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Caudalization of neural fate by tissue recombination and bFGF

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SUMMARY

In order to study anteroposterior neural patterning in *Xenopus* embryos, we have developed a novel assay using explants and tissue recombinants of early neural plate. We show, by using region-specific neural markers and lineage tracing, that posterior axial tissue induces midbrain and hindbrain fates from prospective forebrain. The growth factor bFGF mimics the effect of the posterior dorsal explant in that it (i) induces forebrain to express hindbrain markers, (ii) induces prospective hindbrain explants to make spinal cord, but not forebrain and midbrain, and (iii) induces posterior neural fate in ectodermal explants neuralized by the dominant negative activin receptor and follistatin without mesoderm induction. The competence of forebrain explants to respond to both posterior axial explants and bFGF is lost by neural groove stages. These findings demonstrate that posterior neural fate can be derived from anterior neural tissue, and identify a novel activity for the growth factor bFGF in neural patterning. Our observations suggest that full anteroposterior neural patterning may be achieved by caudalization of prospective anterior neural fate in the vertebrate embryo.

Key words: neural patterning, induction, hindbrain, recombinant explants, bFGF, *Xenopus laevis*

INTRODUCTION

Development of the vertebrate central nervous system (CNS) is characterized by the formation of a neural tube with distinct anteroposterior (A-P) and dorsoventral (D-V) polarity. The origin of this pattern was much studied by classical embryologists, using a variety of approaches (reviewed in Hamburger, 1988), leading to the proposal of several models of regional specification along the A-P axis (reviewed in Doniach, 1993; Ruiz i Altaba, 1993). The common theme among all these models is that anteroposterior specification is imposed by the dorsal mesoderm that comes to underlie the presumptive neuroectoderm during gastrulation. A model proposed by Mangold suggests that qualitatively different regions of the dorsal mesoderm induce specific regions of the CNS in the directly overlying neuroectoderm (Mangold, 1933). Alternatively, the two-step activation-transformation model, suggested by Nieuwkoop et al. (1952) proposes that initially induced neuroectoderm is specified as forebrain, and that more posterior neural structures (midbrain, hindbrain, and spinal cord) are derived from the anterior neuroectoderm that falls under the influence of transforming signals (Nieuwkoop et al., 1952; Eyal-Giladi, 1954; Nieuwkoop and Nigtevecht, 1954). These two successive waves of activity, moving posterior to anterior through the neuroectoderm, first induce anterior neural fate and then modify it to generate A-P pattern (Eyal-Giladi, 1954; Toivonen and Saxen, 1968). A third model proposes that the activity of a single inducer, a low concentration of which induces spinal cord whereas a high concentration yields forebrain, gives rise to neural pattern (Dalcq, 1938). Finally, it has been proposed that the interaction of two inducers distributed along the A-P axis, in opposing gradients, patterns the neuraxis (Toivonen and Saxen, 1955; Saxen and Toivonen, 1961). In this scheme, the neural inducer, which is strongest anteriorly, induces forebrain, whereas the mesoderm inducer, which is highest posteriorly, acts with the neuralizing activity to specify more posterior neural fates such as spinal cord. While these models still guide our understanding of A-P neural patterning, the advent of region-specific neural molecular markers allows us to look more closely at how pattern is established.

The factors involved in defining specific regions of the embryonic CNS have only recently begun to be identified. Many members of the Wnt family of secreted polypeptide growth factors are expressed in the embryonic CNS (reviewed in McMahon, 1993) and mutation of the *Wnt-1* gene in mice disrupts development of the cerebellum (McMahon and Bradley, 1990). This provided the first direct evidence that growth factors are required for the formation of specific regions of the vertebrate CNS. *Dorsalin-1*, a member of the TGF-β family, regulates cell-type differentiation in the D-V axis of the neural tube (Basler et al., 1993). In amphibians, recent studies identified two secreted factors, noggin and follistatin, with direct neural inducing ability (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994). The neural tissue induced by these factors has anterior or forebrain characteristics. This is also true of neural tissue induced by cell dissociation (Grunz and Tacke, 1989; P. Wilson, personal communication) or by expression of a dominant negative activin receptor Δ1XAR1 (Hemmati-Brivanlou and Melton, 1994). Together, these studies suggest that rudimentary neural tissue is of anterior character, lending support to an activation-transformation model of patterning (Nieuwkoop et al., 1952).

In an effort to examine the molecular mechanisms control-

ling A-P neural specification, we have studied the expression of regional neural markers in explants and recombinants of the early neural plate which include axial mesoderm. We show that markers of midbrain and hindbrain are induced in explants of prospective forebrain by recombination with explants of posterior dorsal axis. The growth factor bFGF mimics the induction by the posterior explant, in that it induces the expression of a hindbrain marker in prospective forebrain. bFGF also caudalizes prospective hindbrain, inducing spinal cord. Furthermore, ectodermal explants neuralized by the dominant negative activin receptor and follistatin respond to bFGF by expressing hindbrain and spinal cord markers without mesoderm induction. Interestingly, the competence of the forebrain to respond to both posterior dorsal explant and bFGF is the same, ending at neural groove stages. These findings demonstrate that intermediate regions of the CNS can be induced by conjugation of the anterior and posterior ends of the neural plate and that this induction involves the caudalization of the prospective anterior neural tissue. Furthermore, our results identify a neural patterning activity of bFGF, the capacity to caudalize neural fate. Thus, these findings provide direct evidence that posterior neural fate can be generated from presumptive anterior neural tissue. We discuss our results in light of classical models of neural patterning in the vertebrate nervous system.

MATERIALS AND METHODS

Embryos, microsurgery and culture

Xenopus laevis embryos were obtained as previously described (Thomsen and Melton, 1993) and staged according to Nieuwkoop and Faber (1967). Removal of vitelline membranes and microsurgery of embryos was performed in 0.1× MMR using watchmakers forceps and eyebrow hair knives. Eagleson and Harris's early neurula fate map (Eagleson and Harris, 1989) was used as a guide in the removal of the neural plate and its subsequent subdivision into prospective forebrain, hindbrain, and spinal cord regions. At the early neurula stage 14, the embryo was oriented dorsal side up and the neural plate with the underlying dorsal structures was dissected by trimming around the outer edge of the neural folds with eyebrow hair knives. Thus, all these explants consist of the neural plate with underlying mesoderm and archenteron roof endoderm. With the explant ventral side down and flat to the dish, the prospective forebrain was removed with a transverse cut approximately 250 μm from the anterior edge of the explant. Prospective hindbrain was excised with a transverse cut approximately 500 μm from the anterior edge and 300 μm from the posterior edge of the neural plate. The prospective spinal cord was removed with a transverse cut approximately 250 μm from the posterior edge. The posterior dorsal mesoderm of prospective spinal cord explants was separated from the neuroectoderm using hair knives. Explants were transferred to 0.5× MMR, 0.1 mg/ml gentamycin and allowed to heal for 30 minutes at room temperature and then cultured at 18°C to sibling tailbud stage 28.

