Interactions with mesoderm: neural induction

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Induction of anteroposterior neural pattern in *Xenopus* by planar signals

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Summary

Neural pattern in vertebrates has been thought to be induced in dorsal ectoderm by 'vertical' signals from underlying, patterned dorsal mesoderm. In the frog *Xenopus laevis*, it has recently been found that general neural differentiation and some pattern can be induced by 'planar' signals, i.e. those passing through the single plane formed by dorsal mesoderm and ectoderm, without the need for vertical interactions. Results in this paper, using the frog *Xenopus laevis*, indicate that four position-specific neural markers (the homeobox genes *engrailed-2(en-2)*, *XlHbox1* and *XlHbox6* and the zinc-finger gene *Krox-20*) are expressed in planar explants of dorsal mesoderm and ectoderm ('Keller explants'), in the same anteroposterior order as that in intact embryos. These genes are expressed regardless of convergent extension of the neurectoderm, and in the absence of head mesoderm. In addition, *en-2* and *XlHbox1* are not expressed in ectoderm when mesoderm is absent, but they and *XlHbox6* are expressed in naïve, ventral ectoderm which has had only planar contact with dorsal mesoderm. *en-2* expression can be induced ectopically, in ectoderm far anterior to the region normally fated to express it, suggesting that a prepattern is not required to determine where it is expressed. Finally, the mesoderm in planar explants expresses *en-2* and *XlHbox1* in an appropriate regional manner, indicating that A-P pattern in the mesoderm does not require vertical contact with ectoderm. Overall, these results indicate that anteroposterior neural pattern can be induced in ectoderm soley by planar signals from the mesoderm. Models for the induction of anteroposterior neural pattern by planar and vertical signals are discussed.

Key words: *Xenopus laevis*, *engrailed-2*, *XlHbox1*, *XlHbox6*, A-P patterning, Keller explants, neural induction.

Introduction

The concept that neural development is induced in the ectoderm by the dorsal mesoderm originated from the famous 'organizer' transplantation experiment performed by Hilde Mangold in 1921 (Spemann and Mangold, 1924). In this experiment, the dorsal lip of the blastopore of an amphibian (urodele) early gastrula was found to induce a secondary nervous system in ventral ectoderm of another gastrula. The dorsal lip tissue, composed of dorsal mesoderm, was named the organizer because the secondary axis displayed large-scale anteroposterior and dorsoventral organization. At the time, Spemann proposed two routes by which the inductive signals from the dorsal lip could reach the ectoderm: the planar route, in which signals pass within the continuous plane of tissue (also referred to as tangential or horizontal induction), or the vertical one, in which they pass to the overlying ectoderm after the dorsal lip tissue has involuted (Spemann, 1938).

Support for vertical induction

In 1933, Holtfreter found evidence in favor of the vertical route of induction and against the planar route, observing that urodele exogastrulae lack any histological evidence of neural differentiation. In these abnormal embryos, the mesoderm and endoderm do not involute, but move outwards during gastrulation, thereby precluding vertical interactions while maintaining planar ones. Vertical induction received further support when Otto Mangold (1933) found that dorsal mesoderm (the 'chordamesoderm', or presumptive notochord mesoderm) from early urodele neurulae could induce neural development in ventral ectoderm when inserted into the blastocoel of an early gastrula. Moreover, Mangold discovered that different regions of chordamesoderm along the anteroposterior (A-P) axis generally induced neural tissue of an equivalent A-P level. This led to the model that the chordamesoderm itself contains A-P pattern information in the form of regionalized inducers which induce a parallel pattern in the overlying ectoderm. Many workers have since found that chordamesoderm from different A-P regions of the late gastrula and early neurula can induce different A-P levels of neural differentiation in ectoderm that has been wrapped around it (for examples see Ter Horst, 1948; Sala, 1955; Sharpe and Gurdon, 1990; Hemmati-Brivanlou et al., 1990; Saha and Grainger, 1992). Taken together, the evidence for vertical induction and against planar induction led to common acceptance of ver-

tical induction as the main pathway of neural induction (Spemann, 1938; Hamburger, 1988).

Planar induction revived

It was not until the advent of molecular markers of early neural development that planar neural induction re-emerged as a possibility. Kintner and Melton (1987) made the surprising discovery that *Xenopus laevis* exogastrulae express NCAM, a gene specific to neural tissue at the stages examined. Using in situ hybridization, they found that NCAM RNA was present in the ectoderm next to the evaginated mesoderm. Subsequently, Dixon and Kinter (1989) and Keller et al. (1992b) found that planar explants of dorsal mesoderm and ectoderm ('Keller' explants, see below) express NCAM and another general neural marker NF3, and Savage and Phillips (1989) found that Epi-1, an epidermal marker, is turned off in ectoderm that has had only planar contact with dorsal lip mesoderm. Also, the convergent extension movements associated with the neurectoderm are induced by planar contact with mesoderm (Keller and Danilchik, 1988, Keller et al., 1992b, c). It should be pointed out that this new work has been carried out on *Xenopus* embryos, not on the urodele embryos used by the classical embryologists. It remains to be determined at a molecular level whether planar neural induction occurs in urodeles.

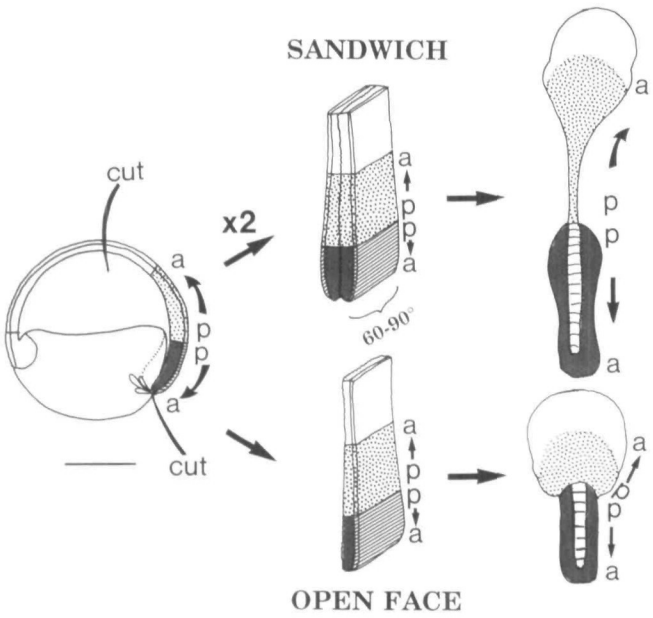


Fig. 1. Regions of early gastrula used to make Keller explants. On left is a stage 10+ embryo in sagittal cross section, dorsal to the right, animal pole up. The shading approximates tissue types predicted by the fate map (Keller, 1975, 1976; Keller et al., 1992a): the animal (upper) hemisphere consists of ectoderm that will give rise to epidermis (white) and neurectoderm (light stippling). Dorsal mesoderm is shown in dark grey and archenteron roof endoderm is striped. Explants were made by cutting out a rectangle of tissue reaching from approximately the animal pole the blastopore, as indicated by the upper and lower 'cuts' respectively, and about 60-90° wide around the equator. The head mesoderm, bounded by the dotted line and the dorsal mesoderm, was removed from explants. The predicted A-P polarity of the mesoderm and ectoderm is indicated. Sandwich explants were made by putting two of these rectangles together with their inner surfaces apposed; these undergo convergent extension (narrowing and elongating) in both the posterior neurectoderm and the meso-endoderm. Open face explants undergo convergent extension only in the meso-endoderm. The layer of endoderm is not shown in the explants depicted on the right. In these, the white column down the center of the mesoderm represents the notochord. Scale bar: 500 μm.

