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Planar and vertical signals in the induction and patterning of the *Xenopus* nervous system

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Summary

The cellular mechanisms responsible for the formation of the *Xenopus* nervous system have been examined in total exogastrula embryos in which the axial mesoderm appears to remain segregated from prospective neural ectoderm and in recombinates of ectoderm and mesoderm. Posterior neural tissue displaying anteroposterior pattern develops in exogastrula ectoderm. This effect may be mediated by planar signals that occur in the absence of underlying mesoderm. The formation of a posterior neural tube may depend on the notoplate, a midline ectodermal cell group which extends along the anteroposterior axis. The induction of neural structures characteristic of the forebrain and of cell types normally found in the ventral region of the posterior neural tube requires additional vertical signals from underlying axial mesoderm. Thus, the formation of the embryonic *Xenopus* nervous system appears to involve the cooperation of distinct planar and vertical signals derived from midline cell groups.

Key words: axial mesoderm, exogastrula, induction, neural, notoplate, patterning, planar signals, vertical signals, *Xenopus*.

Introduction

The development of the vertebrate nervous system is initiated by local signals that induce neural properties in dorsal ectodermal cells, resulting in the formation of a neural plate which displays anteroposterior (A-P) pattern (Spemann, 1938; Roach, 1945; Jacobson, 1964; Hamburger, 1988; Nieuwkoop et al., 1985). Neural induction has been thought to depend on signals transmitted vertically from the dorsal mesoderm to the overlying ectoderm during gastrulation (Spemann and Mangold, 1924; Holtfreter, 1933; Spemann, 1938; Leussink, 1970; Nieuwkoop et al., 1985; Hamburger, 1988). Furthermore, dorsal mesoderm of different A-P character can induce neural structures of equivalent axial character in ventral ectoderm (Mangold, 1933; Ruiz i Altaba and Melton, 1989), leading to the idea that the early regionalization of the neural plate is also imposed by vertical signals from the underlying mesoderm.

Recently, the analysis of exogastrulated embryos, in which involution of mesoderm cannot be detected (Holtfreter, 1933), and of equivalent in vitro explants in *Xenopus* (Kintner and Melton, 1987; Keller and Danilchik, 1988; Dixon and Kintner, 1989; Ruiz i Altaba, 1990), has shown that the dorsal ectoderm can express neural markers in the absence of underlying axial mesoderm. These results raise the possibility that signals, derived from the dorsal blastopore lip or organizer region (Spemann, 1938), can spread in a planar manner through the ectoderm to initiate neural differentiation. Such planar signals have also been proposed to induce the A-P pattern of the neural ectoderm (Ruiz i Altaba, 1990). However, the source, range of action and relative contributions of planar and vertical signals to neural induction and patterning have not been defined.

To address the contributions of planar and vertical neural signals to the formation of the embryonic nervous system, I have examined the development of neural tissue in exogastrulae and mesoderm-ectoderm recombinates. Neural tissue of posterior character displaying A-P pattern is observed in the ectoderm of exogastrulae in which no involution of mesoderm can be detected. In contrast, the forebrain and some ventral neuronal types are not present in exogastrulae and their differentiation depends on additional vertical signals from axial mesoderm. The extensive induction of posterior neural tissue in exogastrulae may be dependent on the notoplate, a midline neural cell group that elongates during gastrulation (Jacobson, 1981; Keller and Danilchik, 1988). The notoplate may serve as a source of planar neural-inducing signals that act on adjacent ectoderm.

Materials and methods

Embryos

Xenopus laevis embryos were obtained by standard procedures and staged according to Nieuwkoop and Faber (1967). Embryos were

incubated in 0.1×MMR (Newport and Kirschner, 1982) after dejellying with 3% cysteine in water. Incubation in 0.1×MMR resulted in normal embryos whereas incubation in higher salt concentrations forced exogastrulation movements. A range of MMR concentrations were tested, from 1× to 1.5×, with 1.3× resulting in over 80% of embryos undergoing complete exogastrulation in most batches. Best results were obtained by incubating early blastula stage (stage 6½-7) embryos in 1.3×MMR in 2% agarose dishes after removing the vitelline membrane manually. Under these conditions, younger embryos tended to disaggregate and older embryos underwent normal gastrulation. Exogastrulae that showed some degree of involution during gastrulation were discarded. Only the exogastrulae displaying a typical head mesoderm protrusion opposite to the ectoderm and a long and thin mesodemal neck were chosed for these studies. Keller sandwiches were made as described (Keller and Danilchik, 1988). All media contained penicillin (100 i.u./ml) and streptomycin (100 µg/ml). Lithium treatment of blastula-stage (stage ~6½) embryos was performed by immersing the embryos in 0.1 M LiCl in tap water for 8-15 minutes. Treatment for 10-15 minutes, routinely resulted in embryos displaying extreme dorsalized/anteriorized phenotypes (Kao et al., 1986).

