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Establishment and maintenance of the border of the neural plate in the chick: involvement of FGF and BMP activity

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Abstract

We have investigated the cell interactions and signalling molecules involved in setting up and maintaining the border between the neural plate and the adjacent non-neural ectoderm in the chick embryo at primitive streak stages. *msx-1*, a target of BMP signalling, is expressed in this border at a very early stage. It is induced by FGF and by signals from the organizer, Hensen's node. The node also induces a ring of *BMP-4*, some distance away. By the early neurula stage, the edge of the neural plate is the only major site of *BMP-4* and *msx-1* expression, and is also the only site that responds to BMP inhibition or overexpression. At this time, the neural plate appears to have a low level of BMP antagonist activity. Using in vivo grafts and in vitro assays, we show that the position of the border is further maintained by interactions between non-neural and neural ectoderm. We conclude that the border develops by integration of signals from the organizer, the developing neural plate, the paraxial mesoderm and the non-neural epiblast, involving FGFs, BMPs and their inhibitors. We suggest that BMPs act in an autocrine way to maintain the border state. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: BMP-4; Noggin; Chordin; FGF-8; Neural crest; Neural plate; Hensen's node; Organizer; msx-1

1. Introduction

The nature of the neural-inducing signals emanating from the organizer has been the subject of intense interest for many decades. However, inducing molecules have started to be identified only recently. The prevailing view of neural induction comes from experiments in *Xenopus*, where it is believed that anterior neural differentiation is a 'default' state of the ectoderm (for review: Wilson and Hemmati-Brivanlou, 1997). Away from the prospective neural plate, cells are normally inhibited from executing this default fate by intercellular signals mediated by Bone Morphogenetic Proteins (BMPs), and specifically BMP-2, -4 and -7. The organizer emits several secreted factors that can bind to, and antagonize, BMPs in adjacent cells, thus releasing them from their inhibition and allowing them to follow their default, anterior neural fate. Six BMP antagonists have been described to date: chordin (Sasai et al., 1995), noggin (Lamb et al., 1993; Zimmermann et al., 1996), follistatin (Hemmati-Brivanlou et al., 1994), Xnr3 (Hansen et al., 1997), cerberus (Bouwmeester et al., 1996) and gremlin (Hsu et al., 1998). All of these are expressed in or close to the frog organizer during gastrulation, and all of them generate ectopic anterior neural tissue or an expansion of the normal neural plate when misexpressed. It is therefore generally believed that newly-induced neural tissue is anterior (rostral) in character, and that some of these cells are subsequently caudalized ('transformed') by other signals from the organizer (Nieuwkoop et al., 1952; Nieuwkoop and Nigtevecht, 1954). Among these later signals are retinoids (Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991), FGFs (Isaacs et al., 1992; Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; Launay et al., 1996) and Wnt3A (McGrew et al., 1995, 1997).

However, recent results from our laboratory suggest that, at least in the chick, additional signals from the organizer

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are required for neural induction prior to BMP inhibition (Streit et al., 1998; Streit and Stern, 1998). First, misexpression of BMP-4 in the prospective neural plate at any stage of development does not prevent neural differentiation (Fig. 1). Second, ectopic expression of either chordin (Streit et al., 1998) or noggin (Streit and Stern, 1998) cannot induce an ectopic neural plate in cells competent to respond in this way to signals from Hensen's node. Third, BMP-2, -4 and -7 are not expressed at detectable levels in the epiblast at stages just preceding neural induction, as they are in amphibians. Fourth, although chordin is not sufficient to elicit the formation of an ectopic neural plate, it can stabilize the expression of the early neural marker *Sox-3* in epiblast cells that have been exposed to signals from the node for a short time (not enough to lead to neural induction by itself). Together, these findings suggest that other signals from the organizer are required upstream of the inhibition of BMPs to cause epiblast cells to follow a pathway towards neural differentiation.

In amphibians, it has been reported that FGF signalling is required for the anterior neural-inducing activity of chordin and noggin (Launay et al., 1996; Sasai et al., 1996; Xu et al, 1997). We therefore started by investigating whether FGF can replace the organizer, providing signals that allow chordin to neuralize epiblast cells. We find that FGF cannot replace the node as a source of upstream signals that allow chordin to act. However, FGF rapidly induces *msx-1*, an early target of BMP signalling (Marazzi et al., 1997; Suzuki et al., 1997). Grafts of Hensen's node also induce the expression of *msx-1*, as well as *BMP-4* itself, a finding that appears paradoxical in the context of the amphibian 'default' model, which proposes that BMP-4 and msx-1 are anti-neuralizing molecules (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou and Melton, 1994; Hawley et al., 1995; Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995; Xu et al., 1995; Suzuki et al., 1997).

The pattern of *BMP-4/msx-1* expression resembles the future boundary between neural and non-neural ectoderm. We therefore investigated whether BMP signalling and its inhibition by chordin and noggin might play a role in the establishment or maintenance of the lateral borders of the neural plate. We find that the border is the only region sensitive to misexpression of BMP-4, chordin and noggin at this stage of development. Finally, we investigate, in vivo and in vitro, whether interactions between newly-induced neural plate and non-induced epiblast are sufficient to generate this border. The results point to complex interactions between these two tissues, whereby the newly-induced neural plate both receives and emits signals that define the lateral borders of the neural plate.

2. Results

In this study, we have used a variety of molecular markers to assess the effects of misexpression of several secreted factors. For guidance, we present the normal patterns of expression of these markers and factors at three different stages of chick development (Fig. 2).

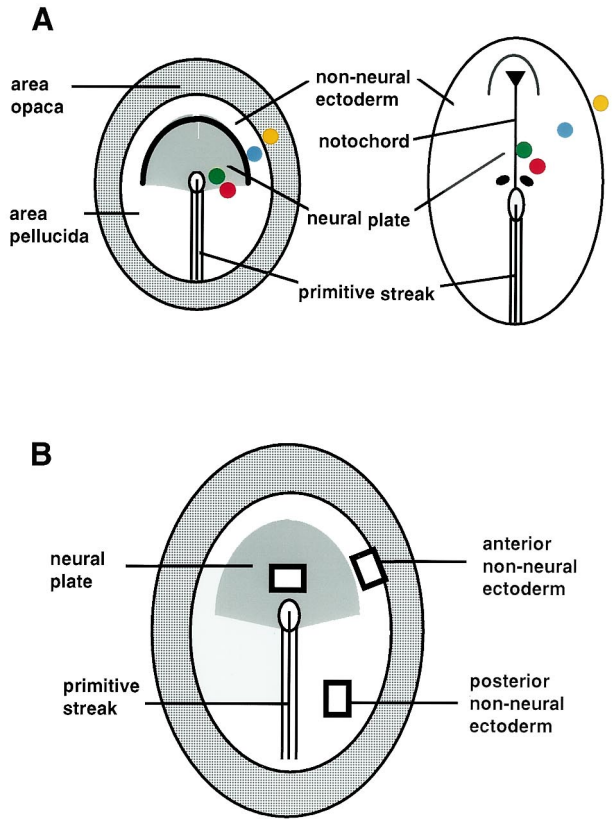

Fig. 1. Diagram of experiments performed. (A) The left diagram shows the positions where various grafts were placed in a stage 4 embryo. Orange, area opaca epiblast; blue, non-neural epiblast; green, prospective neural plate; red, future neural/non-neural border. The diagram on the right shows the final positions of these grafts after incubation to stage 7. (B) Diagram showing the regions (rectangles) from which neural and non-neural epiblast explants were dissected at stage 4. Note that at stage 4, none of these regions are yet committed to their fates (García-Martínez et al., 1997).

2.1. Does FGF signalling sensitize cells to BMP inhibition?

We have recently shown that inhibition of BMP-4 by chordin (Streit et al., 1998) or noggin (Streit and Stern, 1998) is not sufficient to induce neural tissue from non-neural ectoderm in the chick, and that additional upstream signals from the organizer are required. In *Xenopus* it has been suggested that induction of anterior neural tissue by chordin and noggin is only effective if the FGF signalling pathway is intact (Launay et al., 1996; Sasai et al., 1996). Together, these findings raise the possibility that FGFs provide the upstream signals needed for chordin or noggin to act as neural inducers.

