

# Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior-posterior neural pattern

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## SUMMARY

Neural tissue in developing *Xenopus* embryos is induced by signals from the dorsal mesoderm. Induction of anterior neural tissue could be mediated by noggin, a secreted polypeptide found in dorsal mesoderm. We show that bFGF, a known mesoderm inducer of blastula staged ectoderm, induces neural tissue from gastrula stage ectoderm. The type of neural tissue induced by bFGF from stage 10.25 ectoderm is posterior, as marked by Hox B9 expression. When bFGF and noggin are combined on early gastrula stage ectoderm, a more complete neural pattern is generated and no mesodermal tissue is detected. Explants treated with noggin and bFGF elongate and display distinct anterior and posterior ends marked by *otx2* and Hox B9 expression, respectively. Furthermore, treatment of early gastrula ectoderm with noggin and bFGF results in the induction of *En-2*, a marker of the midbrain-hindbrain junction and *Krox 20*, a marker of the third and fifth rhom-

bomeres of the hindbrain. Neither of these genes is induced by noggin alone or bFGF alone at this stage, suggesting a synergy in anterior-posterior neural patterning. The response of later gastrula (stage 11-12) ectoderm to bFGF changes so that *Krox 20* and *En-2* are induced by bFGF alone, while induction of more posterior tissue marked by Hox B9 is eliminated. The dose of bFGF affects the amount of neural tissue induced, but has little effect on the anterior-posterior character, rather the age of the ectoderm treated is the determinant of the response. Thus, an FGF signal may account for posterior neural induction, and anterior-posterior neural patterning could be partly explained by the actions of noggin and FGF, together with the changing response of the ectoderm to these factors.

Key words: FGF, noggin, neural induction, posterior pattern, *Xenopus*

## INTRODUCTION

The embryonic brain and spinal cord arise from dorsal ectoderm that has received a signal from the nearby dorsal mesoderm (prechordal mesoderm, notochord and somites) (Spemann, 1938; Hamburger, 1988). Recent work has shown that neural induction and proper neural patterning occurs in explant culture when competent ectoderm is combined with dorsal mesoderm (reviewed by (Doniach, 1993)). The dorsal mesoderm provides patterning information for both the anterior-posterior (Sharpe and Gurdon, 1990; Hemmati-Brivanlou et al., 1990b; Saha and Grainger, 1992) and the dorsal-ventral (Yamada et al., 1991; Placzek et al., 1991) axes of the neural tube. Signaling between cells within the induced ectoderm is likely to contribute to the final anterior-posterior neural pattern (Doniach et al., 1992). Not surprisingly, the most complete pattern of neural tissue is obtained when the contacts with dorsal mesoderm most closely resemble those within the embryo (Dixon and Kintner, 1989). Thus, a convenient assay for candidate inducers is to treat competent ectoderm and score the response with molecular markers.

This type of assay has identified noggin, follistatin, fibroblast growth factor (FGF) in *Xenopus* (Harland, 1994) and hepatocyte growth factor/scatter factor (HGF/SF) in chick

(Streit et al., 1995) as secreted molecules that could be involved in neural induction and patterning. Noggin is a secreted molecule expressed in the organizer (Smith and Harland, 1992) that has anterior neural inducing activity (Lamb et al., 1993). Noggin cannot be the only organizer signal, since noggin induces only anterior brain tissue (Lamb et al., 1993), whereas the organizer induces the entire anterior-posterior range of neural tissue.

A primary role for follistatin in neural induction at the gastrula stage is unlikely. Although follistatin RNA induces anterior neural tissue from blastula staged ectoderm, perhaps by blocking an activin or an activin-like signal (Hemmati-Brivanlou and Melton, 1992, 1994; Hemmati-Brivanlou et al., 1994), neural induction continues through gastrulation (Sharpe and Gurdon, 1990). Overexpression of follistatin at the gastrula stage does not neuralize ectoderm (F. Mariani, A. Hemmati Brivanlou, personal communication). This suggests that blocked activin signaling cannot account for the organizer-derived signals that mediate neural induction during gastrulation and at later stages. However, although follistatin cannot neuralize autonomously, it may cooperate with other factors in neural induction.

Fibroblast growth factor (FGF), previously studied as a mesoderm inducer (Slack, 1994), has been more recently

proposed to be a neural inducer (Kengaku and Okamoto, 1993). Both aFGF (acidic) and bFGF (basic) induce the expression of a neural marker from ectodermal cells dissociated at the gastrula stage (Kengaku and Okamoto, 1993). Although these experiments support the idea that FGF might induce neural tissue directly, they are also consistent with an interpretation that FGF reinforces the neuralizing effects of cell dissociation (Grunz and Tacke, 1989; Godsave and Slack, 1991), or causes terminal differentiation of neuralized cells. A large body of work implicates FGF in the process of neuronal cell survival and differentiation (Gospodarowicz, 1990; Williams et al., 1994). The expression pattern of FGFs supports a possible role in neural induction or patterning. In *Xenopus*, two forms of FGF have been identified. Embryonic FGF (eFGF) is expressed in tissues known to have neural inducing activity such as the organizer at the gastrula stage, and the posterior notochord during neurulation (Isaacs et al., 1992). bFGF (basic) is unlikely to be involved in neural induction since it lacks a signal sequence and its expression is non-localized; however, it has activity identical to eFGF on blastula ectoderm (Kimelman et al., 1988; Slack and Isaacs, 1989; Isaacs et al., 1992). This suggests that bFGF and eFGF activate the same signal transduction pathway, so that either form may be used to assay FGF activity. In conclusion, the activity and expression pattern of an FGF family member suggests that FGF signaling could be important for neural induction or patterning *in vivo*.

In this paper, we have examined whether bFGF can induce neural tissue from non-dissociated ectoderm. We assess the regional character of the induction at different stages and examine the interaction of bFGF with noggin, an anterior neural inducer.