Antibodies

Monoclonal antibodies (mAbs) Xen1-Xen3 and mAb Xit were derived from hybridomas from mice immunized with dissected formaldehyde-fixed larval brains. Hybridomas were screened by whole-mount immunoperoxidase labelling on tadpole-stage embryos in 48- or 96-well plates. Whole-mount labelling was performed as previously described (Dent et al., 1989; Patel et al., 1989; Hemmati-Brivanlou and Harland, 1989). mAbs HNK-1 (IgM), Xit (IgM) and Xen1 to Xen3 (IgGs) were used as culture supernatants at 1:1. mAb MZ15 (IgG; Smith and Watt, 1985; kindly provided by J. Smith) was used at 1:100. mAb 12/101 (IgG; Kintner and Brockes, 1984; kindly provided by C. Kintner) was used at 1:250. mAb 4D9 (Patel et al., 1989; kindly provided by N. Patel) was used as a protein A-purified IgG at 100 µg/ml. Fluorescein and peroxidase-coupled secondary antibodies were from Tago and Boehringer Mannheim. Peroxidase reactions were carried out in the presence of 0.5 mg/ml diaminobenzidine and 0.003% hydrogen peroxide. Histological sections were obtained by embedding whole-mount labelled embryos in paraplast and cutting 10 µm-thick sections. Staining with hematoxylin and eosin was done by standard methods. Sections were mounted in Permount (Sigma). Photographs were taken with a Zeiss Axiophot microscope and Ilford Pan F of Kodak Ektachrome 50 or 160 Tungsten film.

Whole-mount in situ hybridization

The in situ hybridization method used here was that of Harland (1991; see also Hemmati-Brivanlou et al., 1990a). For detection of *Xenopus brachyury* transcripts by in situ hybridization, pXbra (Smith et al., 1991; kindly provided by J. Smith) was linearized with *Bgl*II and transcribed with T7 RNA polymerase in the presence of dig-11 UTP. The probe was used at 1 µg/ml and was not hydrolyzed since longer probes appeared to give better results.

Lineage tracing

20 nl of rhodamine-lysine-dextran (RLD) of 10,000 M_r (Molecular Probes) in water at 25 mg/ml was injected into a single blastomere B1. The dorsal side of 32-cell embryos was identified by its lighter pigmentation in comparison to the ventral side. Blastomere B1 was identified according to previous fate maps (Dale and Slack, 1987).

Animal cap ectoderm/notoplate-notochord recombinates

The notochord and notoplate were excised from pigmented, RLD-injected embryos in the presence of 0.3% dispase (Boehringer Mannheim) in 0.5×MMR for 1-2 minutes. The excised tissues were checked for contaminating cells and extensively washed in 0.5×MMR containing 0.5% heat-inactivated fetal calf serum for 1-2 minutes and then in 0.5×MMR alone 3-4 times for a total of ~10 minutes. Notochord and notoplate cells have very different morphology and pigmentation permitting them to be clearly distinguished. The midline of the neural plate, the notoplate, can be cleanly separated from the underlying notochord at this stage. Notochord tissue was isolated whole but notoplate explants normally lacked the most anterior and posterior regions. Gastrula-stage (stage 10) ectoderm from albino embryos was excised from the animal cap and immediately used to sandwich the notochord or notoplate to prevent curling of the tissue. Animal caps were obtained from ectoderm that had not been contacted by mesoderm. Recombinates healed within 10 minutes and were incubated in 0.5×MMR at room temperature.