To make tissue recombinants, explants were transferred to an agarose-coated dish containing 0.5× MMR, where the two explants to be joined were oriented next to each other and maneuvered into a pit scooped out of the agarose with watchmakers forceps. The width of the pit was slightly smaller than the recombined tissues and was just deep enough to accommodate the tissue. The two explants were then placed into the pit snugly, and gently covered with a chip of cover slip. After being allowed to heal for 20 minutes, the cover slip was removed and the recombinants cultured to sibling stage 28. Dorsal-ventral orientation was maintained in all recombinants; anterior-posterior orientation was maintained in forebrain-spinal cord recombinants, whereas forebrain pairs were joined at their caudal ends and spinal cord pairs at their cranial ends. Forebrain-posterior dorsal mesoderm recombinants consisted of a piece of posterior dorsal mesoderm sandwiched between two prospective forebrains lacking the head mesoderm. Gastrula stage ectodermal explants were dissected at stage 10 and aged in 0.5× MMR containing 0.05 mM $MgCl_2$ and 0.1 mM $CaCl_2$ until sibling stage 10.5-11 (Nieuwkoop and Faber, 1967) before growth factor treatment. Growth factor treatment of explants was performed in 0.25× MMR, 0.5 mg/ml BSA. Purified recombinant human activin βA was provided by S. Sokol (Harvard) and purified recombinant *Xenopus* bFGF was provided by D. Kimelman (University of Washington, Seattle WA).

In vitro transcription and microinjection

RNAs encoding the truncated activin receptor (Δ1XAR1) and follistatin were generated as described previously (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou et al., 1994). Two-cell stage embryos were injected in the animal pole with 2 ng of either Δ1XAR1 or follistatin RNAs.

RT-PCR

The EF1-α (Krieg et al., 1989), *En*-2 (Hemmati-Brivanlou et al., 1991), *Krox-20* (Bradley et al., 1992), NCAM (Kintner and Melton, 1987), muscle actin (Stutz and Spohr, 1986), and *Xlhbox6* (Wright et al., 1990) oligos used for PCR were as described previously (Hemmati-Brivanlou and Melton, 1994). Oligos for *OtxA*: Upstream: 5′-CGGGATGGATTTGTTGCA-3′, position 252-269; Downstream: 5′-TTGAACCAGACCTGGACT-3′, position 435-452; (Lamb et al., 1993). RT-PCR was performed as described (Wilson and Melton, 1994) with the modification that first-strand cDNA synthesis was primed with random hexamers. PCR was performed as follows: first, a denaturation step of 95°C for 3 minutes; second, 95°C for 30 seconds; third, 60°C for 1 minute; fourth, 72°C for 30 seconds; fifth, repeat second, third and fourth steps 24 cycles; end with 72°C for 5 minutes. For EF1-α, a total of 21 cycles of amplification was performed. The positive control cDNA was prepared using 80% of the RNA isolated from a single whole embryo; the other 20% of the whole embryo was put into the negative control cDNA reaction which contained all of the first strand cDNA synthesis components except the reverse transcriptase.

Lineage tracing and in situ hybridization

For lineage tracing (Doniach and Musci, 1995), one-cell stage albino embryos in 3% ficoll, 0.5× MMR were injected in the animal pole with 5 nl of 25 mg/ml Cascade Blue (Molecular Probes no. D-1976). At neural plate stage 14, forebrain-spinal cord recombinants were made as before using a lineage-labeled spinal cord explant and an unlabeled forebrain explant. Whole-mount in situ hybridization was performed as described by Harland (1991) but using Genius blocking reagent (Boehringer-Mannheim) and the following modifications. At early tailbud stage, the recombinants and control forebrain explants were fixed in MEMFA for 30 minutes whereas middle piece explants were fixed for 1 hour and whole embryo controls for 2 hours. A sibling whole embryo was included with the control forebrain and spinal cord explants to serve as a positive control for the detection. Control forebrain and spinal cord explants and the recombinants were treated with proteinase K for 10 minutes; middle piece explants and whole embryos were treated for 20 minutes. Color development was performed using BM Purple (Boehringer-Mannheim) at 4°C for 48 hours. The color reaction was stopped by a 30 minute fixation in MEMFA. Immunohistochemistry was performed as described by Hemmati-Brivanlou and Harland (1989). The anti-cascade blue antibody (Molecular Probes no. A-5760) was used at a dilution of 1:500. The secondary

antibody was conjugated to Cy3 (Jackson Laboratories no. 111-165-144) and used at a 1:500 dilution.

RESULTS

Expression of regional specific markers in explants of the early neural plate

The basis of our assay is to examine the expression of A-P neural markers in explants and tissue recombinants of the early neural plate. The initial step in our approach was to assess the extent of regional specification in explants of the neural plate using region-specific molecular markers and RT-PCR. The fate map of the open neural plate (Eagleson and Harris, 1989) provided a guide to the regions of prospective forebrain, midbrain, hindbrain, and spinal cord. To show that prospective forebrain and spinal cord tissue could be cleanly separated from hindbrain at the early neural plate stage, the neural plate was separated into prospective forebrain (anterior), hindbrain (middle), and spinal cord (posterior) pieces (Fig. 1A). The explants used in this study are referred to as forebrain, hindbrain, and spinal cord, although they also contain the underlying axial mesoderm and the roof of the archenteron. The explants were cultured to early tailbud stages and the expression of region-specific neural markers examined by RT-PCR. These markers include: *OtxA*, which is expressed in the forebrain, midbrain, and eyes (Lamb et al., 1993); *Krox-20*, which is expressed in rhombomeres 3 and 5 of the hindbrain (Bradley et al., 1992); and *Xlhbox6 (HoxB9)*, which is expressed in the spinal cord and posterior lateral plate mesoderm (Wright et al., 1990). Fig. 1B shows that, in agreement with the fate map, the anterior piece of neural plate (lane 3) gave rise to forebrain, as evidenced by the expression of *OtxA* and the absence of *Krox-20* and *Xlhbox6*. Likewise, the posterior piece (lane 5) expressed the spinal cord marker *Xlhbox6* but not the two more anterior markers. Neither the anterior nor the posterior explant when cultured in isolation expresses the hindbrain marker *Krox-20*. The middle piece (lane 4) was found to express hindbrain-specific *Krox-20* and also *OtxA* and *Xlhbox6*. The latter two markers are expressed in the middle piece because midbrain and spinal cord tissue were intentionally included in the explant to ensure that the entire hindbrain region was excised. These data illustrate that at the early neurula stage, the *Krox-20*-expressing area of the hindbrain can be cleanly separated from the forebrain and spinal cord explants.