## MATERIALS AND METHODS

### Embryo dissection and culture

*Xenopus* eggs from pigmented or albino females were fertilized and cultured as described (Condie and Harland, 1987). Animal cap ectoderm (up to 400  $\mu\text{m}$  across) was isolated at the late blastula stage (stage 9) and aged to the appropriate stage until treatment. If animal caps are allowed to heal, they rapidly lose competence to become neuralized (Sive et al., 1989; T. M. L. and A. K. Knecht, unpublished). Therefore, animal cap ectoderm was prevented from healing in medium with very low calcium and magnesium concentrations (vLCMR; Lamb et al., 1993). Animal cap ectoderm treated in this way did not disaggregate and remained competent to respond to added factors through gastrulation. Expression of XAG-1, a marker of cement gland, in these aged explants shows that this tissue is not completely naive. This raises the question of what can be considered a neutral treatment of animal caps during aging. We note that the more physiological buffers used for culturing explants (Danilchik's; Keller et al., 1985) are even more potent inducers of cement gland (R. M. H., unpublished observation). However, no neural- or mesoderm-specific gene expression is activated, indicating that ectoderm aged in this fashion is still useful to identify neural or mesodermal inducers. Furthermore, we have analyzed the inducing capacity of bFGF on ectoderm aged in a different way (Sharpe and Gurdon, 1990). Animal caps cut at stage 9 and aged under glass coverslips to stage 10.5 in 3/4 NAM respond to bFGF in a similar manner as caps aged in vLCMR (data not shown).

For the purposes of this paper, we consider the turn on of NCAM and *nrp-1*, both pan-neural markers, to be a definitive assay for neural

tissue and we do not consider cement gland to be a neural tissue. Nonetheless, it is possible that cement gland differentiation results from low doses of neuralizing signals (Sive et al., 1989) and that signals that antagonize neural differentiation (such as BMP4; Wilson and Hemmati-Brivanlou, 1995), also inhibit cement gland formation. Thus, if the animal cap heals quickly, neural induction may be prevented because of the accumulation of BMP4. In this case, any treatment that kept the cap open could be considered a partial neuralizing treatment, because BMP4 could diffuse away. However, animal caps aged in vLCMR do not activate neural-specific genes such as NCAM or *nrp-1* above levels seen in explants allowed to heal at stage 8 (data not shown), so we consider aged animal caps to be a rigorous test tissue for candidate neural inducers.

Treatment of ectoderm with added factors was carried out in a low calcium and magnesium Ringer's solution (LCMR; 43 mM NaCl, 0.85 mM KCl, 0.37 mM  $\text{CaCl}_2$ , 5 mM HEPES, pH 7.2, and 50  $\mu\text{g}/\text{ml}$  gentamycin) + 0.5% BSA. Basic FGF (UBI #01-106) produced in yeast was diluted from a 1 mg/ml stock to 1.25  $\mu\text{g}/\text{ml}$ -0.08 ng/ml. Purified human noggin produced in baculovirus (a gift from Regeneron Pharmaceuticals) was diluted from a 1.8 mg/ml stock to 1  $\mu\text{g}/\text{ml}$ . Activin was obtained as a cos cell supernatant (Regeneron Pharmaceuticals) and used at a dilution of 1:10. This is a dose of approximately 20 units/ml (unit defined by Green et al., 1992). Explants remained in their treatment medium until the time of harvest.

### Whole-mount *in situ* hybridization

*In situ* hybridization was carried out essentially as described (Knecht et al., 1995). If analysis of two genes was desired, digoxigenin- and fluorescein-conjugated probes were hybridized simultaneously. After hybridization, samples were treated as described (Knecht et al., 1995). If two probes were used, the first color reaction would be for the least abundant transcript using the darkest chromogen (BM purple, from Boehringer Mannheim (1442 074) or MagentaPhos (Biosynth AG # B-755). After signal reached desired levels, the phosphatase activity was quenched by treating with 10 mM EDTA for 10 minutes at 60°C and subsequent dehydration. Samples were rehydrated, re-blocked and then the second antibody was added. Unbound antibody was washed away as before, and then a different chromogen (BCIP; Sigma) was used to detect the second, more abundant transcript.

Templates for the production of NCAM, En-2, and Krox 20 (Bolce et al., 1992), muscle actin (Hemmati-Brivanlou et al., 1990a), Hox B9 (XIHbox 6, Doniach et al., 1992), collagen type II (Amaya et al., 1993) and *nrp-1* (Knecht et al., 1995) *in situ* hybridization probes have been previously described.

### Marker gene expression

The following genes have been used primarily as markers of mesodermal tissues, however, some also mark neural tissues. *Muscle actin* is expressed in developing somites (Gurdon et al., 1985) *Collagen type II* is expressed in the notochord, floorplate and somites (Bieker and Yazdani-Buicky, 1992; Amaya et al., 1993; Smith et al., 1995). *gooseoid* is expressed in the organizer region at the gastrula stage and then later in the prechordal mesoderm and in neural ectoderm (Cho et al., 1991; Blumberg et al., 1991; Thisse et al., 1994 and RMH unpublished). *Xbra* is first expressed in a ring around the embryo in tissue that is thought to correspond to mesoderm (Smith et al., 1991). Later, *Xbra* is expressed in the posterior notochord and circum-blastoporal tissue (Smith et al., 1991); it is also expressed in regions where orthotopic grafts populate both mesoderm and spinal cord (R. E. Keller, personal communication), so that it is expressed transiently in presumptive spinal cord (see also Fig. 4G, Smith et al., 1991). Thus, while the absence of these markers can be taken as evidence that mesoderm is not formed, their expression does not prove that mesoderm is present.

The following genes have been used as neural markers, although some are expressed at lower levels in mesoderm or epidermis. *NCAM* and *nrp-1* are both expressed throughout the neural tissue (Balak et

al., 1987; Kintner and Melton, 1987; Richter et al., 1990; Knecht et al., 1995). At the late neurula stage, *otx2* is expressed in the anterior brain and shows low level staining in anterior epidermis and mesoderm. At later tailbud stages, *otx2* is expressed exclusively in anterior neural tissue including the forebrain, midbrain, and retina (Lamb et al., 1993; Pannese et al., 1995; Blitz and Cho, 1995). *En-2* is expressed in the midbrain-hindbrain boundary and has lower level expression in anterior mesoderm (Hemmati-Brivanlou et al., 1991; Doniach et al., 1992). *Krox 20* is expressed in the third and fifth rhombomeres in the hindbrain, and in neural crest cells migrating out of the fifth rhombomere (Wilkinson et al., 1989; Bradley et al., 1991). *Hox B9* is expressed in the spinal cord and at lower levels in posterior lateral plate mesoderm (Wright et al., 1990; Godsave et al., 1994).