Exogastrula ectoderm/notochord recombinates

Albino embryos were induced to exogastrulate by incubation in 1.3×MMR. At the end of gastrulation (stage ~13), the ectodermal sacs of total exogastrulae that displayed the greatest degree of elongation and showed a typical anterior mesoderm bud at the anterior end of the endomesodermal region were excised by cutting the ectoderm, avoiding mesodermal contamination. The dorsal and ventral ectodermal layers of the sac were separated and isolated tissues were then inserted into the ectodermal sac between the dorsal and ventral ectodermal layers. The explants healed within ~10 minutes, retaining the implant. Notochords were obtained from pigmented embryos as described above. Anterior mesoderm was excised from the roof of the archenteron from pigmented embryos at stage 12-12½.

Results

Exogastrula ectoderm develops in the absence of involuted mesoderm

Interpretation of the presence of neural tissue in exogastrula ectoderm depends critically on a lack of apposition between the ectoderm and involuted dorsal mesoderm at any stage of gastrulation. The absence of mesodermal cells in the exogastrula ectoderm was assessed in four different ways.

First, the exogastrulae used for analysis were selected as those showing the greatest degree of separation of mesodermal and ectodermal regions during gastrulation. Pigmented exogastrulae were dissected during gastrulation and examined microscopically for the presence of chordamesoderm or head mesoderm cells adhering to the deep ectodermal layer (see Keller and Danilchik, 1988). There was no detectable mesodermal contamination in exogastrulae (n=5, not shown).

Second, the position of mesodermal cells during early stages of exogastrulation was checked by the expression of the frog *brachyury* gene (Xbra, Smith et al., 1991) by whole-mount in situ hybridization (Harland, 1991). Xbra mRNA is expressed in the entire marginal zone of the embryo as an early response to mesodermal induction (Smith et al., 1991; Fig. 1A). As gastrulation proceeds, the ring of mesodermal cells expressing Xbra becomes smaller as the blastopore closes. At this time, Xbra is also expressed in the cells that form the notochord (Fig. 1B). In mid-gas-

trula stage (stage $11\frac{1}{2}$-12) embryos induced to exogastrulate, Xbra mRNA was detected in the mesodermal ring that had undergone constriction movements as a consequence of the exogastrulation movements. Cells expressing Xbra were not detected in the ectoderm (*n*=5; Fig. 1C, D). Moreover, there was a sharp boundary formed by cells positive and negative for Xbra expression coincident with the mesoderm-ectoderm boundary. The forming notochord was detected in the endodermal mass, away from the ectoderm (Fig. 1D). This analysis of the expression of the earliest general mesodermal marker revealed no evidence for involution of mesoderm in exogastrulae.

Third, tadpole-stage exogastrulae were labelled with mAbs directed against antigens characteristic of differentiated axial mesoderm, notochord (mAb MZ15) and muscle (mAb 12/101). Both notochord and somites were excluded from the ectoderm in complete but not in incomplete exogastrulae (*n*=52; Fig. 2E, E' and not shown).

Fourth, if involution of mesoderm were to occur in exogastrulae, it would be likely to represent head mesoderm, which normally is the first mesodermal tissue to involute. This possibility was tested by suppressing the differentiation of anterior mesoderm by retinoic acid (RA at 10^{-8}-10^{-9} M). RA treatment before gastrulation suppresses preferentially anterior mesodermal development in normal embryos (Ruiz i Altaba and Jessell, 1991a) giving rise to headless tadpoles (Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991a). RA-treated embryos induced to exogastrulate lacked the protrusion characteristic of the anterior endomesodermal area suggesting that head mesoderm differentiation was suppressed. Nevertheless, neural tissue was observed in the dorsal ectoderm (*n*=61; not shown). Moreover, cells expressing *Pintallavis*, a recently characterized gene transcribed in all axial mesodermal cells, are not detected in the ectoderm of exogastrulae (see Ruiz i Altaba and Jessell, 1992).

Taken together, these results provide evidence that mesodermal cells do not invaginate for a significant period of time under the ectoderm of complete exogastrulae. Nevertheless, it is difficult to exclude completely the possibility that there is a transient involution of prospective mesodermal cells at the onset of exogastrulation, which could contribute to the inductive events described below.