In *Xenopus* at least, these findings support a role for planar signals in the induction of markers of general neural differentiation, but do not indicate whether A-P pattern is established in this neural tissue. A first indication that some neural pattern may be induced by planar signals came when Ruiz i Altaba (1990) found that exogastrulae express the homeobox gene *Xhox-3* in a restricted pattern in the ectoderm. This gene is normally specifically expressed in the midbrain-hindbrain area at the stages studied. However, with a single marker it was not possible to determine if there was A-P polarity to this neural pattern. Also, results from exogastrulae are difficult to interpret because it is hard to be sure that vertical interactions have not occurred during exogastrulation, since the internal movements of the mesoderm cannot be followed.

In this paper, the expression of four position-specific neural markers has been examined in Keller explants to investigate whether planar signals can induce A-P neural pattern. Keller explants are explants of dorsal mesoderm and ectoderm, made before mesodermal involution (and therefore prior to any vertical interactions), and cultured flat throughout gastrulation and neurulation. Extensive experiments have shown that vertical interactions do not occur in these explants (Keller et al., 1992a,b). The results of the present study (see also Doniach et al., 1992) show that the four genes, *engrailed-2* (Hemmati-Brivanlou and Harland, 1989), *Krox-20* (Wilkinson et al., 1989; Bolce et al., 1992), *XlHbox1* (Oliver et al., 1988) and *XlHbox6* (Wright et al., 1990), each expressed in a specific region along the A-P axis of normal embryos, are expressed in Keller explants in the normal A-P order. These and other experiments presented here indicate that planar signals can induce A-P neural pattern. In addition, there is evidence for autonomous A-P pattern in the mesoderm of these explants.

Materials and methods

Culture of embryos and explants

Eggs were fertilized, dejellied and cultured in 33% modified Ringers (MMR) as in Vincent et al. (1986). All embryos used were albinos. Explants were made in Sater's modified Danilchik's medium (SMDM; A. Sater, R. Steinhardt and R. Keller, personal communication), pH 8.1, using an eyebrow hair knife and a hair loop, and cultured in plastic tissue culture dishes in SMDM until controls reached stage 22-27 (as indicated). Culture temperatures varied from 15°C to 23°C, depending on the rate of development that was required. Embryos were staged according to Nieuwkoop and Faber (1967).

Keller explants

Explants were made at stage 10 to 10+, using regions of

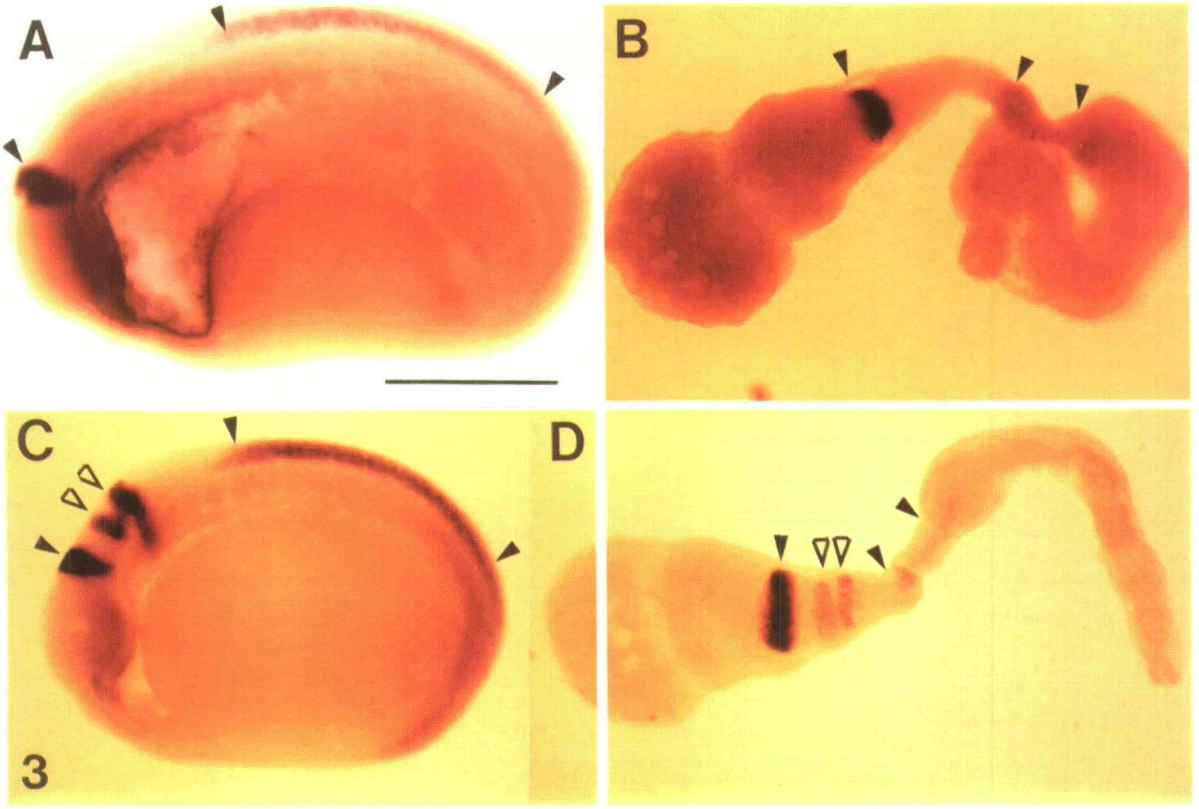


Fig. 3. Double and triple detection of *en-2*, *Krox-20* and *XlHbox6* by whole-mount in situ hybridization in whole control embryos (left side) and sandwiches (right side). Orientation is as in Fig. 2. Digoxigenin probes for the markers were hybridized simutaneously to determine whether the bands of expression seen in Fig. 2 are spatially separate. (A,B) *en-2* and *XlHbox6* expression in stage 22 control embryo and Keller sandwich, respectively. The RNA signal is indicated with arrowheads. There are two distinct regions of expression; comparison with patterns in Fig. 2 implies that the expression in the flared region of the neurectoderm is *en-2* and that in the neck region is *XlHbox6*. There is additional staining in the anterior mesoderm (not marked); this is *en-2* expression (it is also seen in sandwiches hybridized with *en-2* alone but not with *XlHbox6* alone. In A, there is background staining in the archenteron often seen with this technique (Harland, 1991). In B, the mesodermal portion of the sandwich has curled around; this occurred shortly before fixation at stage 22. (C,D) Expression of *en-2*, *Krox-20* and *XlHbox6* in control embryo and Keller sandwich, respectively. Filled arrowheads: *en-2* and *XlHbox6*; open arrowheads: *Krox-20*. The relative position of *Krox-20* expression is confirmed in D by the absence of these bands in B (in situ hybridization in D was kindly carried out by Dr. R Harland). Scale bar 500 μm.