**Reverse transcription-PCR**

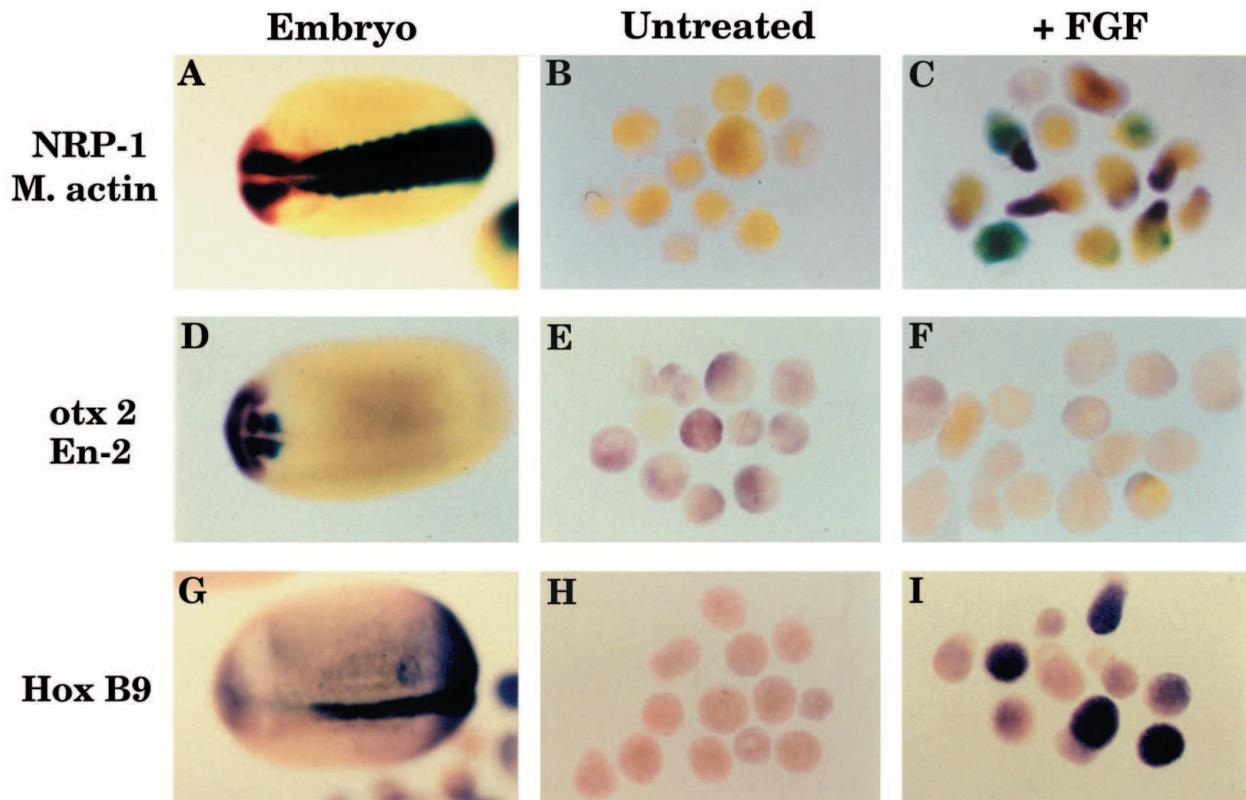
RNA was harvested as described (Condie and Harland, 1987) and subsequently digested with DNase to remove genomic DNA (Wilson and Melton, 1994). RT-PCR was carried out as described (Wilson and Melton, 1994). Primer sets for analysis of *NCAM*, *En-2*, *Krox 20*, *Hox B9* (previously *XIHbox6*) are described by Hemmati-Brivanlou and Melton (1994), while *goosecoid*, *Xbra*, *muscle actin* and *EF-1 $\alpha$*  primer sets are described by Wilson and Melton (1994). New primer sets include *nrp-1* (Richter et al., 1990) U-GGG TTT CTT GGA ACA AGC (943-960), D-ACT GTG CAG GAA CAC AAG (1226-1209), and *otx2* (accession number L26509) U-CGG GAT GGA TTT GTT GCA (252-269), D-TTG AAC CAG ACC TGG ACT (452-435), designed by Paul Wilson; *Collagen type II* (Su et al., 1991) U-GGT ACT GGA TTC TCC TTC (13-31), D-GAA CAG CAC TAG AGT

CCT (185-167) designed by F. Mariani. (U upstream, D downstream and the numbers correspond to the published sequence in the reference cited.)

**RESULTS**

**FGF induces mesoderm and posterior neural tissue from early gastrula ectoderm**

A previous report suggested that FGF has neural inducing activity on gastrula staged cells (Kengaku and Okamoto, 1993); however, alternate interpretations were possible. Dispersed cells were treated with FGF, reaggregated and, after 5 days of culture, were analyzed with antibodies. FGF treatment induced a marker of differentiated neural tissue, but not a marker of mesoderm (Kengaku and Okamoto, 1993). The use of a single mesoderm marker left open the possibility that FGF was inducing a type of mesoderm not detected by the antibody and that this mesoderm could secondarily induce neural tissue. Additionally, it is possible that disaggregation of the ectoderm caused neuralization (Grunz and Tacke, 1989; Godsave and Slack, 1991), but not terminal differentiation of untreated cells. Although Kengaku and Okamoto (1993) disaggregated cells for only 30 minutes, a time found to be insufficient to activate NCAM expression by Wilson and Hemmati-Brivanlou (1995), it is still possible that their culture conditions allowed neuralization. Thus the activity of FGF in these exper-



**Fig. 1.** Neural and mesoderm induction by FGF. In situ hybridization analysis on early gastrula stage treated ectoderm cultured to the early tailbud stage. Untreated ectoderm (B,E,H), FGF-treated ectoderm (C,F,I), and the control whole embryos (A,D,G) were examined for expression of *nrp-1* (purple) and muscle actin (M. actin, blue) (A-C); *otx2* (purple) and *En-2* (blue) (D-F); and *Hox B9* (G-I). FGF induces both muscle actin and *nrp-1*, however, expression of the neural marker is not contingent upon muscle expression. The posterior marker, *Hox B9*, but not the anterior markers, *otx2* or *En-2*, is induced by FGF. In A, the strong expression of muscle actin obscures the *nrp-1* expression in the spinal cord.

iments may have been to differentiate existing neural tissue and not to induce neural tissue from ectodermal tissue.