To establish a baseline for these experiments, we started by investigating whether the early neural markers *Sox3* and *Sox2* are induced by FGF. Heparin beads soaked in FGF4 or

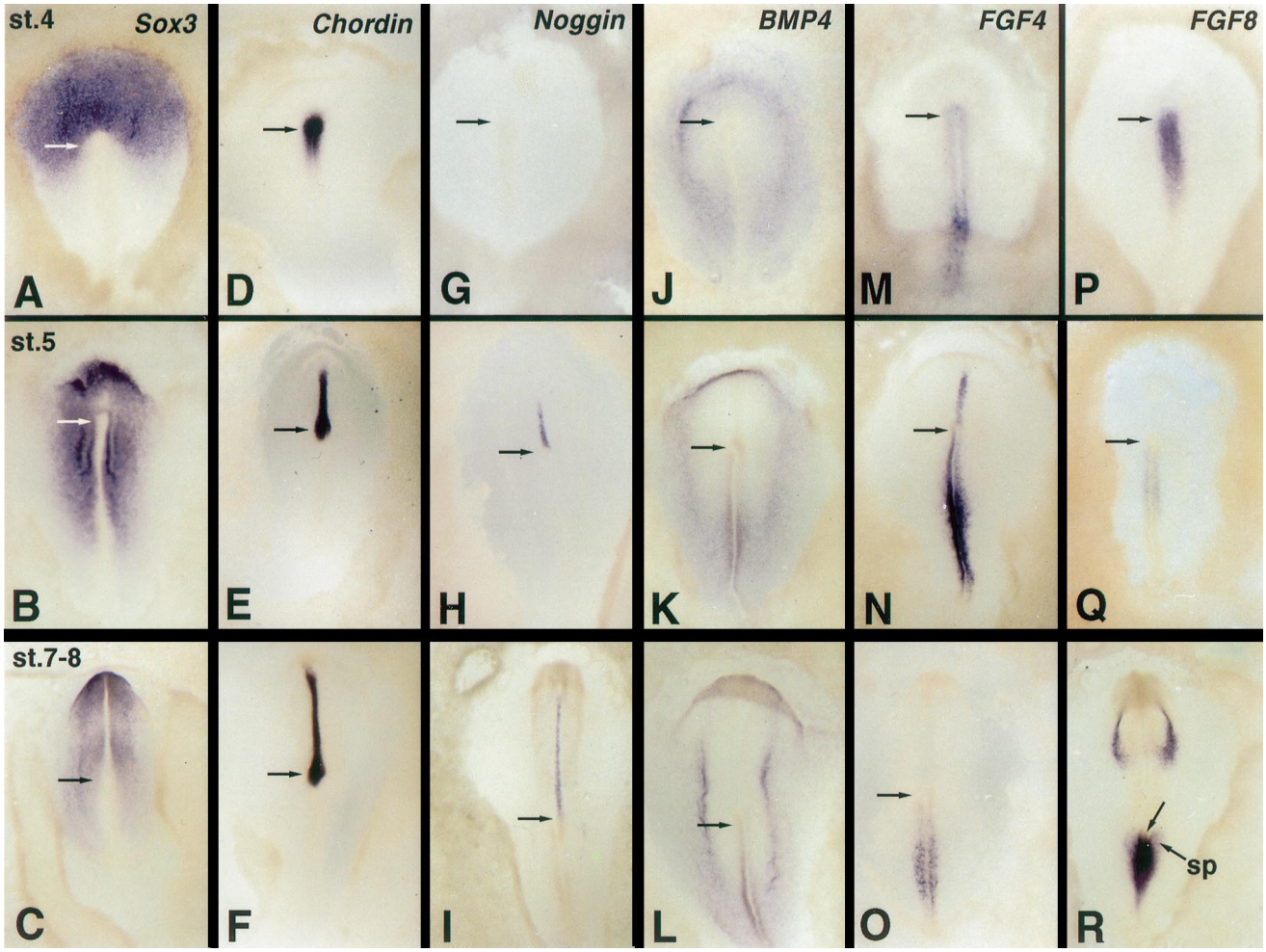

Fig. 2. Expression patterns of six of the markers used in this study at three different stages in development. (A–C) *Sox3* expression. (D–F) *Chordin* expression. (G–I) *Noggin* expression. (J–L) *BMP4* expression. (M–O) *FGF4* expression. (P–R) *FGF8* expression. The upper row of photographs shows embryos at stage 4, the middle row embryos at stage 5, and the lowest row embryos at stages 7–8. The arrow in each panel indicates the position of Hensen's node. In panel R, note the expression of *FGF8* in the forming segmental plate (sp) at stage 8. Note the asymmetric expression of chordin and noggin: both are expressed on the right side of the node (F,H,I). All embryos are photographed from the dorsal side except F and R.

FGF8 were grafted into the area opaca of stage 3^{+}–4 chick embryos (Fig. 1). The two factors differ in their effects. After 5–7 h, FGF4 induces *Sox3* (4/4) and *Sox2* (6/6) as well as the mesodermal marker *brachyury* (4/4) (see also Storey et al., 1998). By contrast, FGF8 induces *Sox3* (5/5), but not *brachyury* (0/8) or *Sox2* (0/6). In agreement with the induction of *brachyury* by FGF4, morphologically recognisable mesodermal tissue is seen surrounding FGF4, but not FGF8 beads (c.f. Fig. 3B,D).

At stage 3^{+}–4, regions that can respond to neural-inducing signals from the organizer have very low, if any, expression of BMP-2, 4 or 7 (Schultheiss et al., 1997; Streit et al., 1998). If FGF sensitizes cells to BMP antagonists, it could do this by first inducing the expression of BMP itself. We first examined the induction of a target of BMP signalling, *msx-1* (Suzuki et al., 1997) by FGF: both FGF8 (6/6; Fig. 3A,B) and FGF4 (5/5; Fig. 3C,D) strongly induce *msx-1* within 5–7 h. However, FGF8 does not significantly upregulate *BMP-4* expression, even after prolonged culture (6/16 with very weak ectopic expression).

These results are not inconsistent with the idea that FGFs might provide the signals required for chordin or noggin to induce neural tissue. As a more direct test of the hypothesis, we investigated whether *Sox2*, which cannot be induced by either FGF8 or chordin alone, can be induced by a combination of these factors. We found that it could not (0/8). We also tested whether FGF4 in combination with chordin can give rise to anterior neural tissue, and found that neither the forebrain marker *Otx2* (0/12) nor the hindbrain marker *Krox-20* (0/6) are induced.

These results suggest that, in agreement with earlier findings (Álvarez et al., 1998; Storey et al., 1998), FGFs can induce the expression of some early neural markers. However, FGF signals are not sufficient to generate a state that is responsive to BMP inhibition by chordin, either for neural induction or for rostrocaudal patterning.

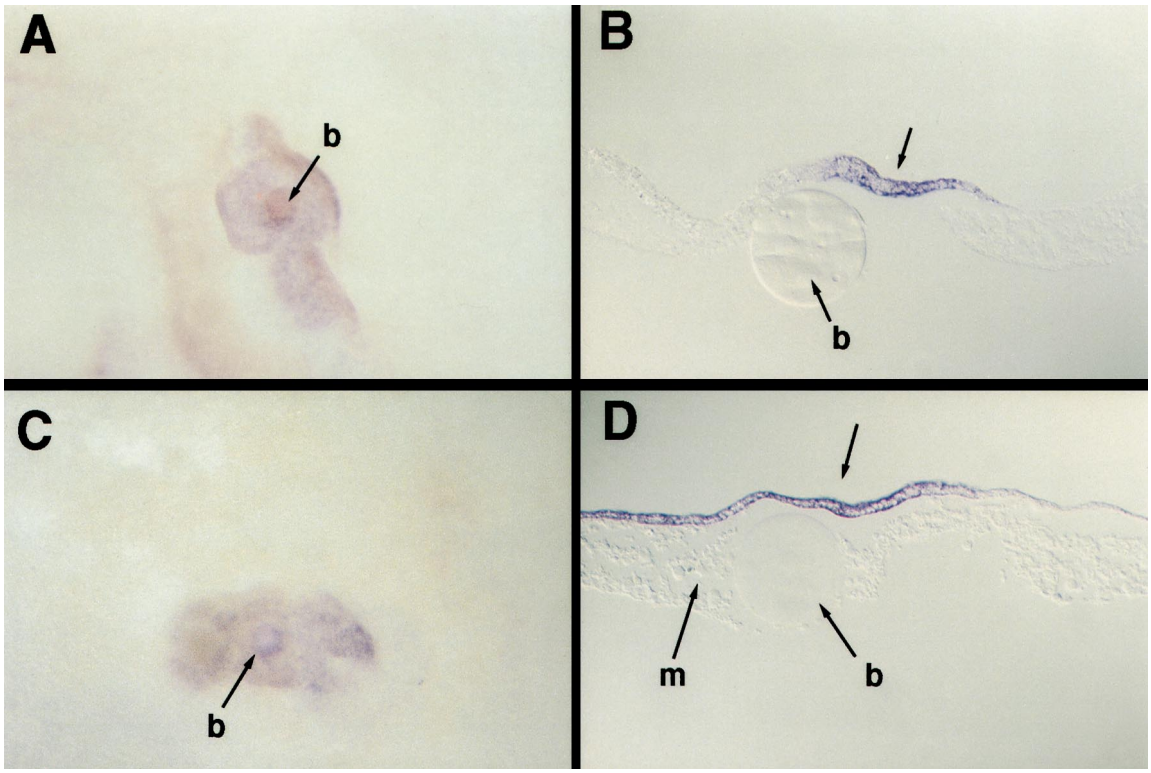

Fig. 3. FGF4 and FGF8 induce *msx-1* expression. FGF8- (A,B) and FGF4- (C,D) coated heparin beads (b) were transplanted into the area opaca of a chick primitive-streak stage embryo. FGF8 induces the expression of *msx-1* (arrow) without intervening mesoderm (B). FGF4 also induces *msx-1* (arrow), but mesoderm (m) is also induced (D). B and D are sections through the same grafts as shown in A and C, respectively.