Recombination of prospective forebrain and spinal cord induces expression of the hindbrain marker *Krox-20*

To determine if it is possible to induce intermediate A-P neural fate from the most anterior and posterior regions of the neural plate, we used a tissue recombination assay and tested for the induction of the hindbrain marker *Krox-20* by RT-PCR. The neural plate of early neurula stage embryos was divided, as before, into prospective forebrain (anterior), hindbrain (middle) and spinal cord (posterior) regions, as shown in Fig. 2A. Explants of the prospective forebrain were then recombined with spinal cord explants such that the two pieces of tissue grew together. Control forebrain and spinal cord explants were cultured separately. When sibling embryos reached early tailbud stage, the recombinants and control explants were assayed for expression of the three regional neural markers. Fig. 2B shows that the forebrain-spinal cord recombinants express the hindbrain marker *Krox-20* (lane 5), whereas the control forebrain and spinal cord explants do not (lanes 3 and 4). These results demonstrate that conjugation of the prospective forebrain and spinal cord tissue causes respecification of the hindbrain.

To assess the specificity of the tissue interaction, forebrain-forebrain and spinal cord-spinal cord recombinants were assayed for *Krox-20* induction. Fig. 2C shows that the conjugation of forebrain to forebrain and spinal cord to spinal cord did not induce *Krox-20* expression whereas the recombination of forebrain to spinal cord did. Thus, the induction of hindbrain by tissue recombination requires the forebrain-spinal cord interaction.

Prospective forebrain tissue is responding to caudalizing signals

To identify the responding tissue in the forebrain-spinal cord recombinant, we performed lineage tracing combined with whole-mount in situ hybridization. The posterior explant, but not the prospective forebrain, in recombinants was derived from embryos that were injected during the first cell cycle with lineage tracer. At early tailbud stage, the recombinants and control explants were fixed and scored for the expression of the midbrain-hindbrain marker *Engrailed*-2 (*En*-2; Hemmati-Brivanlou and Harland, 1989). *En*-2 expression in controls and recombinants is shown in Fig. 3. The recombinants (n=22) showed a strong band or patch of *En*-2 expression in 80% of the cases (3E), while isolated explants never expressed the marker (3B,D). In addition, the lineage tracer revealed that *En*-2 expression in recombinants was always in the unlabeled forebrain component. Fig. 3F-H shows a typical recombinant with a patch of *En*-2 expression in the unlabeled anterior end. Interestingly, the *En*-2 staining was often separated from the posterior explant tissue by a region of *En*-2-negative unlabeled tissue (Fig. 3F-H). All of the middle piece explants showed a single band of *En*-2 staining away from the anterior edge (Fig. 3C), demonstrating the consistent accuracy with which the anterior explant is isolated. These data clearly show that conjugation of prospective forebrain and spinal cord explants induces the forebrain tissue, not the spinal cord, to take on intermediate neural fates that it would not assume in isolation. This suggests that the prospective forebrain tissue is responding to caudalizing signals present in the prospective spinal cord explant.

Posterior dorsal mesoderm alone has caudalizing activity

The prospective spinal cord explant which exhibited caudalizing activity contains posterior dorsal mesoderm. In order to test the source from which the signal emanates, we recombined the mesodermal portion (i.e. separated from the overlying neuroectoderm) of the posterior explants with prospective forebrain explants from which the head mesoderm had been removed. RT-PCR was performed when the recombinants reached early tailbud stage and we assayed for *OtxA*, *Krox-20*, the pan neural marker N-CAM (Kintner and Melton, 1987), and the dorsal mesoderm marker muscle actin (Stutz and Spohr, 1986). Fig. 4 shows that forebrain-posterior dorsal mesoderm recombinants express *Krox-20* (lane 5) while the

two tissues cultured alone do not (lanes 3 and 4). Control posterior dorsal mesoderm explants do not express N-CAM (lane 4), attesting to the fact that the overlying neuroectoderm was entirely removed. Although these results do not rule out a possible role of the presumptive posterior neuroectoderm in caudalization, they indicate that the posterior dorsal mesoderm alone can caudalize prospective anterior neural tissue.

bFGF mimics the activity of the posterior explant by inducing *Krox-20* expression in prospective forebrain

Since the lineage tracing experiment established that the anterior explant was the responding tissue in this induction, we wanted to know if factors present at gastrula and neurula stages could mimic the activity of the posterior piece. This approach

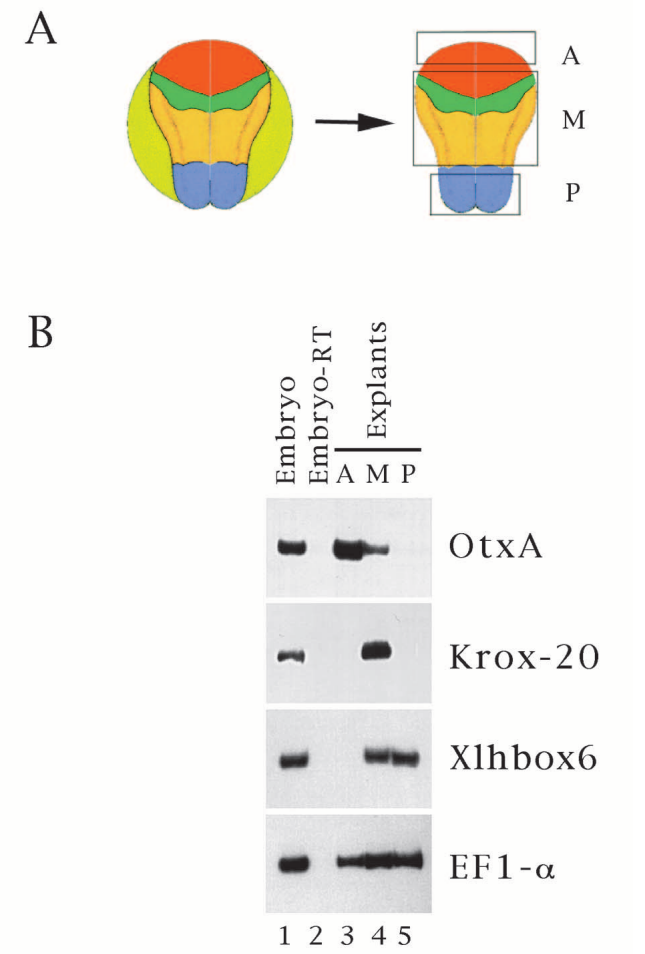

Fig. 1. RT-PCR detection of regionally expressed neural markers in neural plate explants. (A) Schematic diagram of an early neurula stage embryo (stage 14; red=forebrain, green=midbrain, yellow=hindbrain, blue=spinal cord) based on the fate map of Eagleson and Harris (1989). At stage 14, the neural plate was removed (including the underlying axial mesoderm) and divided into three pieces containing, prospective forebrain (A), hindbrain (M), and spinal cord (P) regions. (B) The explants were cultured to tailbud stage and then assayed by RT-PCR for the expression of the forebrain marker *OtxA*, the hindbrain marker *Krox-20*, and the spinal cord marker *Xlhbox6 (HoxB9)*. Lane 1: whole embryo (early tailbud stage). Lane 2: negative control containing all the reagents used in lane 1 except the reverse transcriptase. Lanes 3, 4 and 5 contain RNA derived from explants of presumptive forebrain, hindbrain, and spinal cord respectively. Detection of EF1-α shows that comparable amounts of total RNA were assayed in all of the reactions.