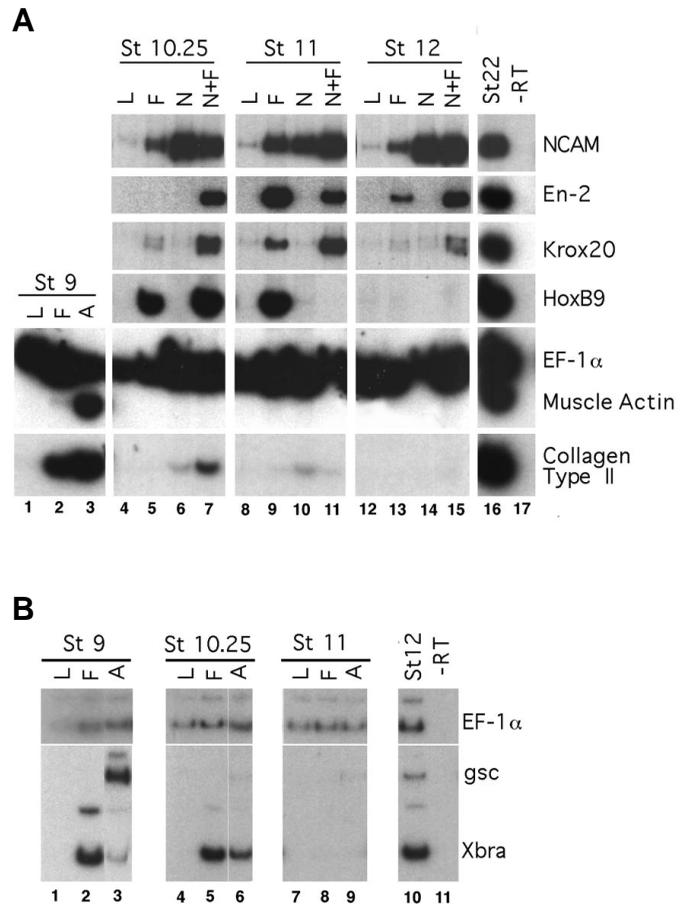
To address these possibilities, we cultured intact gastrula stage animal cap ectoderm in the presence of 50 ng/ml bFGF and analyzed the expression of several neural and mesodermal markers approximately 20 hours after treatment. Fig. 1 shows that explants treated with FGF express both *nrp-1*, a general marker of neural tissue, and muscle actin. However, some explants show expression of only the neural marker, while others show expression of only muscle actin; a third group expresses both markers. Since induction of *nrp-1* by FGF does not correlate with muscle induction, the neural induction may not be secondary. This suggests that FGF could be acting as a primary neural inducer as well as a mesoderm inducer on early gastrula staged ectoderm. The expression of additional markers including NCAM, Krox20, Collagen type II, *Xbra* and *Goosecoid* was analyzed by RT-PCR (see below)

The anterior-posterior character of the neural tissue induced by FGF was examined by analyzing the expression of several region-specific neural markers. A marker of anterior brain tissue, *otx2* (see Materials and Methods for a description of gene expression patterns) is expressed at a low level in untreated explants (Fig. 1E). This expression probably corresponds to the non-neural *otx2* expression found in whole embryos. FGF treatment does not induce *otx2*, indeed it represses the low level expression seen in controls (Fig. 1F). *En-2*, a marker of the midbrain-hindbrain junction is also not induced by FGF (Fig. 1F). The posterior marker *Hox B9*, which is expressed strongly in neural and weakly in mesodermal tissues is strongly induced by FGF (Fig. 1G-I). This suggests that the neural or mesodermal tissue induced by FGF is posterior in character.

### FGF induces neural tissue throughout gastrulation and can act in the absence of mesoderm

Since FGF treatment of early gastrula staged ectoderm still generated mesoderm in some explants, the neural tissue observed may have been induced secondarily. We tested whether older ectoderm, which has lost the ability to respond to mesoderm inducers (Green et al., 1990; Kintner and Dodd, 1991), would still be neuralized by FGF. Animal cap ectoderm aged to stage 11 or stage 12 was treated with FGF and analyzed for the expression of neural and mesoderm markers by RT-PCR at the tailbud stage. Fig. 2A shows that stage 11 and stage 12 ectoderm responds to FGF by expressing the general neural marker NCAM (lanes 9, 13), while failing to express muscle actin or collagen type II, a marker of notochord, floorplate and somites (Bieker and Yazdani-Buicky, 1992; Amaya et al., 1993). Thus, at these later stages of treatment, FGF induces neural tissue in the absence of mesoderm.

However, neural tissue could also be induced by a tissue that transiently took on dorsal mesodermal qualities, but never differentiated into muscle or notochord. Thus, markers of early mesoderm such as *goosecoid* (Cho et al., 1991; Blumberg et al., 1991) and *Xbra* (Smith et al., 1991) are useful to assess whether a transient mesodermalization could account for neural induction. Fig. 2B shows that while ectoderm treated with FGF at stage 9 or stage 10.25 expresses *Xbra* (analyzed at stage 12), treatment at stage 11 no longer induces this early marker of mesoderm (lanes 2, 6, 11). At no stage of treatment does bFGF induce *goosecoid* expression. These experiments



**Fig. 2.** Time course of treatment with factors. (A) Ectoderm taken from late blastula (stage 9) embryos was either treated immediately or aged to early (stage 10.25), mid- (stage 11) or late (stage 12) gastrula stage and treated with 50 ng/ml bFGF (F), 1  $\mu$ g/ml noggin (N), the combination (N+F), activin (A) or with an LCMR control (L). Explants were subsequently cultured to stage 22, and harvested for RT-PCR analysis. Expression of NCAM, *En-2*, Krox 20, *Hox B9*, Muscle actin and Collagen Type II was analyzed. (B) Ectoderm was treated identically as in A except, stage 12 ectoderm was not treated since the explants were harvested at this stage to analyze expression of the early mesoderm markers, *Xbra* and *Goosecoid*. *EF-1 $\alpha$*  expression is shown as a loading control in both panels.

suggest that bFGF has direct neural inducing activity on stage 11-12 ectoderm, since it acts in the absence of mesoderm. Thus, while FGF can act as both a neural and mesoderm inducer on early gastrula ectoderm, it acts only as a neural inducer on later gastrula ectoderm.