Neural differentiation in exogastrula ectoderm

The extent of neural differentiation in *Xenopus* ectoderm in the apparent absence of invaginated mesoderm was determined in exogastrulae by histology and by the expression of five neural antigens detected by mAbs: three novel general neural antigens, Xen1 to Xen3 that appear to be membrane-associated, the HNK-1 carbohydrate epitope (Abo and Balch, 1981), and polysialylated N-CAM (Dodd et al., 1988). In normal tadpole-stage embryos, the HNK-1, Xen1-Xen3 and polysialylated N-CAM antigens are expressed by a large number of neural cells although with slightly different patterns (Fig. 2B-D and not shown; see also Ruiz i Altaba and Jessell, 1991b). Labelling of tadpole-stage exogastrulae with these mAbs revealed the presence of an elongated neural structure in the dorsal ectoderm (*n*=65 embryos for each mAb; Fig. 2B'-D' and not shown). Sections through exogastrula ectoderm revealed the presence of a tubular neural structure surrounded by non-neural ectoderm (Fig. 2H). This tubular structure contained cells with axonal processes and included large HNK-1$^+$ neurons with a labelling pattern and cell body, axon and growth cone morphology characteristic of Rohon-Beard sensory neurons (Hughes, 1957; Nordlander, 1989) (*n*=65; Fig. 2F, G). There were also other HNK-1$^+$ neural cells which presumably represent other classes of neurons. Pigmented neural crest cells were also detected in the exogastrula ectoderm (stage ~36) of heterozygote albino exogastrulae derived from albino females and pigmented males (*n*=20; not shown).

Histological sections of exogastrulae ectoderm (stage ~32) showed no clear organization of neural structures (Fig. 3). In some cases, regions of the ectoderm which contain neural tissue (Fig. 3C, D, see below) exhibited a slight segregation from the surrounding ectodermal cells but often it was impossible to distinguish neural from non-neural ectoderm on the basis of histology alone. In contrast, histological examination of the mesodermal/endodermal region of exogastrulae and of normal embryos showed the presence of recognizable notochord and muscle fibers (Fig. 3A, B). These observations are consistent with the findings that neural cells are present but in a disorganized state in the ectoderm of Keller sandwiches (see below, Keller and Danilchik, 1988). It appears, therefore, that neural specific markers, such as Xen1 (Fig. 3D), are required to detect with certainty the presence of neural tissue in *Xenopus* exogastrula ectoderm.

Neural differentiation in Keller sandwiches

Previous in vitro studies have shown that neural tissue can be induced in the ectodermal portion of Keller sandwiches, explants of early gastrula dorsal tissue that are thought to mimick the convergent extension movements of dorsal cells observed in exogastrulae (Keller and Danilchik, 1988). Several studies have established that, in Keller sandwiches, the mesoderm is never in vertical apposition to the ectoderm but keeps a planar contact with the ectoderm (Keller and Danilchik, 1988; Dixon and Kintner, 1989). Induction of the neural antigen Xen1 was therefore examined in Keller sandwiches and the extent of neural tissue compared to that observed in complete exogastrulae. Whole-mount immunocytochemical labelling of Keller sandwich recombinates showed high levels of Xen1 extending well beyond the original junction area of mesoderm and ectoderm (Fig. 4; *n*=10). These results provide evidence that Xen1 can be induced in ectoderm which has not been exposed to a vertical mesoderm-derived inductive signal to an extent similar to that observed in the ectoderm of exogastrula embryos.

Establishment of A-P pattern by planar signals

Classical transplantation experiments have provided evidence that A-P neural pattern is imposed by underlying mesoderm (Mangold, 1933). However, the high degree of organization of neural tissue in the exogastrula ectoderm (Figs 2, 5) indicated that it may be regionalized. Indeed, the expression pattern of one marker, Xhox3 mRNA, has suggested the existence of A-P neural pattern in exogastrula ectoderm (Ruiz i Altaba, 1990). To examine this further, the expression of the homeobox protein engrailed (en-

2) (Hemmati-Brivanlou and Harland, 1989; Davis et al., 1991; Hemmati-Brivanlou et al., 1991) was monitored by labelling embryos in whole mount with the monoclonal antibody 4D9 (Patel et al., 1989). In normal embryos, en-2 is mainly expressed by cells forming a band at the junction of the midbrain and hindbrain (Hemmati-Brivanlou and Harland, 1989; Davis et al., 1991) (Fig. 5A, B). The highly restricted expression of engrailed makes this a useful molecular marker to test for regionalization of the neural ectoderm (Hemmati-Brivanlou and Harland, 1989).