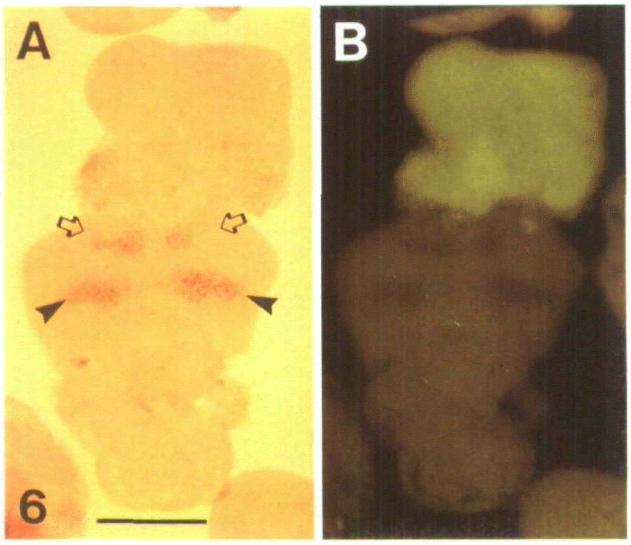


Fig. 6. Ectopic induction of *en-2* expression: Recombinate of FDA-labelled dorsal mesoderm grafted onto the anterior ectoderm of an open-face Keller explant, immunostained for *en-2*. (Orientation: labelled mesoderm, anterior up, posterior down; unlabelled tissue: animal end up, vegetal down, as in Fig. 1) *en-2* expression is in two regions of the unlabelled portion: a 1° region (filled arrows) 2° region (open arrows). Scale bar 250 μm.

the embryo illustrated in Fig. 1. Loose, potentially migratory head mesoderm was gently picked off with an eyebrow hair. Sandwich and open-face explants were then cultured flat under pieces of coverslip resting on silicone vacuum grease (Dow-Corning).

Planar recombinates of mesoderm with ectoderm

In SMDM, the freshly cut edge of a rectangle of dorsal mesoderm from an FDA-labelled (see below) embryo was gently pressed against the freshly cut edge of a piece of ectoderm for a few seconds, and the graft was allowed to heal for 5 minutes. The recombinate was then lightly flattened with a piece of coverslip resting on vacuum grease, cultured in MDM until controls reached stage 22-26, and immunostained as described below.

FDA labelling

Mesoderm donors were lineage labelled by injecting 8 nl of 25 mg/ml fluorescein dextran amine (FDA, a gift of R. Gimlich; Gimlich and Braun,1985) into stage 1 embryos using an air pressure injection system (Tritech Research, Los Angeles) and cultured in the dark throughout development and fixation.

Whole-mount immunocytochemistry and in situ hybridization

Embryos and explants were processed for whole-mount immunocytochemistry (Hemmati-Brivanlou and Harland, 1989), using horseradish peroxidase-tagged 2° antibodies (Bio-Rad or Jackson Immunoresearch), with shortened washes (3×30 minutes) and 0.05% $NiCl_2$ in the diaminobenzidine solution to enhance signal, except when detecting NCAM and 12-101. Whole-mount in situs were done as described in Harland (1991). Digoxigenin-labelled RNA probes were kindly provided by R. Harland and T. Lamb, and the Krox-20 clone was a generous gift of D. Wilkinson. Sequences used as probes are as described in Bolce et al. (1992).

Results

Keller explants

Two types of Keller explants were used (Fig. 1): 'sandwich' explants, in which two sheets of dorsal mesoderm (each covered with a layer of endoderm) and ectoderm are sandwiched together, inner surfaces apposed and 'open-face' explants, in which a single sheet is cultured alone. The involuted head mesoderm (which has not yet come to underly the presumptive neurectoderm) is removed from these explants to avoid potential vertical interactions that might occur, should these highly migratory cells (Winklbauer, 1990; Winklbauer et al., 1991) crawl onto the inner surface of the ectoderm. Keller explants are cultured flat under a coverslip to prevent mesodermal involution. In the absence of such involution, the A-P axes of the mesoderm and ectoderm in these explants point in opposite directions, with the posterior ends of both tissues at their common boundary (Keller, 1975, 1976). In both types of explant, the meso/endodermal portion undergoes essentially normal convergent extension movements (Keller and Danilchik, 1988; Wilson and Keller, 1991), causing elongation along the A-P axis. In sandwiches that are correctly made (i.e. those in which mesoderm has not involuted or migrated between the two sheets of ectoderm (Keller et al., 1992a,b)), the ectodermal portion also converges and extends, with the greatest amount occurring in the presumptive spinal region, producing a long, skinny 'neck'. Progressively less convergent extension occurs in the presumptive hindbrain and midbrain regions, resulting in a gradual widening of the explant towards the anterior end. There is no convergent extension in the ectoderm of open-face explants. Explants that showed irregular or asymmetrical convergent extension were either discarded or not counted in the experiments.