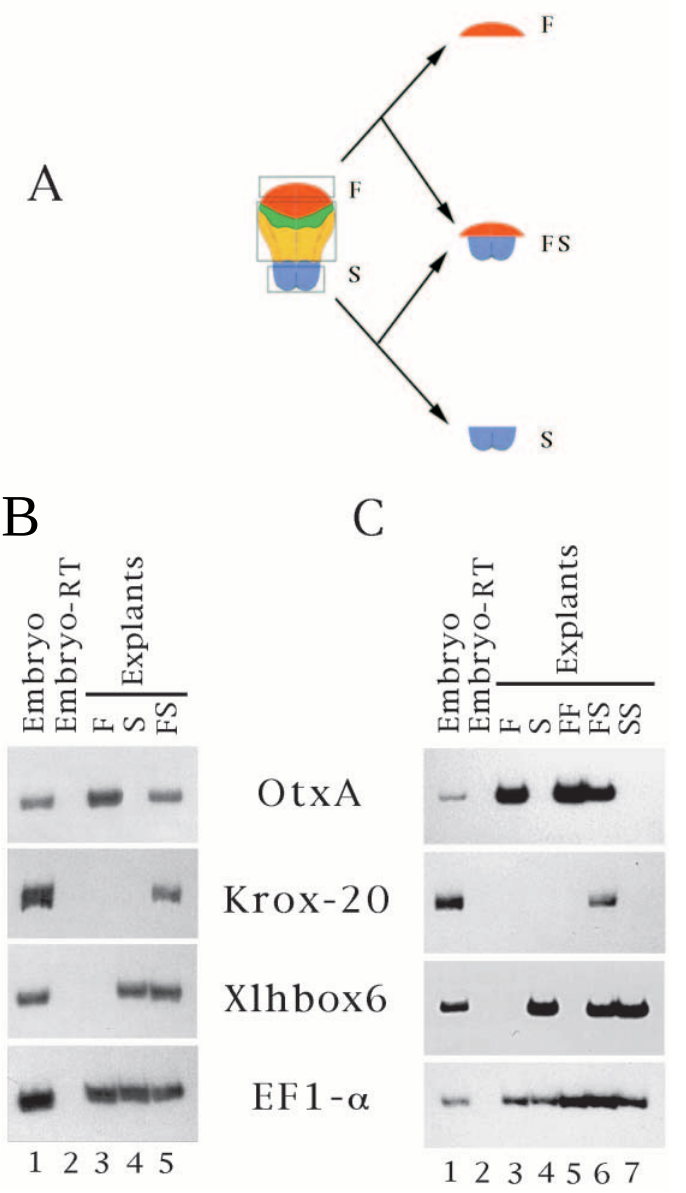

Fig. 2. Recombination of prospective forebrain and spinal cord induces expression of the hindbrain marker *Krox-20*. (A) Schematic diagram of the recombinant strategy used in these assays, prospective forebrain (F) is shown in red and spinal cord (S) in blue. (B) RT-PCR assay for the expression of region specific neural markers in explants and recombinants. *Krox-20* expression was not detected in the control forebrain (lane 3) or spinal cord (lane 4) explants but was expressed in the forebrain-spinal cord recombinants (lane 5). The negative control (lane 2) contained all of the RT-PCR ingredients except the reverse transcriptase. (C) Hindbrain marker induction requires the forebrain-spinal cord interaction. Explants of prospective forebrain and spinal cord regions of the neural plate were recombined forebrain-forebrain (FF), forebrain-spinal cord (FS), and spinal cord-spinal cord (SS) and cultured to tailbud stage for RT-PCR detection of *OtxA, Krox-20 and Xlhbox6. Krox-20* is only induced in the forebrain-spinal cord recombinants. Controls are as described in Fig. 1.

would parallel the one used to identify factors with mesoderm inducing ability. In that case, after it was established that the vegetal pole of the early blastula can induce mesoderm in competent ectoderm (Nieuwkoop, 1969), the animal cap alone was used to characterize mesoderm inducing factors. We thus used anterior explants to screen for factors with hindbrain inducing activity and began with purified bFGF and activin. Early neurula prospective forebrain explants were cultured with either bFGF (5 pg/ml to 5 ng/ml) or activin (0.2-8 ng/ml) and assayed for the expression of *OtxA*, *Xlhbox6*, and *Krox-20* at the early tailbud stage. The posterior piece was used as a control allowing us to discriminate between factors that could induce hindbrain from both tissues. As shown in Fig. 5A, the forebrain explants (lanes 4-7) responded to 5 ng/ml bFGF and expressed *Krox-20* (lane 7). Moreover, we observed a dose response effect such that higher concentrations of bFGF induced stronger *Krox-20* expression in forebrain explants (data not shown). Induction of the spinal cord marker was sometimes observed at 100-150 ng/ml bFGF but the results were not consistent. The explants of prospective spinal cord treated with the same concentrations of bFGF (lanes 9-12) did not express *Krox-20*. Control untreated forebrain and spinal cord explants did not express *Krox-20* (lanes 3 and 8), demonstrating again the accuracy of the dissections. Fig. 5B shows that no concentration of activin had *Krox-20* inducing ability in forebrain explants (lanes 3-7). These concentrations of activin were, however, able to induce mesoderm in animal cap explants demonstrating that our activin was active (data not shown). These results demonstrate that bFGF has a caudalizing effect on prospective forebrain tissue, thus mimicking the induction of hindbrain by the posterior explant.

bFGF also has a caudalizing effect on intermediate neural tissue, inducing spinal cord from hindbrain