2.2. Sequential activation of Sox-2 and Sox-3 and of BMP-4 and its downstream target msx-1 during neural induction

The ability of Hensen's node to induce neural tissue from non-neural ectoderm peaks at full primitive-streak stages (Dias and Schoenwolf, 1990; Storey et al., 1992). At this time, the general neural markers *Sox-2* and *Sox-3* (Rex et al., 1997; Streit et al., 1997) and the competence marker L5 (Streit et al., 1995; Streit et al., 1997) are already strongly expressed in a large region of the area pellucida epiblast and subsequently become restricted to the neural plate. At the same stage, *BMP-4* and *-7* seem to demarcate the border between the forming neural plate and the non-neural ectoderm, where their expression becomes progressively concentrated (Liem et al., 1995; Schultheiss et al., 1997; Streit et al., 1998).

To investigate whether the expression of these genes might characterize different steps following neural induction and whether they are activated sequentially during this process, we compared the onset of expression of *Sox-2*, *Sox-3* and *BMP-4* after exposing non-neural epiblast to organizer signals. A quail Hensen's node (stage 3^{+}/4) was transplanted into the area opaca of a chick host embryo (stage 3^{+}/4). The embryos were fixed either every 1 h from 3–13 h or every 5 h from 15–25 h. *Sox-3* begins to be expressed in chick epiblast overlying the grafted node within 3 h (6/6), and is strongly induced after 4–5 h (8/8) (Fig. 4A), whereas it takes 9–10 h to induce *Sox-2* expression (6/6; $n = 24$ for 3–12 h) (Fig. 4B). Surprisingly, we find that the organizer also induces the neural antagonist *BMP-4*: transcripts first appear after 13–14 h, as a hazy ring of cells some distance away from the node (6/7; $n = 32$ for 3–12 h) (Fig. 4C,D). This ring then intensifies and is refined to a discrete line of *BMP-4* expressing cells (14–15 h; 4/4) (Fig. 4E). After 25 h, *BMP-4* transcripts are detected at the edges of the induced neural plate (5/5) (Fig. 4F). The ring-shaped pattern of induced *BMP-4* expression is remarkably similar to its expression at the border between neural and non-neural epiblast during normal development (Fig. 5D).

To verify the paradoxical finding that BMP-4 (which has been shown to be an epidermal inducer and anti-neuralizing factor in *Xenopus*) is induced by the organizer during neural induction, we investigated the expression of *msx-1*, a target of BMP signalling, in response to an organizer graft. First, we confirm that *BMP-4* and *msx-1* are coexpressed in the early chick embryo by in situ hybridization. At early primitive-streak stages (stage $3–3^{+}$) *msx-1* is strongly expressed in the posterior part of the primitive streak and the epiblast surrounding it (Fig. 5A) and then expands (stage 4; Fig. 5B) progressing anteriorly as two lines that gradually come to surround the future neural plate (stage 5; Fig. 5C,E–H). By stage 8, *msx-1* is expressed in a sharp line at the lateral edges of the neural plate along its entire length. In addition, it is

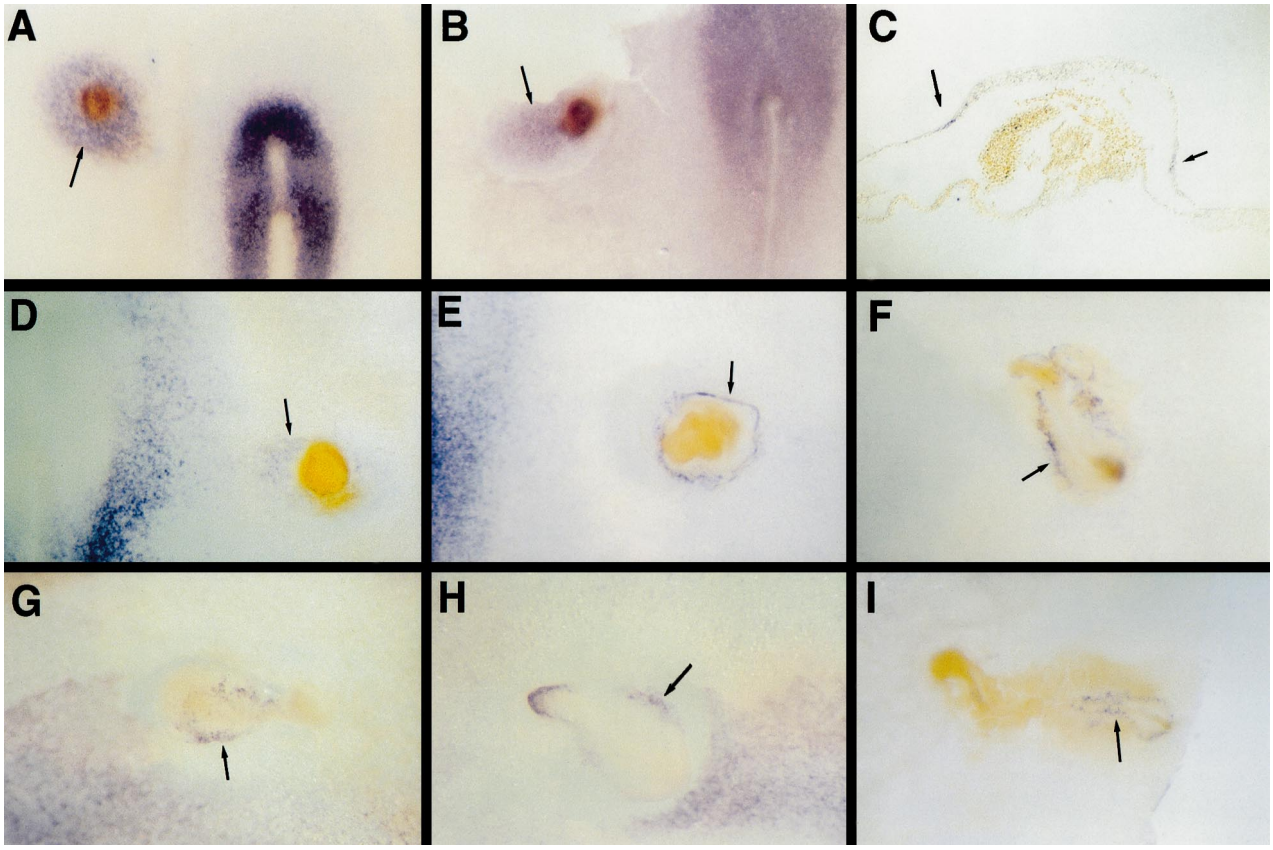

Fig. 4. Induction of *Sox-2*, *Sox-3*, *BMP-4* and *msx-1* by Hensen's node in the area opaca. In each case, the quail node is labelled with QCPN antibody (brown). (A) *Sox-3* is strongly induced after 4 h (arrow). (B) *Sox-2* is induced after 9 h (arrow). (C–F) Time course of induction of *BMP-4*. D, 13 h. E, 15 h. F, 25 h. The arrow shows the developing ring of expression. C is a section through the graft in E. (G–I) time course of *msx-1* induction. G, 13 h. H, 16 h. I, 25 h.

also expressed throughout the width of the neural plate that lies most caudally, immediately adjacent to Hensen's node (Fig. 5D; see also Liem et al., 1995). In agreement with the additional site of expression of *BMP-2* and *BMP-4* in lateral mesendoderm (Watanabe and Le Douarin, 1996; Schultheiss et al., 1997; Tonegawa et al., 1997), *msx-1* transcripts are also detected in lateral mesoderm beginning at stage 4 (Fig. 5G,H). Thus, the *msx-1* and *BMP* expression patterns overlap during early stages of development.

The time-course of *msx-1* expression after a graft of Hensen's node was investigated as described above. Like *BMP-4*, *msx-1* begins to be expressed weakly in host tissue after 13 h in a few cells at a distance from the grafted node (5/10) (Fig. 4G). After 14 h, *msx-1* expression has resolved to a ring, which seems to demarcate the border of the induced neural plate (9/10) (Fig. 4H). By 25 h, *msx-1* expression labels the dorsal aspect of the induced neural tube (6/6) (Fig. 4I).

In summary, Hensen's node induces the expression of the neural antagonist *BMP-4* and its downstream target *msx-1*. This induction occurs at a distance from the organizer and is preceded by the sequential induction of the early general neural markers *Sox-3* and *Sox-2*. These results therefore suggest that the first step to generate neural tissue involves the induction of a young neural plate, and that a specialized border region (characterized by expression of *BMP-4* and *msx-1*) is then established between neural and non-neural ectoderm.

2.3. The border of the neural plate co-expresses neural and non-neural markers

To establish whether the expression domain of *msx-1* overlaps with the region expressing early neural markers, we performed double labelling using *msx-1* and either *Sox-3* riboprobes or L5-antibody. At primitive-streak stages, expression of *msx-1* overlaps with both *Sox-3* (Fig. 5K) and L5 (Fig. 5I) in the posterior epiblast, a region containing presumptive mesoderm and surface ectoderm (García-Martínez et al., 1993; Rosenquist, 1966). Then L5 and *Sox-3* become restricted to the neural plate, while *msx-1* remains in the posterior non-neural epiblast (Fig. 5J,L). However, the border of the neural plate remains unique, as the only region in the embryo where all three markers are still co-expressed.