Total exogastrulae also expressed en-2 within the neural tube with labelled cells forming a discrete band in the anterior region of the neural tube (25/30 embryos; Fig. 5C, D) indicating that en-2$^+$ cells appear in an approximately appropriate A-P position in exogastrulae. The total number of cells and the intensity of en-2 immunoreactivity was, however, reduced in comparison to that detected in normal embryos (Fig. 5B, D), suggesting that additional signals are necessary to generate the normal number of en-2$^+$ cells. In normal embryos, such signals could derive from the underlying mesoderm. Consistent with this, both anterior and posterior notochord have been shown to induce en-2 expression in animal cap ectoderm, albeit with different efficiencies (Hemmati-Brivanlou et al., 1990b). The restricted A-P domains of expression of the homeobox genes engrailed and Xhox3 (Ruiz i Altaba, 1990) in exogastrula ectoderm provides evidence that neural A-P pattern can be induced by planar signals in the absence of involuted mesoderm.

Regionalization of non-neural ectoderm in exogastrula was also observed. In normal tadpole-stage embryos, a cement gland and a characteristic group of cells expressing the HNK-1 (Fig. 5E) but not the Xen1-3 antigens (not shown) is prominent in the anteroventral region of the ectoderm, posterior to the cement gland. Similar cells were also observed in the ectoderm of exogastrulae, anterior to the neural tube (Fig. 5F). Moreover, the positional relationship between these distal cells and the cement gland was conserved in normal embryos and exogastrulae. Neither a cement gland nor distal cells consistently developed in animal cap ectoderm isolated from blastula or early gastrula embryos (not shown). Thus, planar signals appear to contribute more generally to the A-P patterning of the ectoderm.

Neuronal differentiation in the exogastrula neural tube

Neural tissue in exogastrulae contained large Rohon-Beard-like HNK-1$^+$ neurons (Fig. 2B', G) indicating the differentiation of specific neuronal types. However, the analysis of other morphologically and antigenically defined cell types revealed that the pattern of neural differentiation in exogastrula is incomplete.

First, eyes were never observed indicating a lack of certain forebrain cell types in the exogastrula neural tube (Figs 2, 6). Second, a group of neurons found in the ventral spinal cord and hindbrain of normal embryos was identified by expression of the Xit antigen, which appears to be associated with the nuclear envelope (Fig. 6B-D). Although the identity of Xit$^+$ cells is unclear, the position in the ventrolateral basal plate of the neural tube at stage ~36 in normal embryos of some of these cells is similar to that reported

Fig. 1. Position of mesodermal cells expressing Xbra during gastrulation and exogastrulation. (A, B) Whole-mount in situ hybridization of normal embryos at different stages of gastrulation showing the expression of Xbra (Smith et al., 1991). (A) At stage ~11, Xbra is expressed by mesodermal cells all around the marginal zone (mz). The weak, out of focus outline is the contour of the blastocoel (b). (B) At stage ~12-12$\frac{1}{2}$, the entire marginal zone expressed Xbra at higher levels than before. The notochord precursor cells (arrowhead) also express Xbra as they involute away from the closing blastopore (bp). (C, D) Whole-mountin situ hybridization of exogastrulae at stage 12-12$\frac{1}{2}$ showing the expression of Xbra by the mesoderm (mes) constricted in between the ectoderm (ect) and endoderm (end), both delineated by dots (C). (D) High magnification picture of the mesodermal region of an exogastrula showing Xbra expression in the nascent notochord (arrowhead). A shows an animal view of the labelled embryo. B shows a dorsolateral view of the labelled embryo, with the prospective anterior pole at the lower-right end. C, D show lateral views of exogastrulae with the ectodermal sac on top. Scale bar in A = 0.5 mm, valid for A-C. Scale bar in D = 100 μm.