### Combination of FGF and noggin generates a more complete neural axis

FGF induces posterior neural tissue (Fig. 1I) and noggin induces anterior neural tissue (Lamb et al., 1993). In combination, one factor might block the activity of the other, or the combination might generate neural tissues not induced by either factor alone. To address this, animal cap ectoderm aged to the early gastrula stage (stage 10.25) was treated with 2, 10, or 50 ng/ml of bFGF in the presence or absence of 1  $\mu$ g/ml noggin. Ectoderm was treated overnight and fixed for whole-mount in situ hybridization analysis at stage 20. Explants

treated with bFGF showed little morphological change, however in the presence of noggin, the increasing bFGF doses caused the explants to elongate (Fig. 3A-F). To assess the effects on neural patterning, we analyzed the expression of *otx2*, an anterior neural marker, and *Hox B9*, a posterior neural marker. Increasing the dose of bFGF caused increased expression of *Hox B9* (Fig. 3A-C). Noggin treatment induces *otx2* expression, however, as the bFGF dose increases, the fraction of the explant expressing *otx2* decreases with a concomitant increase in *Hox B9* expression. Thus, the ratio of anterior to posterior gene expression appears to be dependent on the ratio of the two factors. Interestingly, *otx2* and *Hox B9* are expressed in non-overlapping domains in the elongated bFGF- and noggin-treated explants. Thus, the combination of noggin and bFGF generates explants with anterior and posterior ends. Since the treatment with noggin and bFGF was simultaneous, it suggests that there is either some heterogeneity in cell response, or that the responding cells can self-organize anterior and posterior ends.

Since the middle region of the elongated structure expressed neither *otx2* nor *Hox B9*, it was possible that intermediate regions of neural tissues were induced. The expression of a hindbrain-specific gene, *Krox 20* (Wilkinson et al., 1989; Bradley et al., 1991) was analyzed in early gastrula staged ectoderm that was treated with bFGF, noggin, or noggin + bFGF and was cultured to an early tailbud stage (stage 24). The explants were simultaneously analyzed for muscle actin expression. Fig. 4A shows the two stripes of *Krox 20* expression in the hindbrain of the control embryo (purple), while muscle actin stains the developing somites (blue). Noggin-treated tissue expresses neither of these genes. bFGF-treated tissue expresses muscle actin (1 of 13 explants) and perhaps a low level of non-localized *Krox 20* (Fig. 4C; see also the RT-PCR analysis in Fig. 2, lane 5). The effect of noggin and bFGF combined is striking. *Krox 20* is induced and expressed in a stripe near the middle of the elongated tissue in some (10 of 13) explants (Fig. 4D). This region probably corresponds to the region that fails to express *otx2* or *Hox B9* (Fig. 3F). Thus, gastrula ectoderm can be induced by the combination of noggin and bFGF to form a hindbrain region that neither noggin nor bFGF induces alone.

The anterior-posterior pattern seen after combined noggin + bFGF treatment could be an indirect consequence of mesoderm contamination, since bFGF can induce muscle from ectoderm taken at stage 10.25. This was a special concern since noggin is a potent dorsalizer of ventral mesoderm (Smith et al., 1993). Thus, it was possible that noggin could act on the mesoderm induced by bFGF to increase the amount of muscle, which might secondarily induce or pattern neural tissue. In contrast to this possibility, we found that the neuralizing effect of

noggin dominated. Fig. 3H-K shows in situ hybridization to *nrp-1* and muscle actin. Explants treated with noggin alone show only *nrp-1* expression. Explants treated with bFGF alone show expression of both *nrp-1* and muscle actin. Explants treated with both noggin and bFGF are elongated and express *nrp-1* throughout the tissue, while completely lacking muscle actin expression. This suggests that noggin blocks the ability of bFGF to induce mesoderm at the gastrula stage, perhaps by neuralizing the tissue and changing its competence to respond to bFGF. This would allow bFGF to act solely to modify the type of neural tissue induced.