2.4. Only the border of the neural plate is sensitive to BMP-4 and its antagonists

The overlapping expression of early neural (*Sox3* and L5)

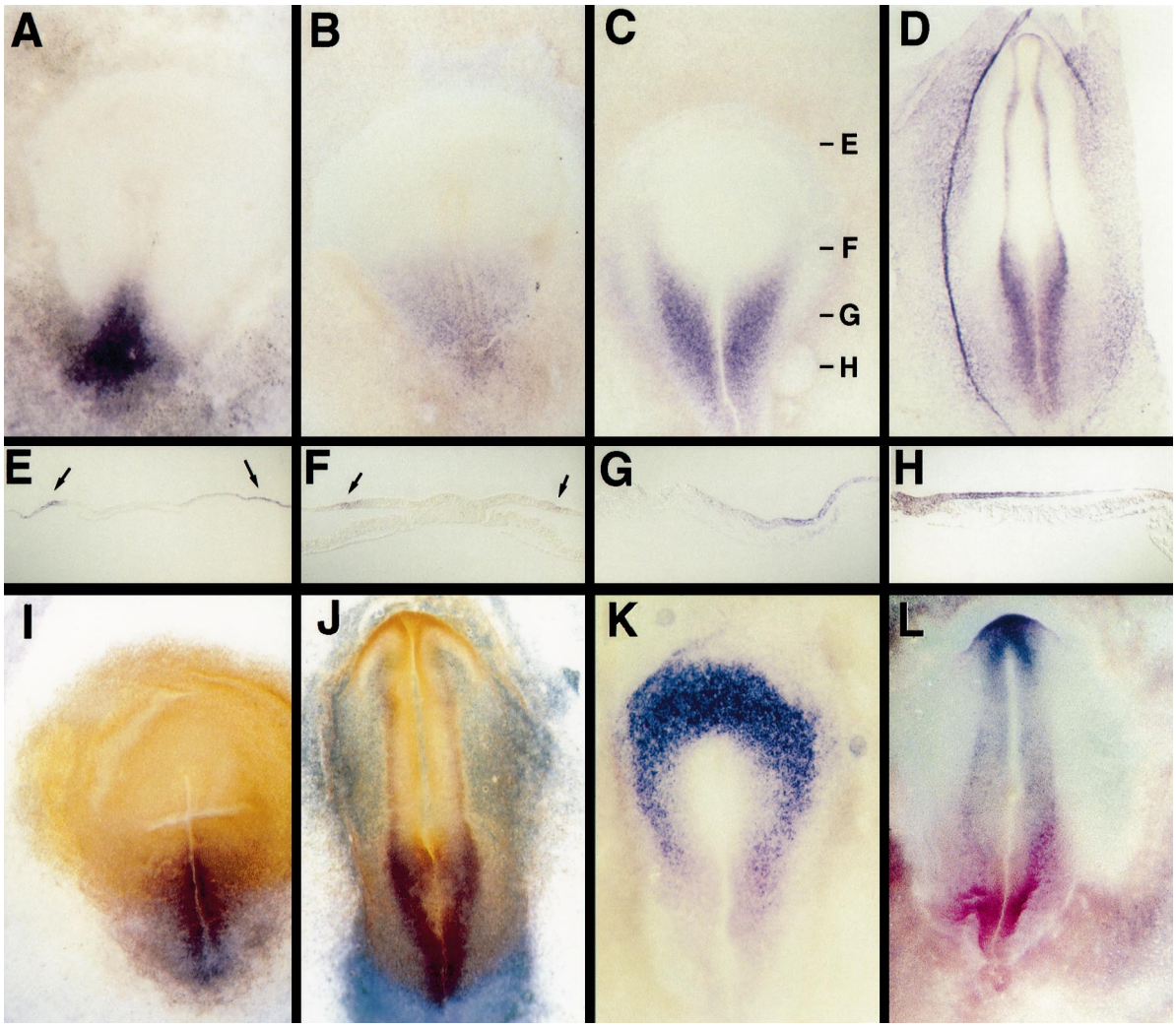

Fig. 5. Comparison of the expression patterns of *msx-1*, *Sox-3* and L5. (A–H) Normal expression of *msx-1*. A, stage 3^{+}. B, stage 4. C, stage 5. D, stage 8. Expression starts in the posterior primitive streak, from where it expands gradually to surround the developing neural plate. E–H are transverse sections through the embryo in C at the levels indicated. (I,J) Combined in situ hybridisation with *msx-1* (blue) and L5 antibody (brown). At stage 4 (I) the two markers overlap around the middle of the primitive streak. At stage 8 (J) the markers overlap posteriorly, as well as along the length of the neural/non-neural border. (K,L) Double in situ hybridisation for *msx-1* (magenta) and *Sox-3* (blue). At stage 4 (K), the two markers overlap close to the middle of the streak. At stage 7 (L), they overlap posteriorly and at the edge of the neural plate.

and 'anti-neural' (*msx-1*, *BMP-4*, *BMP-7*) markers at the edge of the neural plate could indicate that these border cells are particularly sensitive to local signals that determine their final fate. Candidates for such local signals are the BMP antagonists chordin, noggin and follistatin, which are expressed in the node or its derivatives (Connolly et al., 1995, 1997; Levin, 1998; Streit et al., 1998). We used BMP-4-, chordin- or noggin-secreting cells to misexpress these factors at three different positions in primitive-streak stage chick embryos (see Fig. 1A): within the prospective neural plate, at the future border of the neural plate or in the anterior prospective non-neural ectoderm. After overnight culture, embryos were assessed for expression of *Sox-3* and *msx-1*. None of the factors altered the expression of either marker when misexpressed inside the neural plate (BMP-4: $n = 5$; chordin $n = 7$; noggin $n = 4$) or in the non-neural ectoderm ($n = 8$ for each factor) (Fig. 6D,F; see also Streit et al., 1998). In contrast, when expressed at the future edge of the neural plate, BMP-4 causes a narrowing of the neural plate and a medial shift of the border (7/7) (Fig. 6A,C), while both noggin (4/5; Fig. 6E) and chordin (9/10; Fig. 6B,C) misexpression in the same region generate a widening of the neural plate and a lateral shift of the border. The width of the domain expressing *msx-1* remains unchanged.

These results indicate that the balance between BMP-4 and its antagonists is important in the establishment and/or maintenance of the border between neural and non-neural ectoderm, once the neural plate has been induced by signals from the organizer. The co-expression of *msx-1* with early neural markers at this border may indicate an intermediate, labile state. Unlike cells inside the future neural plate or in the prospective epidermis, the border cells appear to be