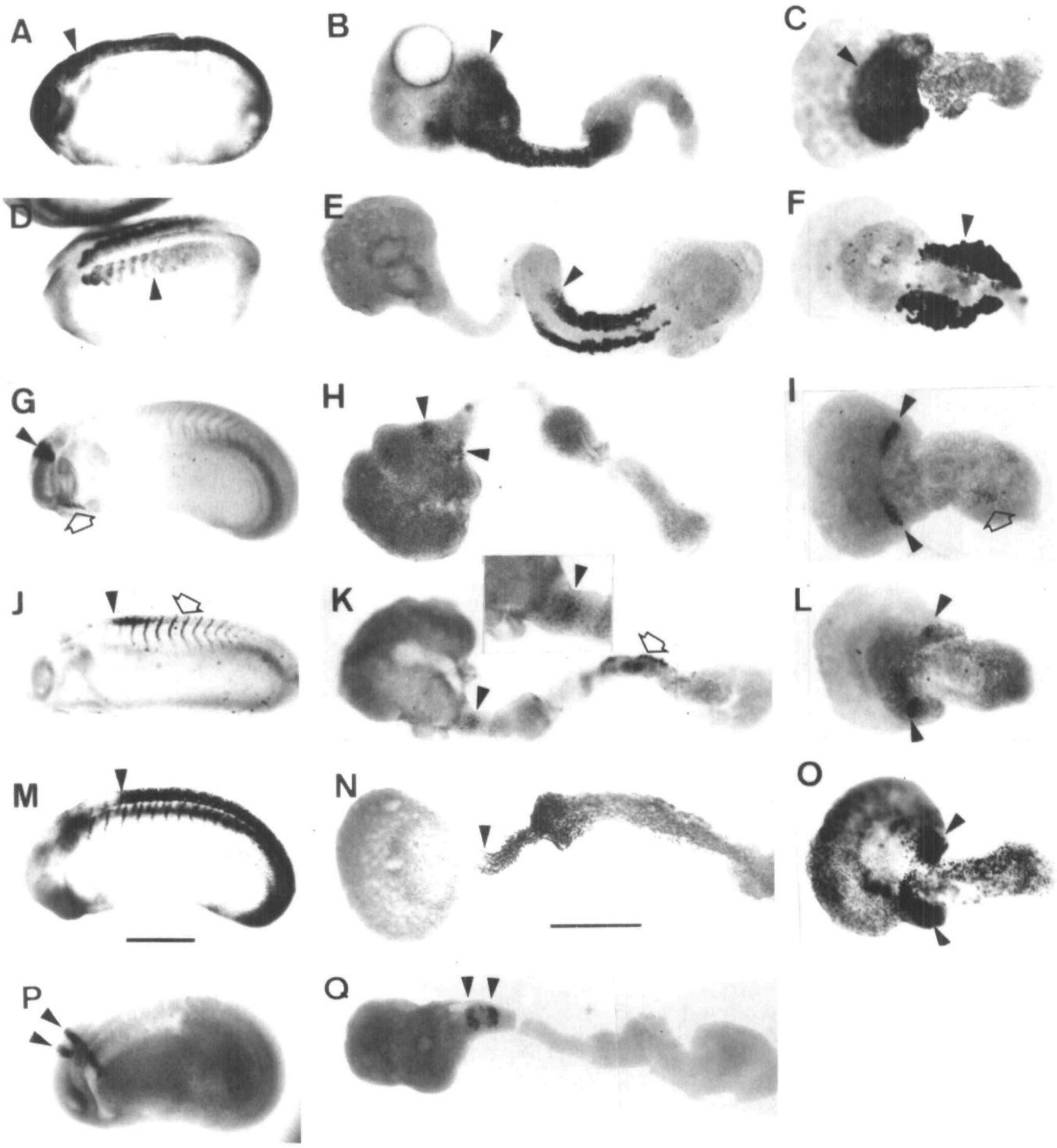
Domains of neural and mesodermal tissue in Keller explants

Dixon and Kintner (1989) showed by RNAase protection assays that there is expression of a general neural marker, NCAM, in Keller sandwiches. When anti-NCAM antibodies (Jacobson and Rutishauser, 1986) are used to stain Keller sandwiches, NCAM protein is detected in the neck region of the ectoderm and more anteriorly, in the flared region beyond the neck (Fig. 2B; Keller et al., 1992b). This defines the neural domain in Keller sandwiches, since NCAM expression is restricted to neural tissue at this stage (Balak et al., 1987; Fig. 2A). NCAM is also expressed in open-face explants (Fig. 2C). Somitic mesoderm, which can be identified using a monoclonal antibody, 12-101 (Kintner and Brockes, 1984), is organized in explants in two blocks flanking the notochord (Fig. 2D-F). The notochord can be identified morphologically, forming a distinctive column of large vacuolated cells.

There is A-P neural pattern in Keller explants

Immunostaining and in situ hybridization were used to localize the expression patterns of three homeobox genes, *en-2*, *XlHbox1* and *XlHbox6*, and a zinc-finger domain encoding gene, *Krox-20* in Keller explants. In intact embryos, these genes are expressed at specific A-P regions in the developing central nervous system (CNS): *en-2* is expressed at the midbrain-hindbrain junction (Fig. 2G; Hemmati-Brivanlou and Harland, 1989); *Krox-20* in the third and fifth rhombomeres of the hindbrain, with expression in the fifth reaching into the neural crest (Fig. 2P; Wilkinson et al., 1989 and Bolce et al., 1992); *XlHbox1* in a broad band in the anterior spinal region (Fig. 2J; Oliver et al., 1988) and *XlHbox6* throughout the spinal region and tailbud (Fig. 2M; Wright et al., 1990). They are also expressed outside the CNS, as will be described later.

These genes are expressed in Keller sandwiches in the normal A-P order: *en-2* protein is detected in the flared region anterior to the narrow 'spinal region' (Fig. 2H); *Krox-20* RNA in two bands posterior to this, still anterior to the spinal region (Fig.2Q); *XlHbox1* protein at the anterior end of the spinal region (Fig. 2K), and *XlHbox6* protein throughout the length of the spinal region, and extending into the posterior end of the mesoderm (Fig. 2N). This latter region consists of posterior mesoderm and presumptive posterior neurectoderm (Keller et al., 1992a,b). The order of expression can be seen more clearly when two (Fig. 3A,B) and three (Fig. 3C,D) of the markers are detected in



the same sandwich. A majority of the explants expressed these markers (95% for *en-2* (n=39); 64% for *XlHbox1* (n=14); 95% for *Krox-20* (n=22); 91% for *XlHbox6* (n=23)), and all of those that were positive showed expression in the respective patterns described above, although there was some variation in the intensity of the signal. This variation could reflect a difference in amount of expression, or in the sensitivity of the detection method.

A potential problem with Keller sandwiches is that expression of the neural markers could result from mesoderm on one face of the sandwich vertically inducing ectoderm on the other face if the two faces are misaligned. This is avoided in open-face explants. In these, all three homeobox genes are expressed: *en-2* and *XlHbox1* each in bilateral stripes a short distance from the mesoderm (Fig. 2I,L, and *XlHbox6* in larger bilateral regions next to and probably extending into the posterior mesoderm/tailbud region (Fig. 2O; *Krox-20* was not tested). Thus, in the absence of the potential vertical interactions that could occur in sandwiches, these genes are still expressed.

Fig. 2. Expression of neural markers in whole embryos and Keller explants. Left panel: whole embryos, stage 22-26 lateral view, anterior left, dorsal up. Center panel: sandwiches, dorsal view, animal pole left. Right panel: open-face explants, dorsal view, animal pole left. Scale bars 500 μm; scale bar in M applies to examples in the left panel, and that in N, center and right panels. Solid arrowheads point to antigen staining in neurectoderm, open arrows indicate putative mesodermal staining. (A,B,C) NCAM detected with rabbit polyclonal antibodies to the *Xenopus* 180×10^3 M_r isoform (a gift of U. Rutishauser; Jacobson and Rutishauser, 1986), assayed at stage 22. (D,E,F) Muscle detected with 12-101, a monoclonal antibody to a skeletal muscle-specific protein (Kintner and Brockes, 1984; obtained from Developmental Studies Hybridoma Bank), assayed stage 20. (G,H,I) *en-2* protein detected with mouse monoclonal antibody 4D9 (arrowheads, Patel et al., 1989; Hemmati-Brivanlou and Harland, 1989), assayed stage 23-24. Open arrows: *en-2* protein in G, the mandibular arch, and I, the distal mesoderm of an open-face explant. *en-2* expression in sandwiches is consistently lower than in open-face explants. (J,K,L) *XlHbox1* protein detected with affinity purified rabbit polyclonal antibodies to the long form (a gift of C. Wright and E. De Robertis; Oliver et al., 1988). Arrowheads: expression in the CNS, and open arrows: expression in J, trunk somites (striped pattern) and K, somitic mesoderm in a sandwich explant. Somitic mesoderm tends to disintegrate by the stage assayed (stage 26), and is not always present. (K) inset: area of neurectoderm that expresses *XlHbox1* in sandwiches, approx. 2× magnification of K. (M,N,O) *XlHbox6* protein, detected with affinity purified rabbit polyclonal antibodies (arrowheads point to anterior boundary in M and N) (a gift of C. Wright; Wright et al., 1990), assayed at stage 24. Some batches of this antiserum cross react with unidentified nuclear antigens in proximal notochord (unpublished observations) and muscle (Wright et al., 1990), and with an extracellular antigen in the cement gland of intact embryos. (P, Q) *Krox-20* RNA detected by whole-mount in situ hybridization, at stage 21-22 (the probe was kindly made by R. Harland; the probe was a gift of D. Wilkinson). Staining is cytoplasmic. (A-O are reproduced with permission from *Science* journal.)