Fig. 3. Histological differentiation of exogastrula and normal embryos. Cross sections of exogastrula (A, C, D) and normal (B) stage 28-30 embryos stained with hematoxylin and eosin (A-C) or labelled with mAb Xen1 by indirect immunofluorescence (D). (A) Cross section through the endoderm-mesoderm region of an exogastrula showing the presence of notochord (n), somite (s) and ventral (v) mesodermal cells surrounded by endoderm (en) which is filled with yolk platelets. The endodermal cells that normally line the archenteron roof (a) are found adjacent to the notochord. Note the absence of an archenteron and the segregation of the different tissues. (B) Cross section of a normal embryo showing the presence and characteristic organization of notochord (n), somite (s), endoderm (en), archenteron roof cells (a), non-neural ectoderm (ec) and neural ectoderm (ne). Note the presence of an archenteron (ar) or primitive gut, pigmented epithelial cells lining the canal of the neural tube and the bilateral segregation of the somites by the notochord. (C, D) Cross sections through the ectodermal sac of exogastrula embryos showing the presence of neural ectoderm, revealed by Xen1 labelling (light green in D), surrounded solely by non-neural ectoderm. The ventral ectoderm (lower regions in C, D) shows the characteristic roughling and heavy pigmentation of atypical epidermis also detected in isolated animal cap ectoderm. Note that histological examination of the ectoderm of exogastrulae does not allow the unequivocal identification of neural ectoderm (compare C and D). In all cases dorsal side is up. Scale bar = 100 μm, for all panels.

Fig. 7. Neural differentiation in embryos treated with high doses of lithium. Normal embryos (A, B) and embryos treated with high doses of lithium (C, D) were labelled at the tailbud stage (stage ~26) in whole-mount with mAb Xen1 (A, C) or mAb MZ15 (B, D). In normal embryos, mAb Xen1 labels most, if not all, of the neural tissue (A) and mAb MZ15 labels the mature notochord (B). In embryos treated with a high dose of lithium, gastrulation is incomplete as the entire marginal zone behaves as dorsal tissue. In these embryos, the blastopore (denoted by arrowheads) does not close completely. Labelling such embryos with mAb Xen1 (C) and mAb MZ15 (D) shows that notochordal tissue is found all around the dorsalized embryo and that neural tissue appears in close proximity. Arrowheads in all panels show the position of the closed (A) or open (C, D) blastopore. All panels show side views. The embryo in A is facing right, while that in B is facing left. In C, D the animal pole is on top. Scale bar in D = 0.5 mm, for all panels.

Fig. 8. Involvement of the notoplate in neural induction. (A-E) Notoplate extension in normal embryos and exogastrulae. (A) Localization of cells derived from blastomere B1. Rhodamine-lysine-dextran (RLD) was injected into a single blastomere B1 at the 32-cell stage. Most RLD-labelled cells were located in the notochord and notoplate, which later becomes the floor plate. (B) Interference contrast photograph of a pigmented exogastrula showing the groove (arrow) in the dorsal ectoderm and the lighter pigmentation of dorsal (neural) ectoderm when compared to ventral epidermis. (C) Fluorescence photograph of a B1-injected exogastrulae showing RLD labelled cells (red) that have undergone extension movements in the dorsal mesoderm (notochord) and ectoderm (notoplate). Note that B1-derived cells extend to the anterior end of the ectoderm. (D, E) The junction region between mesoderm (mes) and ectoderm (ect) of an exogastrula under interference contrast (D) and fluorescence (E) optics. The posterior end of the groove of the notoplate (ntp) and the thin neck region (arrowhead in E) mark the limit of involution, the junction with the notochord (not). In E, the plane of the limit of involution is at a slight angle in relation to the notoplate axis. RLD-labelled cells extend from the limit of involution into both mesoderm and ectoderm. (F-H) Determination of the spatial relationship between Rohon-Beard neurons and notoplate cells by lineage tracing. (F) Fluorescence photograph of a cross section of a B1-injected normal embryo at stage ~28 after whole-mount labelling with mAb HNK-1. RLD-labelled cells derived from B1 are located in the floor plate. Fluorescein-labelled cells are HNK-1^+ Rohon-Beard neurons located in the dorsal neural tube. (G, H) Fluorescence photographs of sections of the ectoderm of B1-injected exogastrulae labelled with mAb HNK-1. RLD-labelled cells are derived from blastomere B1 and fluorescein-labelled cells are HNK-1^+ neurons. Rhodamine and fluorescein-labelled cells comprise distinct populations in normal embryos (F) and exogastrulae (G, H). (I-K) Neural induction in animal cap/notoplate recombinates. (I) Interference contrast photograph of an isolated ectoderm sandwich. HNK-1 labelled cells were not detected after immunoperoxidase labelling. (J, K) Fluorescence photographs of sections of whole-mount-labelled animal cap ectoderm/notoplate recombinates showing the proximity and distinct lineages of RLD-labelled notoplate cells to the induced FITC-labelled HNK-1^+ neurons. The majority of HNK-1-labelled cells in ectodermal recombinates allowed to develop to the early tailbud stage showed the characteristic morphology of Rohon-Beard neurons. A, anterior; An animal pole; B1, blastomere B1; CNS, central nervous system; D, dorsal; Ect, ectoderm; Fb, forebrain; Fp, floor plate; Hb, hindbrain; Mes, mesoderm; Mb, midbrain; Not, notochord; Ntp, notoplate; P, posterior; Sc, spinal cord; V, ventral; Vg, vegetal. In B-E anterior is up. In F-H dorsal is up. Scale bar in B=0.5 mm, for B,C. Scale bar in D=100 μm, for D, E. Scale bar in F=50 μm, for F, G. Scale bar in H=10 μm. Scale bar in I=100 μm. Scale bar in J=50 μm. Scale bar in K=10 μm.