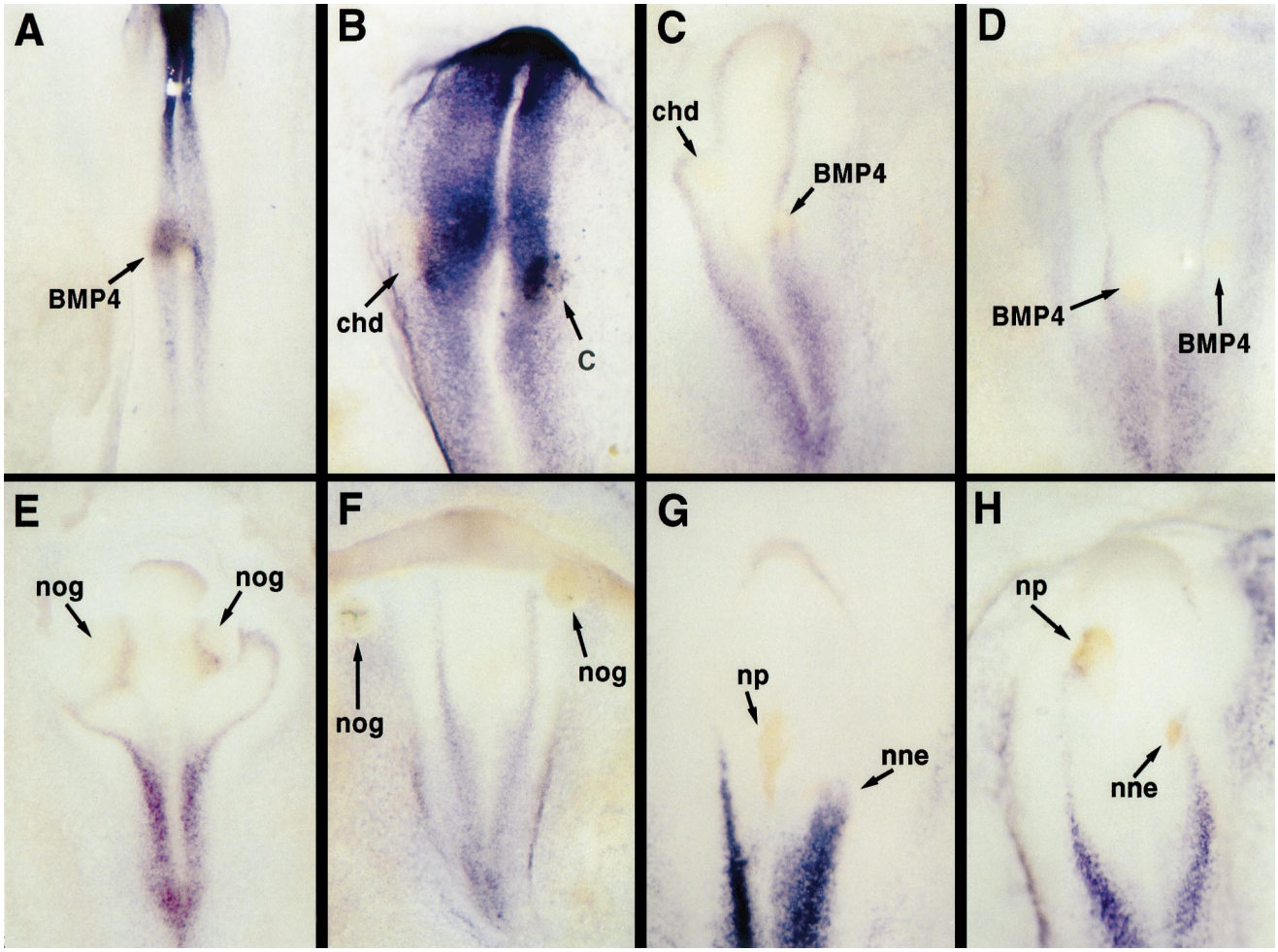

Fig. 6. Signals affecting the position of the neural/non-neural border. (A) Misexpression of BMP-4 results in narrowing of the neural plate (stained for *Sox3*) on the side of the graft. (B) Misexpression of chordin (chd) widens the neural plate. On the contralateral side, a pellet of mock-transfected control (c) cells was grafted. In situ hybridisation for *Sox3*. (C) Misexpression of chordin (chd, left) and BMP-4 (right) shifts the border (marked by *msx-1*) outwards and inwards, respectively. (D) BMP-4 misexpression within the neural plate (left) or in the non-neural ectoderm (right) has no effect on *msx-1* expression. (E) Noggin (nog) shifts the border of the neural plate (*msx-1*) laterally. (F) Noggin misexpressed in the area opaca (left) or non-neural epiblast (right) has no effect on expression of *msx-1*. (G) This embryo received a graft of prospective neural plate (np) and non-neural ectoderm (nne). The neural plate has integrated into the host neural plate. The non-neural ectoderm has interfered with the anterior advancement of the border, and expanded its width (*msx-1*). (H) A neural plate graft (np, left) lengthens the border, while non-neural ectoderm (nne, right) prevents its progression.

unique in that they can be diverted to either a neural or a non-neural fate by BMP and its inhibitors.

2.5. The neural plate mimics the properties of noggin and chordin

What prevents BMP expression at the border from invading the neural plate itself? One possibility is that this is controlled by BMP antagonists expressed in the organizer. However, the border lies more than 250 μm away from the node at most axial levels. An additional possibility is that the young neural plate itself emits BMP antagonists.

We have defined four properties of BMP inhibitors during neural induction (Streit et al., 1998; Streit and Stern, 1998): their inability to induce neural tissue (i) in the area opaca or (ii) in the embryonic non-neural epiblast, (iii) their ability to shift the border of the neural plate laterally and (iv) the maintenance of *Sox3* expression initiated by a graft of the organizer. We have investigated the possibility that the neural plate emits BMP inhibitors by testing all four criteria.

2.5.1. The neural plate does not induce neural tissue in the area opaca

Stage 4 (Fig. 1B) to Stage 6 quail neural plate explants were grafted into the anterior-lateral area opaca of a stage 4 chick host (Fig. 7A–C,E,F). Neither *Sox3* (0/17) nor *Sox2* (0/18) were induced in the host epiblast even after 24 h of culture. We also examined the induction of *msx-1*: no expression was seen in the host up to 17 h after grafting (0/22), but 4/4 embryos showed induction after 24 h. However, despite the expression of *msx-1* at this time, the host epiblast lacked the morphology of an induced neural

plate (Fig. 7F). Therefore the neural plate, like chordin and noggin, is unable to induce neural tissue in the area opaca.

2.5.2. The neural plate does not induce neural tissue in prospective epidermis

Stage 5–7 quail neural plate explants were grafted to the anterior-lateral prospective epidermis (blue position in Fig. 2A). Neither *Sox3* (0/13) nor *Sox2* (0/5) were induced in host epiblast after 18 h.

2.5.3. The neural plate causes a lateral shift of the neural/non-neural border

Young quail neural plate explants from primitive-streak stage embryos were grafted at the future border region of a chick host (red in Fig. 1A). As controls, we grafted quail anterior non-neural epiblast explants (Fig. 1B). The embryos were analysed for *msx-1* expression (as a marker for the border) after overnight culture. Grafts of quail neural plate either integrated into the host neural plate (Fig. 6G; 3/6) or resulted in a lateral shift of *msx-1* expression (Fig. 6H; 3/6). Grafts of non-neural ectoderm, in contrast, seemed to stop the progression of the border (4/4; Fig. 6G,H) and sometimes to enlarge the *msx-1* expressing domain (3/4; Fig. 6G). Therefore, in this assay, the neural plate behaves like chordin and noggin, while the non-neural epiblast behaves like an ectopic source of BMP.

2.5.4. The neural plate weakly maintains expression of Sox3 initiated by a node graft

We tested whether the neural plate (like chordin; Streit et al., 1998) can maintain the expression of *Sox3* initiated by brief (5 h) exposure to organizer signals. First, a chick stage 3^{+}–4 Hensen's node was labelled with the fluorescent tracer CMFDA. It was then grafted to the lateral area opaca of a chick stage 3^{+}–4 host. After 5 h, the node was removed; its complete removal was verified by fluorescence microscopy. At this time, a quail neural plate explant (stage 5–6) was grafted to the same position, and the embryo cultured overnight. In 11/18 cases, *Sox3* expression was seen in the host (Fig. 7D), but this is not as strong or as extensive as when this experiment is done with chordin. Therefore the neural plate resembles chordin in its ability to maintain *Sox3* expression in this assay.

In conclusion, the neural plate appears to contain weak BMP antagonist-like activity, which may play a role in positioning the neural/non-neural border.

2.6. Interactions between neural and non-neural epiblast

While we did not observe induction of neural markers in the area opaca of host embryos after a neural plate graft (see above), we did notice that the grafted quail neural plate itself transiently maintained *Sox2* (7/9 up to 15 h, 0/9 after 24 h) and *Sox3* (10/10 up to 15 h, 0/7 after 24 h) expression and upregulated its expression of *msx-1* (5/10 after 7 h (very weakly) 8/12 (strongly) after 15 h, 4/4 (strongly after 24 h) (Fig. 7B,C,E,F). Therefore, the transplanted neural plate cells can acquire the characteristics of a neural/non-neural border under the influence of signals from non-neural epiblast.

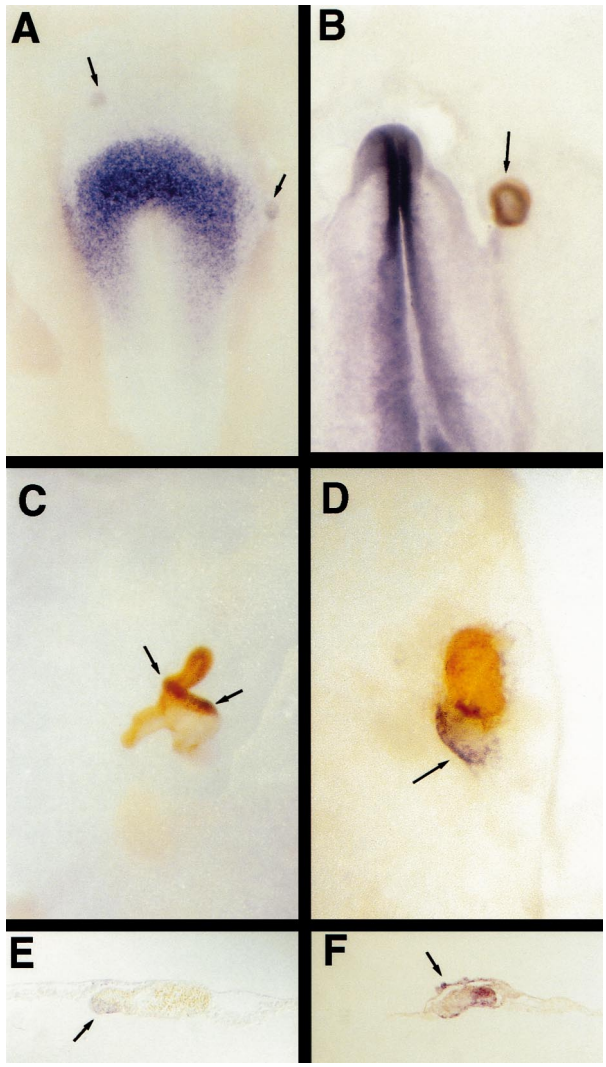

Fig. 7. Neural plate grafts into the extraembryonic region. (A) After 7 h, two neural plate grafts (arrows) continue to express *Sox3* but do not induce expression in the host epiblast. (B) After 15 h, this neural plate graft (arrow) still expresses *Sox2* but has not induced the host. (C) After 15 h, expression of *msx-1* intensifies in the graft but the host still does not express. (D) Embryo that received a graft of Hensen's node in the extraembryonic epiblast; after 5 h, the node was removed and replaced with a quail neural plate explant. The neural plate explant has maintained the expression of *Sox3* (arrow) initiated by the node graft, as does chordin (Streit et al., 1998). (E) Section through the graft in C, showing *msx-1* expression (arrow) confined to the graft. (F) Section through a graft after 24 h, showing expression of *msx-1* in both graft and host tissue, but the latter is barely thickened (arrow). Quail tissue is stained orange/brown.