In the experiments described above, we used the prospective forebrain to demonstrate the caudalizing activity of bFGF. Since the prospective forebrain explant represents the most anterior part of the neural plate it can be argued that this tissue can only be posteriorized and that bFGF may have a more general neural patterning effect. To test this possibility, explants of prospective hindbrain were treated with bFGF and examined for anteriorization and posteriorization by assaying for induction of *OtxA*, *En*-2 and *Xlhbox6*. The prospective hindbrain was excised from the early neural plate and cultured alone or treated with varying concentrations of bFGF (5, 50, 150 ng/ml). Fig. 6 shows that explants incubated alone expressed *Krox-20* but not *OtxA*, *En*-2 or *Xlhbox6* demonstrating the accuracy of the dissections. In the presence of bFGF (lanes 4, 5, 6), however, the hindbrain explants expressed the spinal cord marker *Xlhbox6* but not the forebrain and midbrain markers *OtxA* and *En*-2. Furthermore, as was the case with the forebrain explants, we note that the induction of posterior marker increased with higher concentration of bFGF. These results show that bFGF induces prospective hindbrain

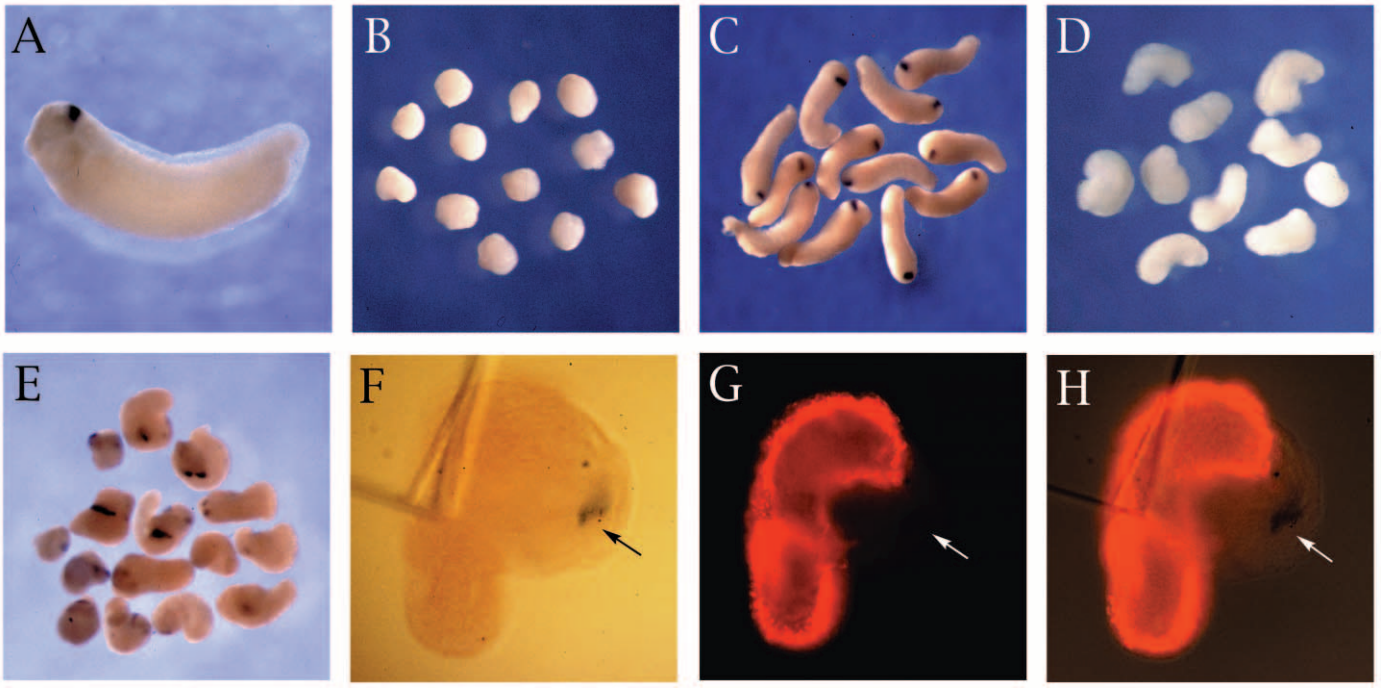

Fig. 3. Prospective forebrain is caudalized by forebrain-spinal cord recombination. Forebrain-spinal cord recombinants were made at the early neural plate stage using lineage-labeled prospective spinal cord explants and unlabeled prospective forebrain explants. The recombinants, control explants and whole sibling embryos were assayed at the early tailbud stage for *En*-2 expression by whole-mount in situ hybridization. Forebrain and spinal cord explants alone, as well as middle piece explants, from which the prospective forebrain and spinal cord regions were removed, served as controls for dissections. (A) Whole embryo. (B) Prospective forebrain explants alone. (C) Middle pieces. (D) Spinal cord explants alone. No *En*-2 expression was detected in the forebrain or spinal cord explants alone whereas whole embryos and the middle pieces showed the characteristic band in the midbrain-hindbrain border. Forebrain-spinal cord recombinants (E) displayed a band or patch of *En*-2 expression. F shows a typical recombinant and the detection of the lineage label is shown in G and H. The patch of *En*-2 staining (arrow) lies outside of the lineage label (red fluorescence, left side, G and H). A chip of cover glass, seen in the upper left, was necessary to position the explant. The *En*-2 staining was always localized to the unlabeled forebrain component of the recombinant.

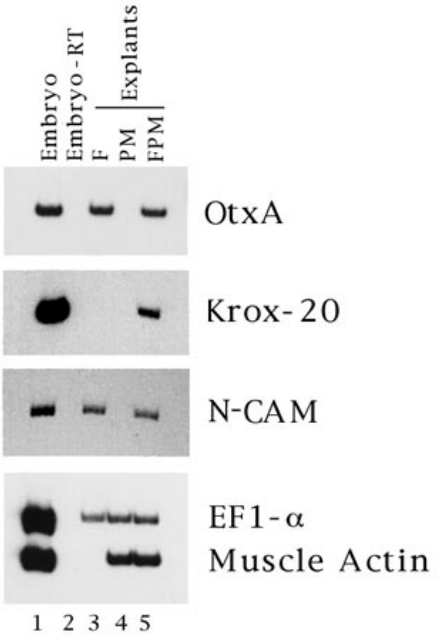

Fig. 4. Posterior dorsal mesoderm alone caudalizes prospective forebrain. Tissue explants are designated F for prospective forebrain, PM for posterior dorsal mesoderm, and FPM for the forebrain-posterior dorsal mesoderm recombinants. RT-PCR examination showed that *Krox-20* is induced in the recombinants (lane 5) but not in either tissue alone (lanes 3 and 4). Muscle actin is a marker of dorsal mesoderm whereas N-CAM is a pan-neural marker. Detection of EF1-α shows that comparable amounts of total RNA were assayed in all of the reactions. The negative control (lane 2) contained all of the RT-PCR ingredients except the reverse transcriptase.

to express a marker of more posterior fate, but not those of more anterior fate, thus demonstrating the true neural patterning activity of bFGF is to caudalize neural fate.