for motoneurons (Nordlander, 1986). The Xit antigen is also expressed in a more diffuse pattern by cells outside the neural tube in the surface ectoderm of the head (Fig. 6C). These two cell types can be distinguished by their position and subcellular localization of the Xit antigen. In over 90% of exogastrulae (48/50 embryos), Xit immunoreactive cells were absent from the neural tube at the early tadpole stage (Fig. 6E) at which time normal embryos displayed a large number of Xit^+ neurons (Fig. 6B, C). However, Xit^+ cells with a diffuse distribution of antigen were present in the superficial ectoderm of both normal embryos and exogastrulae (n=35; Fig. 6F). Consistent with the results with mAb Xit, similar experiments with mAbs directed against restrictin, a motoneuron-associated antigen (Rathjen et al., 1991) show that while the ventral spinal cord and hindbrain of normal *Xenopus* tadpoles were heavily labelled, only trace labelling was detected in the ectoderm of similarly staged exogastrulae (n=56; not shown). The few cells expressing restrictin in the exogastrula neural tube may be primary motoneurons (see Discussion).

Induction of forebrain structures by head mesoderm

Although in exogastrulae a cement gland consistently appeared anterior to the neural tube, eyes were never observed (Figs 2, 6) suggesting that the anterior CNS is absent from exogastrulae. The position of head mesoderm under the anterior neural plate in normal embryos (Keller and Danilchik, 1988) together with the result of transplantation experiments in which anterior mesoderm can induce head structures (Mangold, 1933; Ruiz i Altaba and Melton, 1989) suggest that the induction of the anterior CNS is dependent on vertical signals from head mesoderm. To test this possibility, late gastrula-stage pigmented anterior mesoderm was inserted into the isolated ectodermal sac of albino exogastrulae. Unpigmented eyes developed in 4/5 of these recombinates (Fig. 6J) showing that anterior mesoderm can induce the differentiation of anterior neural structures in exogastrula ectoderm.

Induction of ventral neurons by the notochord

The absence of Xit^+ and $restrictin^+$ neurons in exogastrula suggested that in frogs, as in chickens (Yamada et al., 1991), ventral neurons of the spinal cord and hindbrain may require inductive signals from the underlying mesoderm for their differentiation. To determine whether vertical signals from axial mesoderm are required for the differentiation of Xit^+ neurons, early neurula-stage notochord was inserted into the isolated ectoderm of an exogastrula at the same stage, in effect mimicking the vertical contacts present in vivo (Fig. 6A). These recombinates were then tested for the expression of Xit since this antigen, unlike restrictin, labels the cell bodies. In these recombinates, large numbers of Xit^+ cells were detected within the neural tube (4/5 recombinates; Fig. 6G-I). In contrast, Xit^+ neurons did not differentiate in recombinates of neurula-stage notochord and early gastrula-stage animal cap ectoderm even though large amounts of $Xen2^+$ neural tissue were observed (n=10; not shown). Ectoderm alone did not develop Xit^+ cells (n=10; not shown). These results suggest that the notochord is required for the differentiation of Xit^+ neurons and that it may act only on ectoderm that has previously been exposed to planar inductive signals.