Head mesoderm does not induce en-2

The involuted head mesoderm is nominally removed when Keller explants are made, but it is possible that some of these highly migratory cells are left behind and could migrate into the ectodermal territory, thereby inducing the expression of the neural markers via vertical interactions. To determine whether head mesoderm is actually capable of inducing *en-2* by vertical contact, the most anterior marker used, head mesoderm (see Fig. 1) and a small amount of attached pharyngeal endoderm were wrapped with competent ectoderm (from stage 10 embryos) and cultured until stage 27. *en-2* was not detected in any of these recombinates (n=22). This implies that if contaminating head mesoderm is present in Keller explants and has vertical contact with the ectoderm, it is unlikely to induce *en-2* expression, and perhaps that of the more posterior markers, although they have not been tested.

Contact with dorsal mesoderm is required for expression of neural markers

The ectodermal expression of these genes could be autonomous, rather than being induced by planar contact with dorsal mesoderm. To test this, dorsal ectoderm was explanted without dorsal mesoderm from early gastrulae

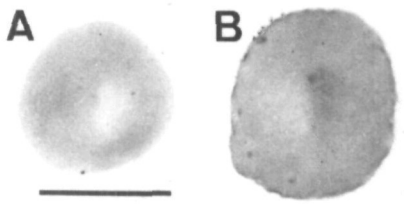


Fig. 4. Isolated dorsal ectoderm, immunostained for (A) *en-2* or (B) *XlHbox1*. Neither marker was detected in these or other explants.

(stage 10+). It does not express *en-2* (0 of 34) or *XlHbox1* (0 of 8; Fig. 4). Therefore, contact with dorsal mesoderm is required for expression of these genes. *XlHbox6* could not be tested because it is expressed in the posterior mesoderm, and *Krox-20* has not yet been tested.

Planar contact is sufficient for neural induction

Is planar contact sufficient to induce these genes in ventral ectoderm, in which they are not normally expressed? Dorsal mesoderm from early gastrulae (stage 10+) that had been labelled with the lineage marker fluorescein dextran amine (FDA; Gimlich and Braun, 1985) was grafted onto the edge of a sheet of unlabelled, stage 10+ ventral ectoderm, creating a planar, open-face recombinate. Normal A-P polarity was preserved by grafting the presumptive posterior

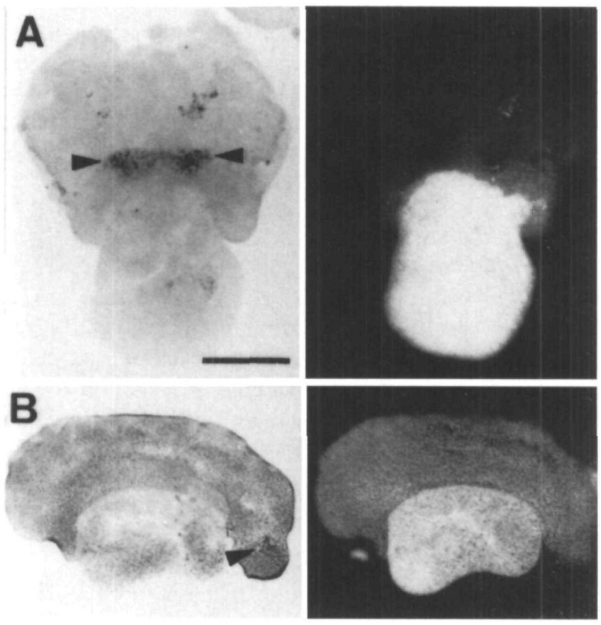


Fig. 5. Induction of *en-2* and *XlHbox1* expression in ventral ectoderm: recombinates of FDA-labelled dorsal mesoderm grafted onto the the edge of unlabelled ventral ectoderm, immunostained for (A) *en-2* (control stage 23) and (B) *XlHbox1* (control stage 26). Left, images viewed with transillumination; right, epifluorescence. Arrowheads point to *en-2* or *XlHbox1*-positive nuclei in the ectoderm. In A, the small patch of staining and fluorescence above the positive nuclei is non-nuclear and is an artifact; also, mesodermal *en-2* expression is visible in the FDA-labelled portion. Scale bar 500μm. (Reproduced with permission from *Science* journal.)

ends of each tissue together. All three homeobox genes were expressed in the unlabelled portion of these recombinates (Fig. 5; 6 of 15 expressed *en-2*, 9 of 18 *XlHbox1*, and 2 of 21 *XlHbox6*). The region of expression, particularly for *en-2*, appears to be closer to the mesoderm than seen in normal open-face explants. These markers were not expressed in control explants of ventral ectoderm alone (0 of 8 expressed *en-2*, 0 of 21 *XlHbox1*, 0 of 13 *XlHbox6*). FDA-labelled mesoderm did not migrate into or mix with the unlabelled ectoderm (Fig. 5A,B), further supporting the notion that contact between mesoderm and ectoderm is exclusively planar. Thus, strictly planar contact is sufficient to induce these genes, even in ventral ectoderm.

Prepattern is not required

The following experiment tests whether *en-2* can be induced ectopically, beyond the latitude predicted by the fate map to express it. A priori, it could be that the A-P expression pattern of genes in the neurectoderm could be dictated by an animal-vegetal prepattern that exists around the whole embryo, including on the ventral side, such that specific genes are only induced in the regions derived from the latitudes fated to express them. To test this, the presumptive posterior edge of FDA-labelled dorsal mesoderm (from stage 10+) was grafted onto the anterior end (near the anterior edge of the presumptive neural plate (Fig. 1)) of an unlabelled open-face explant (also stage 10+). In such recombinates there were two regions of *en-2* expression, one 'primary' region near the mesoderm from the unlabelled portion and a smaller 'secondary' region near the labelled mesoderm, in territory approximately fated to become cement gland or perhaps forebrain (Fig. 6). Keller et al. (1992a) have obtained a similar result using convergent extension as a marker. This demonstrates that a prepattern, in which *en-2* expression is limited to a particular position of the ectoderm along the animal-vegetal axis, does not exist or is at least not required for *en-2* expression.