Ectodermal explants neuralized by Δ1XAR1 and follistatin can be caudalized by bFGF

We have demonstrated above that prospective forebrain explants can be caudalized by bFGF. In order to assess the potential contribution of the prechordal mesoderm in this patterning event, we tested ectodermal explants neuralized by Δ1XAR1 and follistatin for their ability to respond to bFGF. Both Δ1XAR1 and follistatin directly induce neural tissue (i.e. in the absence of mesoderm) that is anterior in character (Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou et al., 1994). Gastrula stage (stage 10.5-11) animal pole ectodermal explants expressing follistatin (Hemmati-Brivanlou et al., 1994) or Δ1XAR1were treated with or without bFGF and then assayed at tailbud stage for A-P neural marker expression by RT-PCR. We used gastrula stage explants because they are no longer competent to respond to the mesoderm inducing effect of bFGF. Ectodermal explants from uninjected embryos served as controls. Fig. 7 shows that in agreement with previous results (Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou et al., 1994) ectodermal explants from follistatin or Δ1XAR1 injected embryos express the anterior neural markers *OtxA* and *En*-2, but not the more caudal neural markers *Krox-20* and *Xlhbox6*. No muscle actin expression was

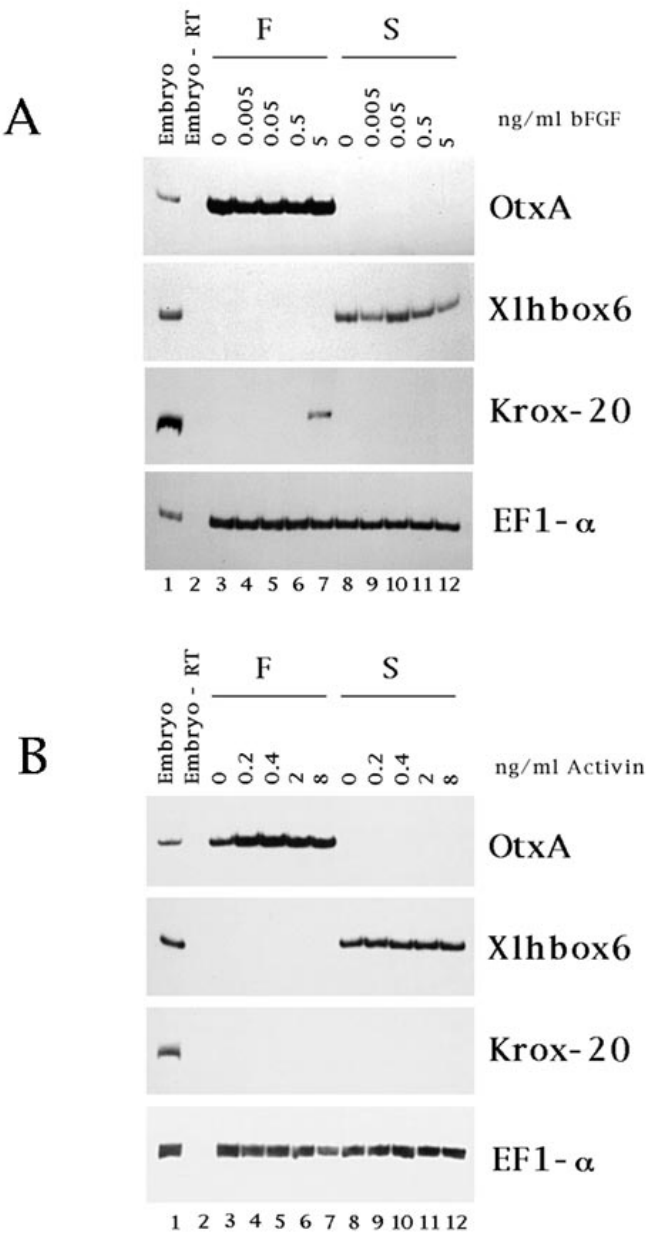

Fig. 5. Forebrain explants express *Krox-20* following treatment with bFGF but do not respond to activin. Early neurula prospective forebrain and spinal cord explants were treated with (A) bFGF (0.005-5 ng/ml) or (B) activin (0.2-8 ng/ml), cultured to tailbud stage, and then processed for RT-PCR detection of *OtxA, Krox-20, Xlhbox6* and EF1-α. (A) Treatment with 5 ng/ml bFGF induced *Krox-20* expression in forebrain explants (lane 7) while lower doses (lanes 3-6) did not. Posterior explants, treated and untreated, did not express *Krox-20* (lanes 8-12). (B) Activin treatment did not induce *Krox-20* in explants of prospective forebrain (lanes 3-7) or spinal cord (lanes 8-12). Detection of EF1-α shows that comparable amounts of total RNA were assayed in all of the reactions. The negative control (lane 2) contained all of the RT-PCR ingredients except the reverse transcriptase.

detected, verifying that the neural tissue was induced in the absence of dorsal mesoderm. In response to 5 ng/ml bFGF, *En*-2 expression was enhanced and *Krox-20* and *Xlhbox6*

expression was induced in follistatin and Δ1XAR1 explants (lanes 7 and 8). A similar but stronger effect was observed in the treatment with 50 ng/ml bFGF (lanes 10 and 11). Control explants, treated or not, did not express any of the neural markers (lanes 3, 6 and 9) and the lack of muscle actin expression in all of the explants verified that no dorsal mesoderm was induced. These data show that in the absence of mesoderm, neural tissue induced by follistatin and the dominant negative activin receptor, responds to the caudalizing effect of bFGF, expressing hindbrain and spinal cord markers.

The competence of forebrain to be caudalized ends by neurula

Inherent to any example of embryonic induction is the temporal competence of the responding tissue. We thus wanted to assess the competence of the forebrain explants to respond to caudalizing signals in the context of the recombinants and bFGF. Forebrain explants were taken at successive stages of development (open neural plate stage 14, neural groove stage 18, closed neural tube stage 20, tailbud stages 25 and 30, and tadpole stage 35) and either conjugated as heterochronic grafts to early neurula (stage 14) posterior explants or treated with bFGF (5 ng/ml). Explants were grown overnight and then assayed by RT-PCR for the expression of *Krox-20*. As shown in Fig. 8A, *Krox-20* expression was induced by spinal cord recombination in forebrain explanted at stage 14 (lane 5) but not from later stages (lanes 7, 9, 11, 13, 15). Fig. 8B shows that the competence of the prospective forebrain to be caudalized by bFGF is also present in stage 14 forebrain explants (lane 4) but not in any of the older stages. Thus, the competence of forebrain explants to become caudalized in response to either recombination with prospective spinal cord or bFGF exists at early neural plate stages and is lost by neural groove stage (stage 18), the time when neural folds in the trunk region are very close but not yet touching (Nieuwkoop and Faber, 1967).