### The age of the ectoderm affects the A-P character of the neural tissue induced by bFGF (+/-noggin)

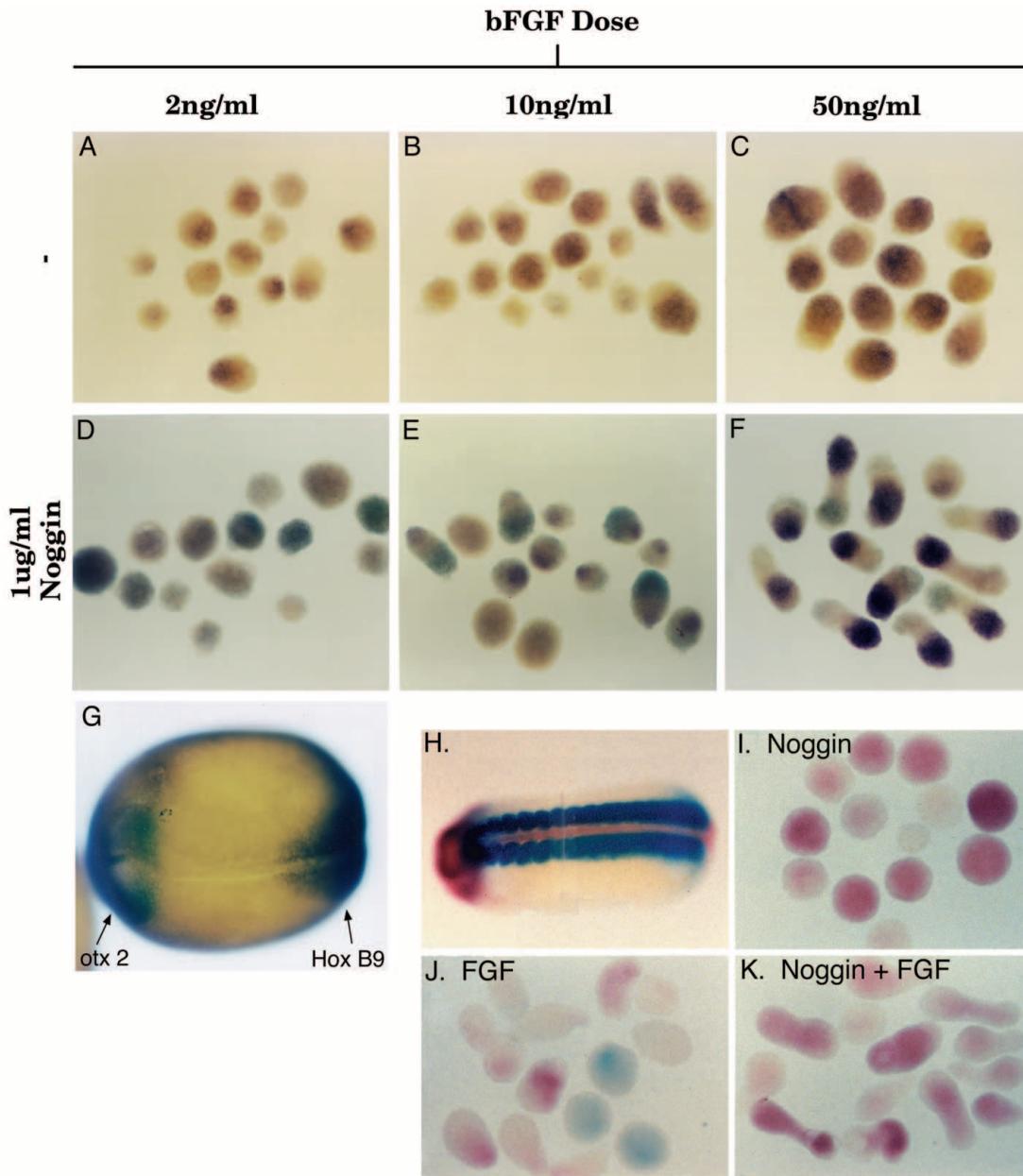
One model of neural induction and patterning proposes that the prospective neural ectoderm may respond to the same signal differently at different times (Doniach, 1993). A complex pattern could arise after exposure to one factor, since the tissue will activate different developmental pathways at different times. Fig. 2 shows that treatment with bFGF (with or without noggin) results in a variety of different outcomes depending on the stage of treatment (see Table 1 for a summary). Stage 10.25 ectoderm treated with bFGF expresses the posterior marker *Hox B9* (Fig. 2, lane 5) and weakly expresses *Krox 20*, but does not express the more anterior marker *En-2*. Thus, early gastrula ectoderm responds to bFGF by generating mainly posterior tissues. When stage 11 ectoderm is treated with bFGF (Fig. 2, lane 9), however, *En-2*, *Krox20* and *Hox B9* are all expressed, indicating the generation of a more complex neural pattern. Finally, bFGF treatment at stage 12 (Fig. 2, lane 13) induces *En-2* expression, but not *Krox 20* or *Hox B9*. Thus, there appears to be a posterior to anterior progression in the type of neural tissue induced by bFGF as the responsive tissue ages.

Addition of noggin to the bFGF treatments shifts the response so that at the stage 10.25 treatment (Fig. 2, lane 7), *En-2* and *Krox 20* are induced in addition to *Hox B9*, like the stage 11 ectoderm treated with bFGF alone. Although *Krox 20* expression in these explants can be localized to a stripe (Fig. 4D), *En-2* expression was not found in a localized pattern (data not shown). Additional signals may be required for the localized expression of *En-2*, and for the expression of *Krox 20* in two stripes. Stage 11 treatment (Fig. 2, lane 11) with noggin + bFGF results in *En-2* and *Krox 20*, but not *Hox B9* expression. This suggests that the presence of noggin hastens the loss of competence to generate posterior neural tissue. Treatment with noggin + bFGF at stage 12 (Fig. 2, lane 15) continues to induce *Krox 20*, as well as *En-2*, even though bFGF alone only induces *En-2* at this stage. Thus, noggin treatment may enhance and prolong the ectodermal com-

**Table 1. Effects of ectodermal age on anterior-posterior neural patterning by FGF and noggin**

	+ FGF				Noggin + FGF		
	St. 10.25	St. 10.5	St. 11	St. 12	St. 10.25	St. 11	St. 12
<i>otx2</i>	-	-	ND	ND	+	ND	ND
<i>En2</i>	-	-	+	+	+	+	+
<i>Krox20</i>	-/+	+	+	-	+	+	+
<i>Hox B9</i>	+	+	+	-	+	-	-

Data compiled from Figs 1, 2, and 5. (ND, not determined; +, expresses indicated marker, +/- weak expression, - no expression.)



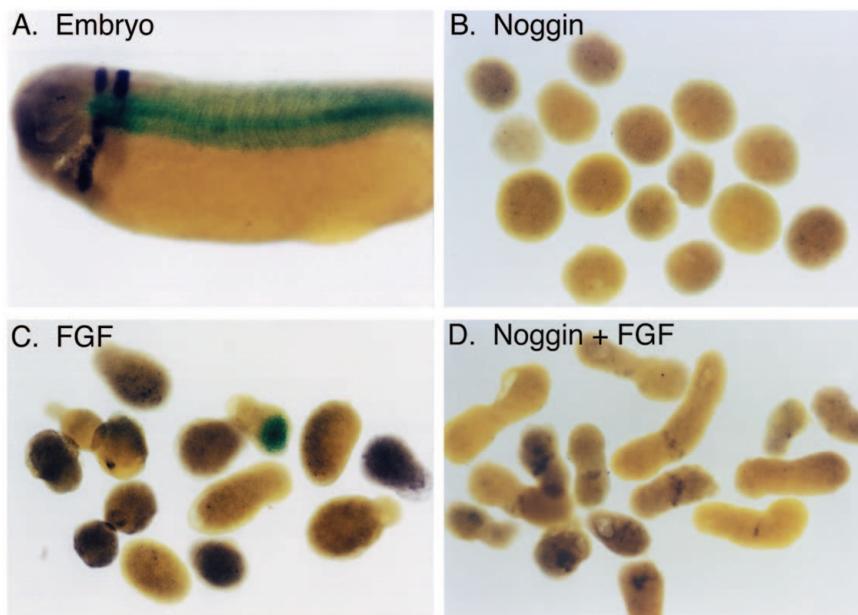
**Fig. 3.** Noggin + FGF generates neural tissue with anterior-posterior polarity. Ectoderm from stage 10.25 embryos was treated with increasing doses of bFGF (2 ng/ml, 10 ng/ml, 50 ng/ml) either in the absence (A-C) or presence (D-F) of 1 µg/ml noggin. Explants were cultured until control whole embryos (G) reached the late neurula stage (stage 18). Expression of *otx2* and *Hox B9* were analyzed by in situ hybridization. *Otx2* (light blue) is expressed at the anterior end, and *Hox B9* (purple) is expressed at the posterior end of the control embryo (G; the dark color at the anterior end is an effect of the embryo edge, and is not *Hox B9* staining). Increasing bFGF dose increases *Hox B9* expression (A-C). In the presence of noggin, increasing bFGF dose (D-F) causes the explants to elongate and to express *otx2* and *Hox B9* on either end of the elongated structure. Expression of a general neural marker, *nrp-1* (purple) and muscle actin (blue) was examined in whole embryos (H) and in explants treated with 1 µg/ml noggin (I), 50 ng/ml bFGF (J), and the combination of 1 µg/ml noggin and 50 ng/ml bFGF (K). Noggin alone induces *nrp-1* expression, bFGF alone induces both muscle actin and *nrp-1*, and the combination of the two induces only *nrp-1*, no muscle actin expression is observed.