A-P pattern in the mesoderm

In intact embryos, *en-2*, *XlHbox1* and *XlHbox6* are also expressed in specific regions outside the CNS. *en-2* protein is detected bilaterally in nuclei of loose clusters of cells in the mandibular arch (Fig. 2G; Hemmati-Brivanlou and Harland, 1989; Hemmati-Brivanlou et al., 1991); *XlHbox1* protein is detected in nuclei of somites in the mid-trunk (Fig. 2J) and lateral plate mesoderm of the mid trunk (Oliver et al., 1988); and *XlHbox6*, in nuclei of trunk lateral plate mesoderm and tailbud (posterior) mesoderm (Wright et al., 1990).

In Keller explants, in addition to the expression in the neurectoderm, *en-2* protein is detected in loose, bilateral clusters of nuclei in the anterior (distal) part of the meso-endoderm. In histological sections (not shown), it can be seen that these nuclei are in the deep layer of tissue, suggesting that the respective cells are not from the layer of superficial endoderm (Keller, 1975). The clustered pattern of these nuclei in explants resembles that of those expressing *en-2* in the mandibular arch (Fig. 2I). It has been suggested that the *en-2*-expressing cells in the mandibular arch are derived from the neural crest, so the presence of similar cells in the mesodermal portion of explants was surprising. The location of these cells raised three questions. First, are they derived from the mesoderm, or from neural crest that has migrated from the ectoderm into the mesoderm? Second, if this *en-2* expression is mesodermal, is it autonomous within the anterior mesoderm, or is contact with posterior mesoderm and/or ectoderm required? Third, is the presence of anterior, *en-2* expressing, mesoderm required for the expression of *en-2* in the ectodermal portion of explants, or is posterior mesoderm a sufficient source of inducer?

To answer the above questions, Keller sandwiches were cut into two portions, a 'lower' one consisting of anterior mesoderm, and an 'upper' one consisting of ectoderm and posterior mesoderm, at stage 10+. The two portions were cultured separately but under the same coverslip so as to keep track of each pair of complementary portions from each sandwich. When controls reached stage 26, individual pairs were stained with antibodies to *en-2*. Mesoderm was detected with antibodies to muscle (using mAb 12-101) and notochord (using Tor-70, a monoclonal antibody against the notochordal sheath (Bolce et al., 1992). Only those pairs in which the upper portion also contained a substantial amount of muscle and notochord were scored for *en-2* expression, in order to avoid possible contamination of the lower portion with neural crest from the ectoderm of the upper portion. *en-2* was detected in all of the lower portions (n=9), in loose clusters of nuclei, and it was detected in 3 of 9 of the upper portions of the same pairs, in a band of nuclei characteristic of the neural expression pattern. Therefore, (1) the expression in the lower portions is not neural crest derived, it is mesodermal. This suggests that a substantial portion of the *en-2*-expressing cells in the mandibular arch are mesodermal, although it remains possible that additional ones are neural crest-derived. (2) *en-2* expression in the anterior mesoderm is autonomous, indicating that contact with ectoderm or posterior mesoderm is not required for this regional expression; and (3) the anterior mesoderm, notably that which expresses *en-2*, is not required for expression of *en-2* in the neurectoderm.

XlHbox1 protein is also detected in the nuclei of somites in the middle of the mesodermal portion (Fig. 2K) of Keller sandwiches. Thus, the mesoderm of planar explants exhibits A-P pattern, suggesting that vertical interactions with the ectoderm are not required for patterning of the mesoderm.

Discussion

Position-specific molecular neural markers have been used here to determine whether A-P neural pattern can be induced in planar explants of dorsal mesoderm and ectoderm. All four of the markers (*en-2*, *Krox-20*, *XlHbox1*, and *XlHbox6*) are expressed in these explants, and in the normal A-P order. Both of the markers that were tested (*en-2* and *XlHbox1*) are not expressed in ectoderm in the absence of mesoderm, and they and *XlHbox6* are induced in ventral ectoderm when placed in planar contact with dorsal mesoderm. Based on the above results, it is concluded that planar signals alone are sufficient to induce A-P neural pattern. These results corroborate those of Keller et al. (1992b), who found that the convergent extension movements associated

with the neurectoderm are induced by planar signals. They are also in agreement with those of Ruiz i Altaba (1990), who found that the anterior neural marker Xhox-3 is expressed in the ectoderm of exogastrulae. In addition, the results indicate that the mesoderm exhibits some A-P pattern of its own in planar explants.

The results are in apparent conflict with the finding that *en-2* protein is often not detected in the ectoderm of *Xenopus* exogastrulae (Hemmati-Brivanlou and Harland, 1989), in which planar interactions presumably occur. It is possible that the ectoderm of extreme exogastrulae is mesodermalized, owing to the absence of the blastocoel, and is therefore no longer competent for neural induction. This needs to be examined. Alternatively, inhibitory interactions may occur in exogastrulae but not in Keller explants. The mixed results obtained with exogastrulae suggest that these abnormal embryos are not a reliable system in which to examine planar induction.

Do Keller explants eliminate vertical interactions?

The conclusions in this study largely depend on evidence that Keller explants truly eliminate vertical interactions. There are four lines of evidence that support this contention. First, extensive experiments by Keller et al. (1992a), in which individual cell movements were traced in Keller sandwiches, have established that full convergent extension of the spinal neurectoderm only occurs in those sandwiches in which there is virtually no mesoderm forming vertical contacts with the ectoderm. When there is vertical contact between ectoderm and mesoderm in Keller explants, convergent extension is reduced and irregular. In this paper, only Keller explants that showed symmetrical and complete convergent extension were considered as usable samples. Second, experiments in this paper indicate that the head mesoderm, which is highly migratory and therefore the most likely to form vertical contacts with ectoderm in Keller explants, is unable to induce *en-2*, the most anterior marker, when placed in vertical contact with animal cap ectoderm. Other experiments (Dixon and Kintner, 1989; T. D. and C. R. Phillips, unpublished) indicate that this tissue is also unable to induce NCAM expression. Thus, even in the event that some vertical interactions occurred between head mesoderm and ectoderm in Keller explants, it is unlikely that expression of the markers studied would result from these interactions. Third, the neural markers were also expressed in open-face explants. These explants avoid the potential problem that exists in sandwiches, in which vertical interactions could occur between ectoderm and mesoderm from different layers of the sandwich. Finally, lineage labelling experiments in this paper show that mesoderm can induce expression of all three of the genes tested without migrating into or beneath the responding ectoderm.

How far can planar signals go?

The four position-specific genes tested are markers for the middle portion of the central nervous system along the A-P axis, from the midbrain-hindbrain junction to at least the anterior spinal cord. At a minimum, the results presented here indicate that the A-P pattern induced by planar signals is complete to this extent. To determine whether full neural pattern from anterior to posterior is induced, it will be necessary to use markers specific to the forebrain and the posterior spinal cord. Although it is not possible to say whether there is any neural pattern anterior to *en-2* in planar explants, it is clear that there is some kind of neural tissue beyond this, because NCAM is detected in the anterior portions of Keller explants beyond *en-2*.