DISCUSSION

We have described the use of tissue recombination to examine the specification of anteroposterior neural fate. Using this assay, we show that both explants of posterior dorsal axis and bFGF can caudalize anterior neural tissue. Prospective forebrain can give rise to midbrain and hindbrain and prospective hindbrain can give rise to spinal cord. Furthermore, ectodermal explants neuralized by Δ1XAR1 and follistatin can be caudalized by bFGF in the absence of mesoderm. The competence of forebrain explants to be caudalized in response to

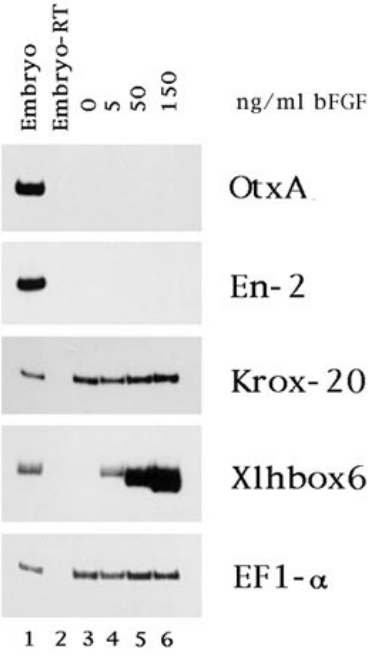

Fig. 6. Treatment of prospective hindbrain explants with bFGF induces spinal cord but not forebrain-midbrain marker expression. Explants of the prospective hindbrain were treated with bFGF (5, 50, 150 ng/ml) and at early tailbud stage assessed for anteriorization and posteriorization by detection of the forebrain and midbrain markers *OtxA* and *En*-2 and the spinal cord marker *Xlhbox6*. Treated explants (lanes 4, 5, 6) expressed *Xlhbox6* but not *OtxA* or *En*-2. Untreated explants (lane 3) did not express *OtxA*, *En*-2, or *Xlhbox6*. Detection of EF1-α shows that comparable amounts of total RNA were assayed in all of the reactions. The negative control (lane 2) contained all of the RT-PCR ingredients except the reverse transcriptase.

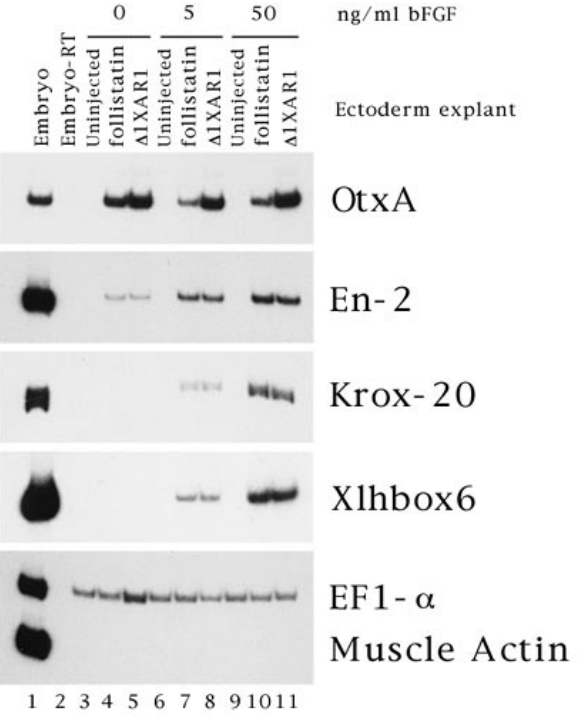

Fig. 7. Neural tissue induced by follistatin and dominant negative activin receptor is caudalized by bFGF. Gastrula stage (stage 10.5-11) ectodermal explants from uninjected embryos or those injected with 2 ng of either follistatin or dominant negative activin receptor (Δ1XAR1) RNA were treated with 0, 5, or 50 ng/ml bFGF, cultured to tailbud stage and then assayed by RT-PCR for the expression of A-P neural markers. Mesoderm induction was assayed by muscle actin expression. Levels of EF1-α shows that comparable amounts of total RNA were assayed in all of the reactions. The negative control (lane 2) contained all of the RT-PCR ingredients except the reverse transcriptase.

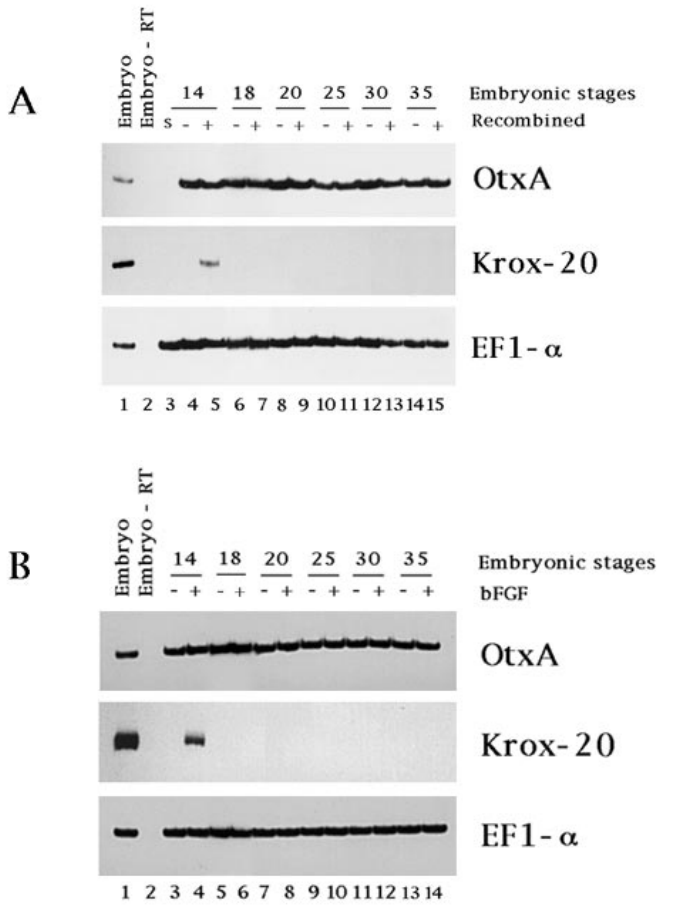

Fig. 8. The competence of forebrain explants to express *Krox-20* in response to prospective spinal cord explant recombination and bFGF. 14, 18, 20, 25, 30 and 35 are embryonic stages representing: open neural plate, neural groove, neural tube, tailbud and tadpole stages. (A) Competence of forebrain to respond to the prospective spinal cord in recombinants. s, spinal cord alone; +, recombinants; -, forebrain explants alone. (B) Competence of forebrain explants to respond to bFGF. +, forebrain cultured in the presence of bFGF at 5 ng/ml; -, forebrain explants cultured in buffer alone. Detection of EF1-α shows that comparable amounts of total RNA were assayed in all of the reactions. The negative control (lane 2) contained all of the RT-PCR ingredients except the reverse transcriptase. The competence of prospective forebrain explants to respond to either the posterior piece or to bFGF ends by neural groove stage (stage 18).

both posterior dorsal axis explant recombination and bFGF is lost by neural groove stage. These findings identify a novel neural patterning activity for bFGF and suggest that caudalization of an initial anterior neural fate may be a mechanism by which anteroposterior patterning is achieved in vivo.