To study this in more detail, we set up in vitro cultures of prospective neural plate and non-neural epiblast alone, or of a combination of the two. The neural plate explant was labelled with the fluorescent dye CMFDA to distinguish it from the non-neural epiblast explant. Non-neural epiblast

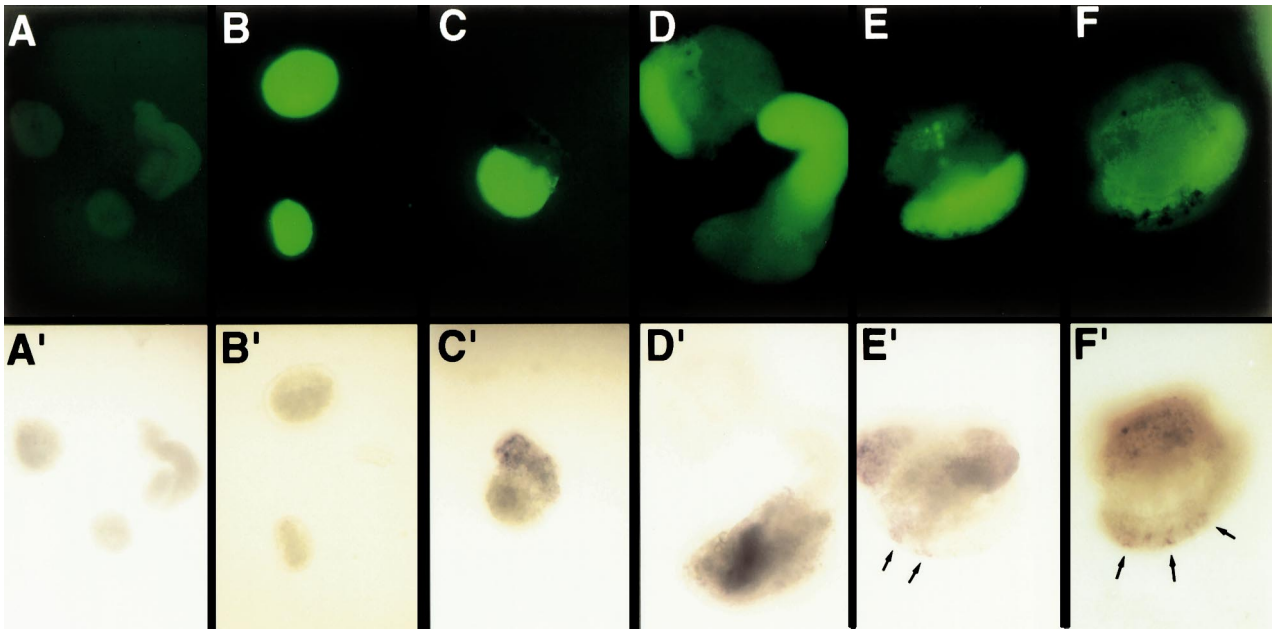

Fig. 8. Expression of *msx-1* in neural/non-neural co-cultures. (A–F) show fluorescence views of the same cultures seen in bright field (after in situ hybridization for *msx-1*) in A′–F′. In each case, the neural plate was labelled with CMFDA. Neither non-neural ectoderm (A/A′) nor neural plate (B/B′) express *msx-1* when cultured alone. C/C′. In combination with posterior non-neural ectoderm, expression is seen in the non-neural ectoderm only. D/D′–F/F′: combinations of neural plate with anterior non-neural ectoderm. *msx-1* expression is seen in non-neural ectoderm (D/D′), in both tissues (E/E′) or in neural plate only (F/F′).

was obtained from two regions, shown in Fig. 1B. After overnight culture, *msx-1* is not detected in any of the tissues cultured in isolation (Fig. 8A,A′,B,B′), except in a few posterior non-neural ectoderm explants (2/15). In contrast, 60–75% of the neural/non-neural recombinants express *msx-1* strongly (Fig. 8C–F,C′–F′) (15/20 in the combination with anterior non-neural ectoderm; 7/12 in co-cultures with posterior non-neural ectoderm). Examination of the source of the cells expressing *msx-1* in these co-cultures revealed a surprising result. When neural plate tissue is combined with anterior non-neural ectoderm, *msx-1* expression is found in either the neural plate (6/15) (Fig. 8F,F′), or in the non-neural ectoderm (4/15) (Fig. 8D,D′) or in both (5/15) (Fig. 8E,E′). In contrast, when combined with posterior non-neural ectoderm, *msx-1*-positive cells are exclusively derived from the non-neural tissue (7/7) (Fig. 8C,C′).

In conclusion, neural plate/non-neural ectoderm interactions appear to play a role in positioning the border. Different regions of non-neural ectoderm differ in their effectiveness in this process.

3. Discussion

3.1. Roles of BMP and its inhibition in neural induction and border formation

3.1.1. BMP-4 may act in an autocrine way at the border of the neural plate

We have shown in the present results and in Streit et al. (1998) and Streit and Stern (1998) that ectopic expression of BMP-4 or its antagonists in the prospective neural plate or non-neural ectoderm does not affect neural or epidermal development in the early chick embryo. We now show that there is only one region where BMP-4 and two of its antagonists (chordin and noggin) do have an effect: the prospective boundary between neural and non-neural ectoderm. When misexpressed at this prospective border, BMP-4 narrows the neural plate and shifts the border medially, while noggin and chordin expand the neural plate and shift the border laterally. Since expression of *BMP-4* and its target gene *msx-1* is only detectable at this border, these results strongly suggest that local regulation of BMP activity is involved in positioning of this boundary.

During neurulation, new neural plate is generated continuously in the region adjacent to Hensen's node (see Sausedo and Schoenwolf, 1994; Catala et al., 1995, 1996; García-Martínez et al., 1997; Knezevic et al., 1998). In this posterior region, *msx-1* expression spans the width of the young neural plate (Fig. 5D), a domain that also expresses the transcription factors *Cash-4*, *Sax-1* (Henrique et al., 1997; Storey et al., 1998) and *Pax-3* (Bang et al., 1997). In older (more rostral) neural plate, *msx-1* expression becomes confined to the border itself. The sharpening of expression of *BMP-4* and *msx-1* at the border of the neural plate may be the result of two events: a medial-to-lateral clearance of BMP activity under the influence of the node and its derivatives (which emit BMP antagonists), and the positive feedback that BMP activity exerts upon its own transcription (see Biehs et al., 1996). Since in

the chick the border is the main site of *BMP-4* expression at this stage, it is not surprising that this is the only region responsive to BMP antagonists. What is more surprising is that this region is the only one responsive to BMP-4 itself: this suggests that BMP-4 acts in an autocrine way to maintain the border, and also that other factors or constraints confine responsiveness to BMP-4 to the border region.

3.1.2. A gradient of BMP activity is not sufficient to induce the border

A recent view is that different concentrations of BMP-4 specify, respectively, epidermis (high concentrations), neural crest/placodes/neural border (intermediate) or neural plate proper (very low concentration) (Morgan and Sargent, 1997; Neave et al., 1997; Wilson et al., 1997; Marchant et al., 1998; reviewed by Baker and Bronner-Fraser, 1997). Two of our results argue directly against this view. First, the expression of *BMP-4* and its target gene *msx-1* is highest, not intermediate, at the border of the neural plate. Second, when posterior non-neural epiblast (which expresses high levels of *BMP-4*) is co-cultured with neural plate, *msx-1* is not induced in the neural plate but continues to be expressed in the non-neural portion. By contrast, anterior non-neural epiblast (which expresses low levels of *BMP-4*) does induce *msx-1* in the neural plate as well as upregulates its own expression of this marker. This is not consistent with the proposal that different threshold levels of BMP are sufficient to determine, in ascending order: neural plate, neural crest, and epidermis. If this were correct, the higher level of *BMP* expression in posterior epiblast would be expected to counteract the neural state of the neural plate, and to generate a border at some distance from the junction, while the lower level of *BMP* expression by the anterior epiblast should also induce a border, but closer to the junction. This is not the case.

Together, our observations suggest that BMP-4 activity plays a role in positioning the border, but that other factors are also required. The Wnts and/or FGFs are good candidates. It was recently proposed that in *Xenopus*, a Wnt activity (which may be Wnt8, LaBonne and Bronner-Fraser, 1998or Wnt7B, Chang and Hemmati-Brivanlou, 1998) acts together with maintained BMP inhibition to define the territory that will give rise to the neural crest (the border of the neural plate). It has also been proposed that FGF activity plays a role in neural crest/border development (Mayor et al., 1995; Mayor et al., 1997), although this effect may be indirect, by induction of Wnt activity (LaBonne and Bronner-Fraser, 1998).

3.2. Roles of FGF in neural induction and border formation

Our experiments address two questions relating to the role of FGF in early neural development: (a) is FGF the upstream signal from the organizer that allows BMP inhibitors to induce nervous system?; and (b) does FGF activity cooperate with BMP activity to induce the border of the neural plate?

3.2.1. Does FGF cooperate with BMP antagonists to generate the neural plate?

In *Xenopus*, BMP inhibition leads to the induction of anterior nervous system (reviewed by Sasai and De Robertis, 1997; Wilson and Hemmati-Brivanlou, 1997). However, our previous studies have revealed that ectopic expression of chordin or noggin in competent epiblast is not sufficient to initiate any neural development, but that other upstream signals from the organizer are required for chordin to stabilize the expression of early neural markers (Streit et al., 1998; Streit and Stern, 1998). Could FGF be the signal required before chordin/noggin can act to generate or to pattern the CNS?