In open-face explants, in which convergent extension does not occur, it is possible to make a rough comparison between the A-P extent of NCAM expression and that of the presumptive neural region at the beginning of gastrulation (Keller, 1975). Both of these cover approximately 50% of the distance between the mesoderm and the animal pole. Thus, the extent of planar induction of neural differentiation in explants is comparable to that in normal embryos. When translated into cell diameters, planar signals would have to travel approximately 50 cell diameters to induce the entire A-P extent of the neurectoderm (R. Keller, personal communication). It is interesting to note that the positional neural markers are expressed in distinct stripes in open face explants, even in the absence of morphogenesis, indicating that morphogenesis itself is not required for patterning (although it presumably contributes to the separation of the stripes). Moreover, the pattern is compressed, much as is the fate map of the early gastrula, implying that the pattern is set up prior to or independently of morphogenesis.

In the experiments in which dorsal mesoderm was grafted onto the edge of ventral ectoderm, the distance between the markers expressed and the mesoderm was apparently shorter than in normal open-face explants. This is analogous to the results of Keller et al. (1992b), in which the region that converged and extended in sandwich explants of equivalent tissues to those above was much shorter than in recombinates using dorsal ectoderm. Keller et al. (1992b) propose that planar signals cannot pass as far in ventral ectoderm as they can in dorsal ectoderm, perhaps reflecting the predisposition of dorsal ectoderm towards neural induction that has been much discussed lately (Sharpe et al., 1987; London et al., 1988; Sokol and Melton, 1991; Otte et al., 1991). The greater distance that planar signals apparently pass in dorsal ectoderm may be a result of such priming by signals prior to neural induction, perhaps going as far back as the cortical rotation during the first cell cycle (London et al., 1988; Gerhart et al., 1989).

A-P pattern in the mesoderm of planar explants

Two of the markers used, *en-2* and *XlHbox1*, are expressed in specific regions outside of the central nervous system (CNS): *en-2* in the mandibular arch and *XlHbox1* in the somites and lateral plate mesoderm of the mid-trunk. Results presented here suggest that at least some of the *en-2*-expressing cells in the mandibular arch are derived from mesoderm anterior to the notochord, rather than from neural crest as has been suggested (Hemmati-Brivanlou et al., 1991). Both *en-2* and *XlHbox1* appear to be expressed in appropriate regions of the mesoderm of planar explants. These results concur with much earlier results of Holtfreter (1933) who observed that the evaginated mesoderm and endoderm of exogastrulae display marked A-P morphology and differentiation. As predicted by the fate map, A-P polarity of the mesoderm is a mirror image to that of the ectoderm. Clearly, the mesoderm does not require vertical con-

tact with ectoderm to develop its A-P pattern. Taken together with the results that *en-2* and *XlHbox1* are also expressed in the ectoderm of planar explants, it appears that these two homeobox genes do not require vertical alignment of the ectoderm and mesoderm to be expressed in the correct position, contrary to recent proposals (DeRobertis et al. 1989; Frohman et al. 1990).

Additional results here indicate that *en-2* expression in the mesoderm does not require contact with ectoderm, at least after the early gastrula stage, since removal of these tissues at the beginning of gastrulation does not prevent *en-2* expression in anterior mesoderm.

Applying vertical induction models to planar induction

The previous models that have been proposed assume neural induction is primarily vertical, and that the dorsal mesoderm provides regionalized pattern information. The simplest model is based on the classical finding that different A-P regions of the dorsal mesoderm from the early neurula can induce corresponding A-P levels of neural tissue (Mangold, 1933). In this model, each level of A-P pattern in the neurectoderm is induced by a specific signal passing vertically from the mesoderm lying directly below it (Fig. 7A). On its own, this model is hard to reconcile with planar induction for two reasons. First, the ectoderm depends on the vertical proximity of each qualitatively different inducing signal to develop a pattern; this is lost when the signal is planar (Fig. 7B). Second, even if it is assumed that there was some A-P information in the ectoderm before neural induction, it is hard to imagine how, for example, an anterior-inducing planar signal originating in the most anterior mesoderm would find its way to its target in the anterior neurectoderm after passing all the way through posterior mesoderm and then posterior neurectoderm.

There have been a variety of gradient models proposed for vertical induction in amphibians. These generally require two inducers originating in the mesoderm, one arranged in an A-P gradient with a high point in the posterior mesoderm, and another that is at a constant level

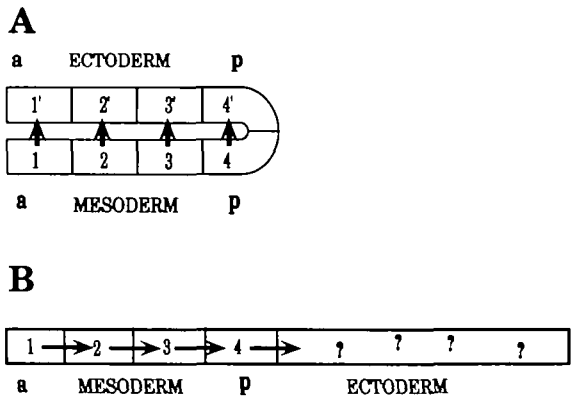


Fig. 7. (A) Schematic representation of a simple model for vertical induction of A-P neural pattern. Involuted mesoderm contains regionalized inducers that induce pattern in ectoderm directly above. (B) When the same inducers use the planar route (indicated by unfolding the sheet of mesoderm and ectoderm shown above), there is no longer any positional information for the ectoderm.

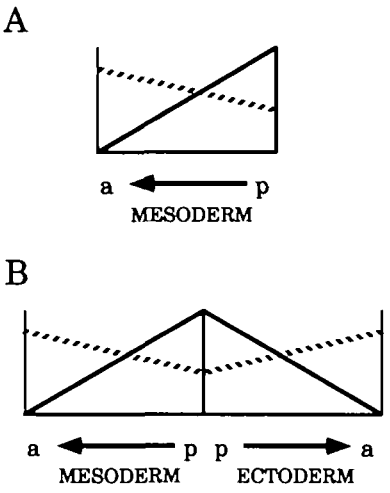


Fig. 8. (A) Simplified distribution of inducers of A-P neural pattern in models proposed by Saxen and Toivonen (Saxen, 1989) or Nieuwkoop (Nieuwkoop and Albers, 1990). *x*-axis, A-P axis; *y* axis, concentration of inducers. In the Saxen-Toivonen model, the dashed line represents the 'neuralizing inducer', which alone induces forebrain development and the black line is the 'mesoderm inducer', which, in combination with neuralizing inducer causes posterior neural development. In the Nieuwkoop model, the dashed line is the 'activator', which induces forebrain, and the black line is 'the transforming agent', which posteriorizes previously activated tissue. (B) Adaptation of the above model for planar induction.

along the A-P axis, or is higher at the anterior end of the mesoderm (Fig. 8A; Saxen, 1989; Nieuwkoop and Albers, 1990). It is tempting to try to adapt these models so that the same signals are used for vertical and planar induction. However, it is difficult to envision how an inducer emanating from the mesoderm could spread through the plane of the ectoderm and end up with an even distribution, let alone a higher amount at the anterior end (Fig. 8B). It is possible that vertical and planar induction use the same inducers, but if so, it seems unlikely that the previous gradient models will apply without additional assumptions.

