 DNA (note that Bmp-4 has no effect); 8, full-length antisense Bmp-4 RNA and control plasmid; 9, antisense Bmp-4 RNA and pCSKA–Bmp-4 DNA; 10, truncated ΔBmp-4 antisense RNA from which the mature growth factor portion was deleted is still able to induce neural differentiation; 11, ΔBmp-2 antisense RNA (lacking the growth factor region) does not induce neural differentiation; 12, control antisense *gsc* mRNA, which is not expressed in the animal cap; 13, antisense β-gal RNA; 14, *noggin* mRNA and control pCSKA–β-gal; 15, *noggin* mRNA and pCSKA–Bmp4 DNA, showing that Bmp-4 counteracts neurogenesis by noggin; 16, *follistatin* mRNA (*XFS*) and control plasmid DNA; 17, *XFS* mRNA and pCSKA–Bmp-4 DNA.

METHODS. Embryo manipulations and RNA analyses were as in Fig. 1. For CMV–*chd*, pCMV–β-gal, pCSKA–β-gal and pCSKA–Bmp-4 DNAs, 0.1 ng per blastomere were injected; for DNBMPR, 0.5 ng; for antisense Bmp-4, antisense ΔBmp-4, antisense ΔBmp-2, antisense *gsc* and antisense β-gal RNAs, 0.6 ng; for *noggin* mRNA, 0.1 ng; and for *XFS* mRNA, 0.15 ng. All antisense RNAs were as described (H.S., A. Fainsod, C. Niehrs, Y.S. and E.M.D.R., submitted). To obtain pSP35–DNBMPR, cDNA encoding residues 1–174 of Bmp-2/4 receptor[17] (followed by a termination codon) was amplified by PCR and subcloned into the *Eco*RI–*Sal*I sites of the mRNA expression vector[6]. For pSP35–*noggin* and pSP35–*follistatin* the entire coding sequence was amplified by PCR, adding *Nco*I and *Xba*I restriction sites. To make synthetic mRNA, vectors were linearized with *Eco*RI, except for pSP35–DNBMPR, which was linearized with *Pst*I, and transcribed with SP6 RNA polymerase.

4 might inhibit neural differentiation caused by *noggin* and *follistatin* mRNAs. Interestingly, CSKA–Bmp-4 blocked neural induction by noggin and follistatin (Fig. 3*c*, lanes 14–17).

This raises the question of whether any differences exist in the function of *chd*, *noggin* and *follistatin*. With *chd* and *Bmp-4*, there is good genetic evidence linking their *Drosophila* homologues, *sog* and *dpp*, to the formation of CNS[1–4]. No *Drosophila* homologues have been reported for *noggin* and *follistatin*. A particularly interesting observation is provided by *lim-1*, an organizer-specific homeobox gene that in mouse is required for the formation of all brain structures anterior to rhombomere 3 (ref. 21). *Xenopus* cells injected with activated *Xlim-1* mRNA are able to induce neural tissue in neighbouring cells in the absence of noggin or follistatin expression[22], but do express *chd* mRNA (M. Taira and I. Dawid, personal communication). Dominant-negative receptors can interact with multiple signalling pathways[19], and it may be worthwhile to explore in future whether the neuralizing effects reported for activin receptor mutants[23,24] are mediated by a block of Bmp-4 signalling.

In this study we have shown that Chd, like its homologue sog, leads to the formation of neural tissue and that Bmp-4 can antagonize this effect. Bmp-4 transcripts present endogenously in the animal cap are required to prevent neural differentiation in *Xenopus* explants. An antagonism between Chd and Bmp-4 is consistent with the roles of their homologues in *Drosophila*. On the other hand, the inhibition by Bmp-4 of the neural induction caused by noggin and follistatin is not easily explained. One hypothesis that may help reconcile the data is that, in both *Drosophila* and vertebrates, neural induction may be specified by the dorsal–ventral positional values of the ectoderm. In *Xenopus* ventral values would be induced by high concentrations of Bmp-4 (and presumably other factors) and dorsal values by antagonistic signals emanating from the organizer. It is not known how noggin, follistatin and Chd function from a biochemical viewpoint; for example, they may exert their activity through their own receptor-mediated pathway, affect Bmp-4 processing or bind directly to Bmp-4 or other ventralizing factors. However, the three molecules are able to dorsalize *Xenopus* embryos. Thus, the same dorsal–ventral patterning signals are used by the mesoderm and by the ectoderm. A similar situation has been reported recently in *Drosophila*, although in this case the ectoderm is the sole source of the signal[25]. Finally, it is conceivable that the dorsal–ventral system is involved only in the specification of the most anterior (archencephalic) neural tissue, and that the key to the differentiation of the posterior CNS will be found in an independent anterior–posterior patterning system. □

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