petence to generate brain structures, while reducing competence to generate spinal cord.

#### FGF dose response

We carried out a dose-response analysis to determine the minimal dose of bFGF sufficient to induce neural tissue and to learn whether different doses of bFGF could induce neural

tissue with different anterior-posterior character. Stage 10.5 ectoderm was treated with increasing doses of bFGF and then cultured until stage 20, when explants were harvested for RT-PCR analysis. Fig. 5 shows that expression of the general neural marker *nrp-1* increases with the increasing bFGF concentration up to 50 ng/ml, which appears to induce neural tissue maximally. *Hox B9* is weakly induced by 2 ng/ml and



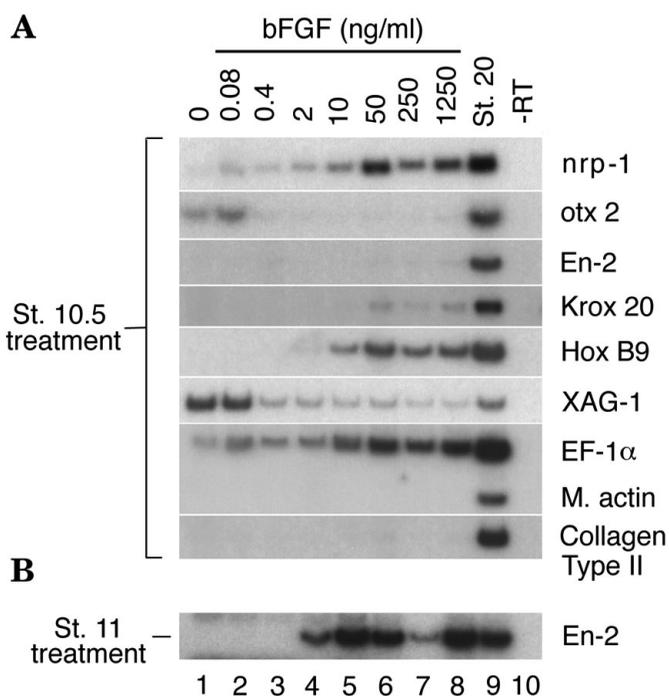
**Fig. 4.** Induction of hindbrain by noggin and FGF. Expression of Krox 20 (purple) and Muscle actin (blue) in stage 24 control embryos (A) and in stage 10.25 ectoderm treated with 1 µg/ml noggin (B), 50 ng/ml FGF (C), and the mixture of noggin and FGF (D) after culture to stage 24. Whole embryos express Krox 20 in two stripes in the developing hindbrain, while muscle actin is expressed in the somites. Noggin does not induce either gene. FGF can induce muscle actin (1/13 in this experiment); induction of muscle is highly stage-dependent and thus shows some variability between experiments (compare to Fig. 1). FGF may induce a low level of unlocalized Krox 20. FGF + noggin explants show Krox 20 expression localized to a stripe in the central region of the elongated structure, and no muscle actin expression.

Krox 20 is weakly induced by 10 ng/ml bFGF. Higher concentrations induce expression more strongly, but no dose induces En-2, and expression of the more anterior markers otx2 and XAG-1 are repressed by bFGF. Although there may be some effect of dose to differentially induce spinal cord (Hox B9) versus hindbrain (Krox 20), the effect is not clear since Krox 20 is a less abundant transcript. While stage 10.5 ectoderm is not competent to express En-2 in response to any dose of bFGF, stage 11 ectoderm expresses En-2 in response to a broad range of concentrations, starting at 2 ng/ml (Fig. 5B). This result strengthens the argument that increasing ectodermal age results in competence to generate progressively more anterior tissues. In conclusion, the anterior-posterior character of neural tissue induced by FGF appears to be primarily dependent upon the age of the tissue treated and not on the dose of FGF.

**DISCUSSION**

**FGF is a direct neural inducer**

The possibility that FGF could act as a neural inducer on *Xenopus* ectoderm was only recently suggested (Kengaku and Okamoto, 1993), since previous experiments mainly concentrated on the mesoderm inducing activity of FGF at the blastula stage. Our experiments show that bFGF induces the pan-neural markers NCAM and nrp-1 from mid- (stage 10.5-11) and late- (stage 12) gastrula ectoderm (Figs 2, 5), without inducing mesoderm either early, as marked by goosecoid and Xbra, or late, as marked by muscle actin or collagen type II. Although ectoderm isolated at stage 9 may have already received inducing signals, FGF clearly induces the expression of general and region-specific neural markers. If FGF were simply acting to differentiate cells that were already neuralized, expression of the general neural markers would not be induced above the level seen in untreated tissue. This suggests that bFGF is not causing differentiation of tissue that has already been neuralized, but rather it is an inducer of neural tissue. Also, because