Mechanisms for planar induction of A-P neural pattern

Many mechanisms have been proposed over the years to explain pattern formation. Some will be reconsidered here for planar induction, along with some ways of testing them. There have been several excellent reviews recently about mechanisms of pattern formation (Wolpert, 1989) and neural induction and A-P patterning in amphibians (Jacobson and Sater, 1988; Saxen, 1989; Slack and Tannahill, 1992) that can be referred to for more details.

Concentration gradient

As mentioned above, gradient models have been proposed to explain vertical neural induction. Regardless of whether such gradients operate for vertical induction, it is possible that there is a gradient system for planar induction. Inducer(s) could emanate from the mesoderm, forming a concentration gradient within the plane of ectoderm, with high levels inducing posterior pattern and low levels anterior pattern. A gradient model would be supported if pos-

terior pattern elements are lost when the amount of inducer is reduced (e.g. by cutting off some mesoderm). Results of a similar type have been taken as support for a gradient model for digit patterning in the chick limb bud (Tickle, 1981).

One possible problem with a gradient model is that experiments with *Xenopus* have shown that any reduction in the size of the organizer results in reduction and loss of anterior pattern elements, while those in the posterior are increased (Gerhart et al., 1989). This is the reverse effect of that predicted above for reductions in amount of neural inducer. Not enough is known about how these changes in organizer function or size affect the system that induces neural pattern. However, it seems somewhat contradictory that reduction in the organizer could cause an increase in the amount of neural inducer, thereby leading to an excess of posterior neural pattern.

Prepattern

In recent years molecular evidence has accumulated that there is a bias or predisposition towards neural development in the dorsal but not ventral ectoderm (Sharpe et al., 1987; London et al., 1988; Otte et al., 1991). Taken to an extreme, there could be a precise A-P neural pattern which is laid out cryptically in the ectoderm before induction, and the inductive signal from the mesoderm could be merely permissive for the expression of the pattern. Such a bias, or a prepattern, should it exist, is not strictly required for patterned neural development, as shown in the very first organizer graft (Spemann and Mangold, 1924), in which a secondary nervous system was induced in ventral ectoderm. Results in this paper do not support a strict requirement for a prepattern, either in the dorsal side, or in the latitudes fated for mid-brain, since three of the positional neural markers can be induced in ventral ectoderm, and *en-2* can be induced in ectoderm anterior to its normal position of expression. Thus, if there is a prepattern, it can be overridden by signals from the mesoderm.

Ectodermal competence

It has been proposed that ectodermal competence plays a role in determining the lateral boundaries of the neural plate and the induction of placodes beyond the boundary (such as the lens; Albers, 1987; Servetnick and Grainger, 1991). There are two features to this model: (1) a neural inducer spreads laterally through the neurectoderm (via homeogenetic induction) from the dorsal midline of the neural plate at a certain velocity, and (2) the competence of the ectoderm changes with time, with a series of different responses. Thus, as the signal spreads laterally, medial ectoderm receives the neural inducer early, and develops as CNS; progressively more lateral ectoderm receives the signal progressively later, and responds by becoming neural crest, placode or epidermis, the latter developing as a default after competence to respond to the inducer runs out. An equivalent process could determine A-P pattern in the neurectoderm, if a neural inducer spreads into the ectoderm from the dorsal mesoderm, and if the age of ectoderm when the inducer was received determined the level of A-P neural pattern. This model could be tested using heterochronic planar recombinates, for example dorsal mesoderm and presumptive posterior neurectoderm with younger presumptive anterior neurectoderm. The model would be supported if the posterior pattern is repeated in the presumptive anterior neurectoderm.

Timing gradient

This model is related to a model proposed by Nieuwkoop (1985), in which the duration of vertical contact with involuted dorsal mesoderm determines A-P pattern. To apply this to planar induction, the inducer could instead spread through the plane of the ectoderm from the mesoderm at a given velocity, such that the ectoderm closer to the source (i.e. posterior) is exposed to the inducer for longer than that further away (i.e. anterior). The final amount of time exposed to the inducer could be determined either by a general change in competence or the cessation of the signal. This model is partly supported by the results of Sive et al. (1989). They found that posterior presumptive neurectoderm would express cement gland-specific RNA (cement gland is a tissue normally made beyond the anterior edge of the neural plate), when isolated early in gastrulation, while the same tissue would make NCAM, a neural marker, when isolated later in gastrulation. These results were interpreted in terms of the vertical induction model proposed by Nieuwkoop (1985). However, there is some evidence that cement gland can be induced by planar signals (unpublished results). When applied to planar induction of A-P neural pattern, this model would be supported if progressively longer planar contact between dorsal mesoderm and ectoderm induced progressively more posterior neural markers.

'Phase-shift model'

Two signals could propagate through the ectoderm via homeogenetic induction, each with a different velocity. The time interval between receipt of the signals at a given point in the ectoderm could determine the level of A-P development. A model with these features, referred to as the 'phase-shift' or 'thunderclap' model, was proposed by Goodwin and Cohen (1969). To test this model, recently induced posterior neurectoderm could be assayed for its ability to induce anterior neurectoderm in planar recombinates.

Self organization

In normal neural induction, it is possible that the mesoderm provides minimal pattern information to the ectoderm. Two signals could emanate from the mesoderm, one giving polarity to the ectoderm and the other stimulating general neural development. These signals could reach the ectoderm via either a planar or a vertical path, or both. A-P neural pattern could then self-organize as a result of subsequent cell-cell interactions within the plane of the ectoderm. The ectoderm, of urodeles at least, does have a capacity for some neural self-organization: when exposed to an exogenous neural inducer, it can develop into semi-organized forebrain and eyes. The whole A-P range of neural types, albeit only partly organized, will develop when varying amounts of a second inducer are added (see Saxen, 1989 for review). It is possible that a purified endogenous neural inducer would give a full A-P neural axis, but such an inducer remains to be isolated, in *Xenopus* at least. In the absence of purified inducer, it is difficult to test this model.

However, it may end up as a default model if there is no positive evidence for any of the above models.

Clearly, many experiments need to be done before induction of A-P neural pattern is understood. Most of the experiments suggested above depend on having regional molecular markers, and the existence of a reliable double or triple labelling method in order to distinguish between them. The markers used in this paper are suitable for such experiments, although they are expressed rather close together, especially in the absence of convergent extension. This makes it difficult to explant or recombine tissues fated to express only one of these markers. Markers more widely spaced, such as those specific to the forebrain or posterior spinal cord would be particularly useful for extending studies of A-P patterning.

The role of planar and vertical induction in A-P patterning

The results presented here show that A-P neural pattern can be induced in planar explants, and it is proposed that this occurs in normal development. There has been a lot of previous evidence indicating that vertical signals can also induce neural pattern. The two induction pathways must somehow coordinate in order to achieve a single pattern, whether they use the same or different inducer molecules. It remains to be seen exactly which functions the vertical and planar pathways of induction share, and which are unique. Those functions that overlap may guarantee that the elaborate process of A-P patterning happens correctly every time, as originally proposed by Spemann (1938).

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