In vivo, the expression of various members of the FGF family in *Xenopus*, chick and mouse embryos is consistent with this proposal: *FGF-2, -3, -4* and *-8* have all been reported to be expressed in mesoderm of the organizer and adjacent regions during gastrulation (Isaacs et al., 1992; Tannahill et al., 1992; Riese et al., 1995; Mahmood et al., 1995a,b; Bueno et al., 1996; Storey et al., 1998). Also consistent with this proposal is the finding that FGF signalling is required for neural induction in *Xenopus* (Launay et al., 1996; Sasai et al., 1996; Xu et al., 1997). In the presence of dominant-negative FGF receptors, the BMP inhibitors chordin and noggin are unable to induce the expression of anterior neural markers. In addition, several reports implicate FGF as a direct neural inducer in both *Xenopus* and chick (Lamb and Harland, 1995; Álvarez et al., 1998; Storey et al., 1998). In the chick, this effect has not been clearly dissociated from the mesoderm inducing effects of FGF (Storey et al., 1998), or the assays have been performed too close to the prospective neural plate to allow recruitment of cells to be ruled out definitively (Álvarez et al., 1998). In the area opaca, where recruitment does not complicate the experiment, FGF only induces posterior neural tissue (Storey et al., 1998).

In our experiments, we show that even a combination of FGF and chordin does not lead to stable expression of either general (*Sox2*) or anterior (*Otx2* or *Krox20*) neural markers in area opaca epiblast. This finding suggests that FGF is not sufficient to confer competence to epiblast cells so that BMP antagonists can generate or pattern the nervous system.

3.2.2. FGF may cooperate with BMP signalling to induce the border of the neural plate

One of the surprising findings in the present study is that the organizer induces the expression of a ring of *BMP-4*, which appears to demarcate the limits of the newly-induced neural plate. This was unexpected because the ‘default model’ in *Xenopus* (see above) proposes that signals from the organizer should inhibit BMPs. Why should the organizer also induce *BMP-4* expression? What are the signals responsible?

The results of our experiments and others lead us to propose a model, in which FGF and BMP activities cooperate to set up and maintain the border between neural and non-neural ectoderm (Fig. 9). We find that FGF8 rapidly and strongly induces *msx-1* expression in area opaca epiblast in the absence of mesoderm. Several *FGFs* are expressed in the organizer during gastrulation and neurulation (Isaacs et al., 1992; Tannahill et al., 1992; Riese et al., 1995; Mahmood et al., 1995a, 1995b; Bueno et al., 1996; Storey et al., 1998). Consistent with these observations, *msx-1* is expressed in the young (posterior) neural plate adjacent to the node in the normal embryo (Fig. 5D). This raises the possibility that signals from the organizer induce *msx-1* in the adjacent epiblast. A recent study has shown that BMP-4 expression both regulates, and is regulated by, *msx-1* in tooth development (Bei and Maas, 1998), defining a positive feedback loop for *BMP* transcription in response to BMP activity (see also Biehs et al., 1996). We might therefore expect *BMP-4* to be expressed in the young neural plate; however, this is not the case. One reason could be that BMP inhibitors (noggin, chordin, follistatin) secreted by the organizer (and/or the low level of BMP inhibition by the neural plate itself, as shown in this study) rapidly sequester any BMP produced and therefore cause the downregulation of *BMP-4* transcription. Regions of the epiblast more distant from the organizer still receive *msx-1*-inducing signals from the paraxial mesoderm (FGF; Mahmood et al., 1995b; Storey et al., 1998). In addition, our finding that a bead of FGF can induce *msx-1* expression over a distance of 300 μm (almost twice the width of the open neural plate; Fig. 3A,C) suggests that FGF produced at the midline may diffuse further than the BMP inhibitors. Together, these mechanisms might lead to *msx-1* expression becoming concentrated at the lateral border of the neural plate. In turn, the strongly *msx-1*-expressing cells in this region would produce high levels of BMP-4, generating a positive feedback mechanism that maintains the high expression of *msx-1* (Fig. 9).

Grafts of Hensen's node do induce *msx-1*, but much more slowly than does FGF. One possible reason is that the node only produces low levels of FGF, and that this signal is transmitted to more distant regions by a relay mechanism travelling through the neural plate. In fact, the young neural plate itself expresses FGFs (Tannahill et al., 1992; Storey et al., 1998), and various members of this family can induce each others' transcription (Niswander et al., 1994; Storey et al., 1998).

3.3. Interactions between neural and non-neural epiblast in border formation

If signals are transmitted through the neural plate by a relay mechanism, some other mechanism is required to stop their propagation in the tissue. This led us to investigate whether confrontation between young neural plate and non-neural epiblast from a remote region might lead to the establishment of a boundary expressing *msx-1*. The idea that

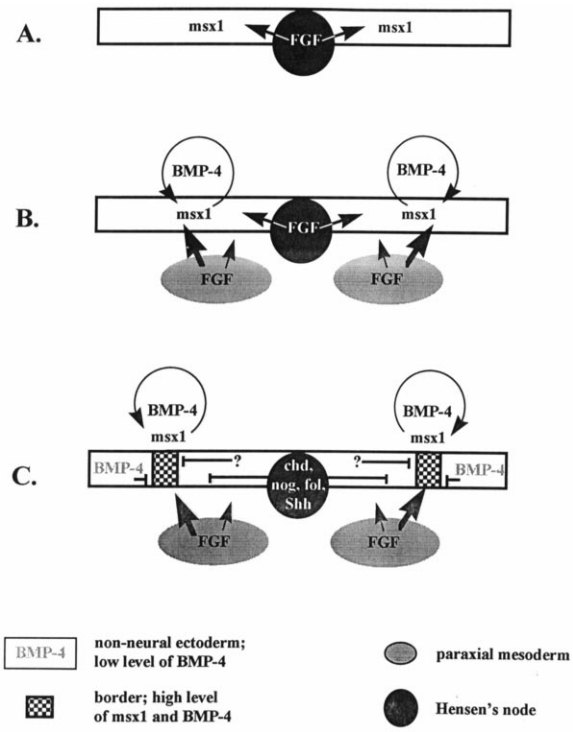

Fig. 9. A model for the interactions that set up and maintain the border of the neural plate. (A) Initially, FGF emitted by the organizer induces *msx-1* in the adjacent epiblast. (B) *msx-1* activates *BMP-4* transcription, which enhances *msx-1* expression. At the same time, the underlying paraxial mesoderm also expresses FGF, which contributes to enhance *msx-1*. (C) However, BMP inhibitors (chd, nog, fol) produced at the midline rapidly sequester any secreted BMP. Sonic hedgehog (Shh) may also contribute to the downregulation of *msx-1*. This leads to downregulation of both *msx-1* and *BMP-4* except in a ring, at a distance from the organizer (checker pattern). This is reinforced by FGF from the paraxial mesoderm, and by a low level of BMP activity from the lateral ectoderm. This leads to a situation where BMP-4 acts in an autocrine way at the border itself. In addition, the neural plate itself may produce other antagonists of BMP signalling and/or FGF.

the boundary of the neural plate is established by interactions between neural and non-neural ectoderm was first proposed by Moury and Jacobson (1990), and supporting evidence was subsequently obtained in both *Xenopus* and chick embryos (Dickinson et al., 1995; Liem et al., 1995; Selleck and Bronner-Fraser, 1995; LaBonne and Bronner-Fraser, 1998). It appears from these experiments that both the non-neural ectoderm and the neural plate contribute to neural crest (Moury and Jacobson, 1990; Liem et al., 1995). Our experiments reveal further complexity to this interaction. When prospective neural plate is grafted into non-neural epiblast of a host embryo, *msx-1* starts to be induced in the grafted neural plate after just 7 h under the influence of signals from non-neural epiblast, but induction of the same marker in the non-neural epiblast of the host requires 20–25 h. This could suggest either that young neural plate does not yet emit the signals required to induce *msx-1* in non-neural epiblast, or that longer exposure to signals from the neural plate are required for this induction.

Our experiments reveal that grafts of neural plate placed

at the edge of the host neural plate behave like misexpression of chordin or noggin: they shift the border laterally. Grafts of non-neural epiblast, on the other hand, are comparable to misexpression of BMP-4: they interfere with progression of the boundary. BMPs are known to be expressed in non-neural ectoderm in frog and chick embryos (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Streit et al., 1998), albeit at low level. Our findings indicate that the young neural plate itself is a source of as yet unidentified BMP inhibitors (Fig. 9C).

In addition, we have revealed that anterior and posterior prospective epidermis behave differently in combination with neural plate explants in vitro. Posterior epidermis seems unable to induce *msx-1* expression in the neural plate explant, while anterior epidermis can. This is difficult to explain, but it is worth noting that there are several known differences between these two regions of epiblast. First, the region used as a source of posterior epidermis normally never comes into apposition with neural plate in normal development, while the anterior-lateral epiblast does. It is conceivable that the epidermal signals that delimit the neural plate are confined to the region of epidermis that normally adjoins the neural plate. If so, this property should extend into the area opaca, which can induce *msx-1* in neural plate grafts. A second possibility is related to the pattern of expression of *BMPs* in these two areas: the posterior epiblast expresses high levels of *BMP-4* and *-7*, while the anterior region expresses them at a lower level. This may commit the posterior ectoderm to a border state maintained by the neural plate, but inhibit other neighbouring cells from becoming boundary-like. A third difference is that the posterior epiblast probably contains some cells fated to contribute to lateral mesoderm (Rosenquist, 1966; García-Martínez et al., 1993). It is worth pointing out that most previous studies of interactions between neural and non-neural epiblast have used this posterior region (Dickinson et al., 1995; Liem et al., 1995; Selleck and Bronner-Fraser, 1995).