**Fig. 5.** FGF dose response. (A) Stage 10.5 ectoderm was treated with increasing doses of bFGF (0.08, 0.4, 2, 10, 50, 250, 1250 ng/ml) and then cultured to the early tailbud stage (stage 20). RT-PCR analysis of nrp-1, otx2, En-2, Krox 20, Hox B9, Muscle actin, Collagen type II shows that when stage 10.5 ectoderm is treated with increasing amounts of bFGF, nrp-1 expression increases. At 2 ng/ml Hox B9 and 10 ng/ml Krox 20 are induced, while En-2 or otx2 is not induced by any dose, and the low level expression of otx2 seen in untreated explants is repressed by 0.4 ng/ml or more of bFGF. bFGF treatment on St 10.5 ectoderm did not induce muscle actin or collagen type II mesoderm, and repressed XAG-1 expression. EF-1α is a loading control. (B) When ectoderm is treated at stage 11, En-2 is induced by a wide range of doses, with 2 ng/ml being the minimal dose.

we have failed to detect the expression of any mesoderm-specific expression, it suggests that neural induction by bFGF is direct. This makes FGF a candidate to be one of the neural inducing signals released by dorsal mesoderm, especially since one form, eFGF, is expressed in dorsal mesoderm. Thus, by examining the expression of early markers of neural tissue and several markers of mesoderm, we have clarified the mechanism of neural induction by FGF initially proposed by Kengaku and Okamoto (1993).

At early gastrula stages, when the ectoderm is competent to form both mesoderm and neural tissue, bFGF can induce either type of tissue. The loss of ectodermal competence to form mesoderm in response to bFGF parallels closely the loss of competence to respond to activin. A previous comparison of the loss of competence to respond to activin or bFGF suggested that the ectoderm lost competence to respond to bFGF at the late blastula stage (Green et al., 1990). This may have been an effect of the assay used (tissue elongation) or the dose given. Here we have shown that animal cap ectoderm maintains sensitivity to bFGF, but progressively changes the way it responds.

### Mechanism of neural patterning

eFGF RNA, and probably protein, is localized to the posterior region of the developing body axis, so it is made in the right place to be involved in induction of the posterior neural plate (Isaacs et al., 1992). We have found that bFGF has neural inducing activity at the appropriate time. Although the dose of bFGF has little effect on the regional character of the inductions, the age (or competence) of the ectoderm has a profound effect (Fig. 6). bFGF induces early gastrula (stage 10.25) ectoderm to express only Hox B9, a marker of posterior neural tissue. Later treatment (stage 10.5) induces the more anterior marker Krox 20, as well as Hox B9. Treatment at stage 11 induces the expression of the even more anterior marker En-2, while Krox 20 and Hox B9 expression is maintained. However, the latest stage of ectoderm (stage 12) treated with bFGF expresses En-2, but posterior tissue is absent. A simplistic model emerges that could partially explain neural patterning in vivo. eFGF begins to be expressed at the start of gastrulation, with the highest expression at the posterior end. At this time, the ectoderm will respond to FGF by adopting a posterior neural fate. Secreted eFGF may diffuse from its originally posterior location, thus exposing progressively more anterior regions to the signal. While the signal diffuses, the ectoderm ages, and the competence of the ectoderm to respond to the signal changes. Thus, the region of the embryo that receives the signal late in gastrulation would respond by inducing the more anterior genes like En-2.

However, it is unlikely that FGF is the only factor involved in neural induction and patterning, since other factors with neural inducing capacity are known to be present. Although it is not clear what activity follistatin has on gastrula neuroectoderm, noggin is known to be present and is capable of acting as an anterior neural inducer (Smith and Harland, 1992; Lamb et al., 1993). We have shown that the combination of noggin and FGF generates a more complete range of neural tissues than either factor alone can induce from early gastrula ectoderm. However, the anterior-posterior neural axis generated is incomplete, since it does not display the full extent

of neural pattern seen in vivo. For example, Krox 20 is expressed in two stripes of the hindbrain in embryos, but when early gastrula ectoderm is treated with FGF and noggin, Krox 20 is only sometimes expressed in a single stripe. Furthermore, although the midbrain-hindbrain marker, En-2 is induced by the combination, it does not appear to be localised. Thus it seems that other factors would cooperate to produce a more complete pattern. Such factors may be identified by exploiting assays similar to the one used here.

The effect of ectodermal age on neural patterning has been addressed previously. One study with urodeles suggests that age has an effect on the anterior-posterior regional character of induced neuroectoderm (Nieuwkoop and Albers, 1990). Furthermore, changes in age have been observed to affect medio-lateral patterning of the neural plate and induction of lens and ear vesicles (Albers, 1987; Servetnick and Grainger, 1991). Another study with *Xenopus* (Sharpe and Gurdon, 1990) concluded that age had little effect on A-P pattern; however, only two neural markers that have some overlapping expression were examined. Since both genes shared a region of expression, a sharp change in competence to induce one neural region, and not the other, may have been difficult to detect. Although the effect that ectodermal age has on the anterior-posterior character of induced neural tissue in vivo is not entirely clear, the evidence does point to age as a factor in differential responsiveness. Thus, the change in the ectodermal response to FGF observed in our experiments may be relevant to in vivo neural patterning.

### Requirement for FGF signaling in neural induction or patterning?

There has been no appropriate test for the necessity of FGF signaling for neural induction or patterning. FGF gene disruptions in mouse result either in early death (FGF-4, Feldman et al., 1995) or in no major embryonic phenotype (FGF-5, Hebert et al., 1994; and FGF-3, Mansour et al., 1993). Overexpression of a dominant inhibitory FGF Receptor Type I in *Xenopus* (Amaya et al., 1991, 1993) or gene disruption of FGFR-1 (Yamaguchi et al., 1994; Deng et al., 1994) in mice have a similar phenotype of disrupting mesoderm formation and patterning. These studies show no defect in anterior neural tissue formation when FGF signaling is compromised. This suggests that if FGF signaling is important, it is probably only needed for posterior neural tissue formation. By injecting the dominant inhibitory FGF receptor RNA into the prospective posterior neural region in *Xenopus*, it will be possible to determine whether FGF receptor signaling is required. Whatever the result of this experiment, our results suggest that signalling by a receptor tyrosine kinase could be an important component of neural induction and patterning.

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