Support for models of A-P neural patterning

The activation-transformation model proposed by Nieuwkoop et al. (1952) suggests that during gastrulation, initially induced neuroectoderm is specified as forebrain, and that only under the influence of subsequent modifying signals are more posterior neural fates generated, by transformation of anterior neural fate (Nieuwkoop et al., 1952; Eyal-Giladi, 1954; Nieuwkoop and Nigtevecht, 1954). In a series of experiments in which folds of competent ectoderm were inserted into the dorsal axis of gastrula and neurula stage newt embryos, Nieuwkoop et al. (1952) observed that the folds developed posterior to anterior neural characteristics along their proximal to distal axis. In addition, posterior folds displayed forebrain, hindbrain and spinal cord characteristics whereas anterior folds developed mostly anterior neural structures. Nieuwkoop interpreted these results in a two-step model involving an activation of the neuroectoderm followed by a transformation into cranio-caudal regional fates. Activation not only induces the neuroectoderm but also specifies it with anterior neural fate such as forebrain. The transforming action, which is strongest posteriorly, is superimposed on the activated neuroectoderm and modifies it to generate more posterior neural fates. Thus, without the influence of the transforming action, the activated neuroectoderm develops solely as forebrain. Recently, several treatments have been shown to induce neural tissue directly (i.e. in the absence of mesoderm induction; Grunz and Tacke, 1989; Lamb et al., 1993; Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou et al., 1994). All of these treatments, cell dissociation, noggin, follistatin, and the dominant negative activin type II receptor give rise to neural tissue which is anterior (forebrain) in character. No direct neural inducer isolated so far has the ability to induce posterior neural tissue such as spinal cord. These observations are consistent with the concept of the primary neural inducing signal (activation) in the Nieuwkoop model.

The results reported here demonstrate that it is possible to derive posterior neural fates from prospective anterior regions of neural tissue, thus providing evidence for the second signal (transformation). We find that recombination of anterior and posterior neural plate explants, containing prospective forebrain and spinal cord respectively, together with underlying mesoderm, induces intermediate regions of the CNS, midbrain and hindbrain. Lineage tracing combined with in situ hybridization proves that it is the prospective forebrain that responds to the recombination, indicating that posterior neural fates can be induced from prospective anterior neural tissue. The growth factor bFGF can mimic the effect of recombination, inducing prospective forebrain to express the hindbrain marker *Krox-20*. bFGF also induces explants of prospective hindbrain to express the spinal cord marker *Xlhbox6*, but not the forebrain-midbrain markers *OtxA* or the midbrain-hindbrain marker *En*-2, further illustrating that bFGF has a caudalizing effect on neural fate. Moreover, in the absence of mesoderm, neural tissue induced by follistatin and the dominant negative activin receptor responds to the caudalizing effect of bFGF, expressing hindbrain and spinal cord markers. Thus, the results presented here, together with the fact that all known treatments that induce neural tissue directly give rise to anterior fate, provides strong support for both steps of the activation-transformation model.

The posterior dorsal axis explant: a source of caudalizing signal(s)

We have established here that the posterior dorsal axis explant used in our recombinant assay has caudalizing activity. Since these posterior pieces include both ectoderm and axial mesoderm, the activity revealed by our experiments may originate in either tissue. These signals may in turn act directly on the forebrain explant neuroectoderm or indirectly through the underlying head mesoderm. The importance of axial

mesoderm in the A-P and D-V patterning of the early neural plate has been established by both classical and modern studies (e.g. Mangold, 1933; Holtfreter, 1936; Hemmati-Brivanlou et al., 1990; Yamada et al. 1993). bFGF mimics the caudalizing activity of the posterior axial explant. In the embryo, bFGF is present in the posterior explant at the time when our recombinants are made (Song and Slack, 1994). However, the role of bFGF as a signaling molecule, either in mesoderm induction or in the context of neural patterning, is clouded by the absence of a functional signal sequence for secretion (Kimelman and Maas, 1992). Thus, it is possible that in our assay bFGF mimics the activity of a related FGF factor. In agreement with this hypothesis, we note that XeFGF and FGF-3, both secreted factors, are strongly expressed in the posterior end of the embryo from gastrula stages onward (Isaacs et al., 1992; Tannahill et al., 1992). At the stages when our explants are made the strongest expression of both XeFGF and FGF-3 is in the mesodermal and ectodermal components of the posterior dorsal axis, suggesting that both germ layers might be involved.

Progressive determination of neural fate

Recent studies in *Xenopus* have indicated that neural determination along the A-P axis occurs progressively and becomes fixed at the early neural plate stage (Sive et al., 1989, 1990; Sharpe and Gurdon, 1990; Sharpe, 1991; Saha and Grainger, 1992). The competence of the ectoderm to respond to these signals also becomes temporally and spatially restricted (Sive et al., 1989; Sharpe and Gurdon, 1990; Saha and Grainger, 1992). Our findings illustrate that at the early neural plate stage, neural fate can still be modified toward a more posterior fate. Interestingly, however, we found that while it is possible to derive hindbrain from forebrain, we have not been consistently successful in obtaining spinal cord from the same tissue. Moreover, although we were able to induce midbrain and hindbrain from forebrain, and spinal cord from hindbrain, we could not generate anterior neural tissue. Thus, while it appears that neural fate along the A-P axis is not irreversibly determined at the open neural plate stage, we have only been able to show that it can be modified progressively in a posterior direction. These observations parallel those made in the chick embryo where it has been suggested that the expression of *En-2* in the midbrain-hindbrain region may be regulated by signals originating from more caudal tissue (Martinez and Alvarado-Mallart, 1990; Gardner and Barald, 1991).

In summary, our findings suggest that caudalization of prospective anterior neural fate may be a mechanism by which anteroposterior neural pattern is specified. Furthermore, members of the FGF family of growth factors may be involved in this modification of neural fate. These results provide evidence for the existence of the second type of signal in the activation-transformation model originally proposed by Nieuwkoop et al. (1952).

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