3.4. Signals from the mesoderm?

In the normal embryo (from about the time of appearance of the first somite), the border of the neural plate lies at about the same mediolateral level as the lateral edge of the paraxial mesoderm. Could prospective somite tissue play a role in establishing the border? There is some evidence that this is the case in both amphibians and chick (Raven and Kloos, 1945; Bang et al., 1997; Bonstein et al., 1998; Marchant et al., 1998). We find that FGF8 can induce *msx-1* in the area opaca, and prospective paraxial mesoderm has been reported to be a source of FGF-3, -4 and -8 (Niswander and Martin, 1992; Tannahill et al., 1992; Mahmood et al., 1995b; Bueno et al., 1996; Storey et al., 1998). Therefore, the involvement of paraxial mesoderm in border formation or maintenance may be mediated by FGF.

3.5. A ‘pre-border’ state?

During normal development of the neural plate from stage 5 onwards, the early neural markers *Sox3* and L5 share a domain of expression with *msx-1* adjacent to the node (Fig. 5). As the neural plate extends away from the node, the two sets of markers diverge. The entire length of the neural/non-neural border retains its expression of all three markers, as does the most caudal neural plate that remains close to the node; the older (rostral) neural plate loses *msx-1* expression but retains neural markers. This raises the possibility that one of the earliest states in response to neural-inducing signals is an unstable state, defined by the co-expression of neural and border markers, as well as *Cash-4*, *Sax-1* (Henrique et al., 1997; Storey et al., 1998), *Pax3* (Bang et al., 1997) and *Wnt8C* (Hume and Dodd, 1993). It is only in this state that cells appear to be responsive to BMP-4 or its antagonists, and they may develop in one of three possible directions: they may join the neural plate and lose *msx-1* expression but retain *Sox3* and L5, they may lose all markers and develop into epidermis, or they may remain in the border region and perhaps retain their responsiveness to BMP and its antagonists for some time.

It should be pointed out, however, that this situation only appears to apply to the neural plate developing in caudal regions of embryos at stage 5 and older. The epiblast fated to contribute to CNS rostral to the hindbrain does not co-express *Sox3* and *msx-1* at primitive-streak stages. Perhaps the mechanisms by which the neural/non-neural border is established differ in cranial and caudal regions of the nervous system. It has even been proposed that the initial neural-inducing signals for these two regions of the CNS may derive from different tissues (Eyal-Giladi and Wolk, 1970; Thomas and Beddington, 1996). Alternatively, it is possible that rostral parts of the nervous system are established even before primitive streak formation.

3.6. Homoiogenetic induction

One deficiency of the ‘default model’ is that it does not easily explain the phenomenon of homoiogenetic neural induction. This is defined as the induction of neural plate by already induced neural plate, and was first reported in amphibians (Mangold and Spemann, 1927), but has also been studied in avian embryos (Rasilo and Leikola, 1976). To date, no BMP antagonists have been shown to be expressed in the newly-induced neural plate of either species. Chick noggin appears to be expressed in the neural plate but only in anterior regions and after stage 8 (Connolly et al., 1997) (see Fig. 2). We have shown here that the neural plate has BMP-antagonist-like activity before this stage. Despite this, grafts of neural plate into competent epiblast do not induce any early neural markers even after 24 h of contact, and at this time only *msx-1* expression is seen and the host epiblast is not significantly thickened (Fig. 7F).

Neural plate grafts only seem to affect cells at the border. Our experiments therefore argue that homoiogenetic induction does not exist in the chick in regions outside the border.

3.7. Conclusions

Our results indicate that several tissues and signalling pathways cooperate in the initial establishment, and in the maintenance of the neural/non-neural boundary. Signals from the organizer, the developing neural plate, the paraxial mesoderm and the non-neural epiblast converge at this boundary. The molecules involved appear to include FGFs acting in concert with BMP activity. The responsiveness to BMP and its inhibitors is confined to the border region itself. We suggest that BMPs act mainly in an autocrine way to maintain the border state.

4. Materials and methods

Fertile hens' eggs (White Leghorn; Spafas, MA) and quails' eggs (Karasoulas, CA) were incubated at 38°C for 8–30 h to produce embryos between stages 3 and 9 (Hamburger and Hamilton, 1951).

4.1. cDNA clones

A myc-epitope tagged version of BMP-4 (Liem et al., 1995) in pMT-23 and a HA-epitope tagged version of chordin (Streit et al., 1998) in pMT21 were used for expression in COS cells. *Sox-2* and *Sox-3* plasmids were provided by Drs. R. Lovell-Badge and P. Scotting (Uwanogho et al., 1995; Collignon et al., 1996). Chick *msx-1* was obtained from Dr. K. Liem; chick *brachyury* was provided by Dr. J. Smith, *Otx2* from Drs. L. Bally-Cuif and E. Boncinelli (Bally-Cuif et al., 1995) and *Krox20* from Dr. D. Wilkinson (Wilkinson et al., 1989).

4.2. Cells and transfection

COS-1 cells were grown in DMEM containing 10% new born calf serum. Transfection with *BMP-4*, and *chordin* was performed using lipofectamine-plus (Gibco BRL). 24 h after transfection, pellets containing 1000 cells were generated by setting up hanging drop cultures. The pellets were transplanted into embryos 48 h after transfection. The presence of BMP-4 and chordin in conditioned medium collected from COS cells was confirmed by Western blots using anti-myc or anti-HA antibodies. A stable cell line expressing noggin (CHO-B3) and parent CHO cells were a gift from Dr. R. Harland; secretion of noggin protein was confirmed in Western blots using noggin specific antibodies (Smith et al., 1993). To obtain pellets for grafting CHO-B3 and CHO cells were trypsinized mildly, resuspended in serum containing medium and centrifugated at 200× g. The resulting pellets were transferred to a medium containing petridish and then cut into smaller pellets (500–1000 cells) using fine steel needles.

4.3. FGF beads

Heparin beads (Sigma) were incubated in 50 μg/ml FGF4 or FGF8 (R&D Systems) in PBS or in PBS only (control) for 2 h on ice. The beads were then extensively washed in PBS before grafting into the embryo.

4.4. Dissection and grafting techniques

Chick host embryos were explanted and maintained in New culture (New, 1955), modified as described by Stern and Ireland (1981), for 3–24 h. Quail donor embryos were immersed in Pannett-Compton saline (Pannett and Compton, 1924), Hensen's node was excised and transplanted into the inner margin of the area opaca of a chick host as described previously (Storey et al., 1992). To isolate neural plate from stage 4 quail donors, the hypoblast and endoderm layers were carefully removed from the ventral side of the embryo and a piece of neural plate immediately anterior to Hensen's node was dissected using fine steel needles (see Fig. 1B). Neural plate from older embryos was dissected with the aid of 0.08% Trypsin in Tyrode's saline. Non-neural ectoderm was isolated from the positions indicated in Fig. 1B. Grafts were placed into the inner margin of the area opaca of a chick host. Cell pellets (500–1000 cells) were grafted into different regions of chick host embryos as indicated in Fig. 1A.

For node removal experiments, an excised chick Hensen's node was labelled with 50 μM 5-chloromethylfluorescein diacetate (CellTracker^{TM} Green CMFDA; Molecular Probes) in Tyrode's saline for 15 min and washed extensively in Tyrode's before grafting. After 5 h, the node was removed and its complete removal checked by fluorescence microscopy. A quail neural plate (stage 5–6), freed from adjacent mesendoderm, was then grafted to the same site and the embryo incubated overnight.

4.5. Explant culture

Neural plate, anterior and posterior non-neural ectoderm was isolated from stage 4 chick embryos from the regions indicated in Fig. 1B. Embryos were collected in Tyrode's saline and the endodermal or mesodermal layers were carefully removed prior to dissecting the epiblast layers. Neural plates were labelled with CMFDA as described above. Explants of non-neural ectoderm and neural plate were cultured alone or in combination on Millicell-CM filters (Millipore; Millicell-CM, PICM 03050) in medium 199 (Gibco, BRL) as described in detail by Krull and Kulesa (1998). This technique allows us to set up the recombinants with planar contact initially. For in situ hybridisation explants

were removed from the filters in phosphate buffered saline using bent steel needles and transferred into collagen gel matrices (Streit et al., 1995) for further processing.

4.6. Immunocytochemistry and in situ hybridisation

To visualize quail tissue we used the monoclonal antibody QCPN (Developmental Studies Hybridoma Bank, maintained by the Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205 and the Department of Biological Sciences, University of Iowa, Iowa City 52242, under contract N01-HD-2-3144 from NICHD). The staining was performed as described before (Streit et al., 1997).

Whole-mount in situ hybridisation using DIG-labelled RNA-probes and histology were performed as described by Streit et al. (1997). Double in situ hybridisation was performed using DIG-labelled or FITC-labelled RNA-probes, and combination of whole-mount immunostaining with monoclonal L5 antibody and in situ hybridisation was performed as described in Stern (1998).

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