The effects of RA on cell patterning in *Xenopus* embryos (Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991a, b) together with evidence that the chick notochord (Hornbruch and Wolpert, 1986) and floor plate may be sources of retinoids (Wagner et al., 1990) and that these tissues can induce the differentiation of ventral neuronal cell types (Yamada et al., 1991) suggested that RA might promote the differentiation of Xit^+ neurons in exogastrulae. However, RA treatment of exogastrulae (10^{-7}-

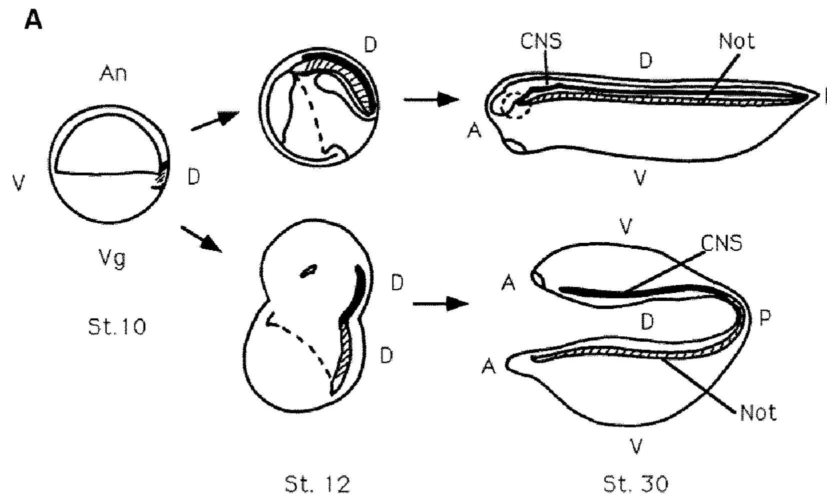


Fig. 2. Exogastrula embryos display a patterned neural tube. (A) Diagram showing the location of midline mesodermal (hatched) and neural (filled) structures in normal embryos (top figures) and embryos induced to exogastrulate (lower figures). The evagination of mesoderm in exogastrulae results in the connection of this ectodermal sac to the endomesodermal mass only by the posterior junction (P) or limit of involution. (B-B') Early tadpole-stage (stage 28-30) normal (B) and exogastrulated (B') embryos display HNK-1^+ cells including Rohon-Beard neurons (RB) in the neural tube, distal cells (dc) located anteroventrally and posterior to the cement gland (cg) and big fat cells (bfc) in the ventral endodermal region. The head mesodermal region also labels with HNK-1 in normal (not shown) and exogastrulated embryos (B'). Rohon-Beard neurons can be identified by their large size and labelling of the cell body and axon, including a large growth cone, with mAb HNK-1. Immunoreactivity in the cell body is concentrated in the plasma membrane and in a cytoplasmic inclusion. (C-D') The neural markers Xen1 (C, C') and Xen2 (D,D') are expressed in the neural tube of normal (C, D) and exogastrulated (C', D') tadpole-stage embryos (stage 34-36). (E, E') Expression of MZ15 antigen by the notochord and otic vesicle (ov) in normal (E) and exogastrulated (E') tadpole stage-embryos (stage 34-36). The notochord does not enter the ectoderm in exogastrulae. Arrowheads in D' and E' point to the limit of involution. (F) A section through the neural tube of a normal embryo showing Rohon-Beard neurons (arrowheads) identified by their characteristic morphology and the expression of the HNK-1 epitope in dorsal regions at the early tadpole stage (stage 28-30). HNK-1^+ cells with a morphology characteristic of Rohon-Beard neurons are also present in the neural tube of exogastrulae (G). (H) A section showing the general expression of Xen1 immunoreactivity in the exogastrula neural tube. In B-E' anterior is to the left except in C' where anterior is up. In F-H dorsal side is up. Labelling with each of the different Abs was performed on 50-70 exogastrulae derived from at least three independent batches of embryos. A, anterior; An, animal, bfc, big fat cells; bl, blastopore lip; cg, cement gland; CNS, central nervous system; D, dorsal; dc, HNK-1^+ distal cells; Not, notochord, ov, otic vesicle; P, posterior; RB, Rohon-Beard neurons; V, ventral; Vg, vegetal. Scale bar in E' = 0.5 mm, for B-E'. Scale bar in F=20 μm, for F, G. Scale bar in H=20 μm.

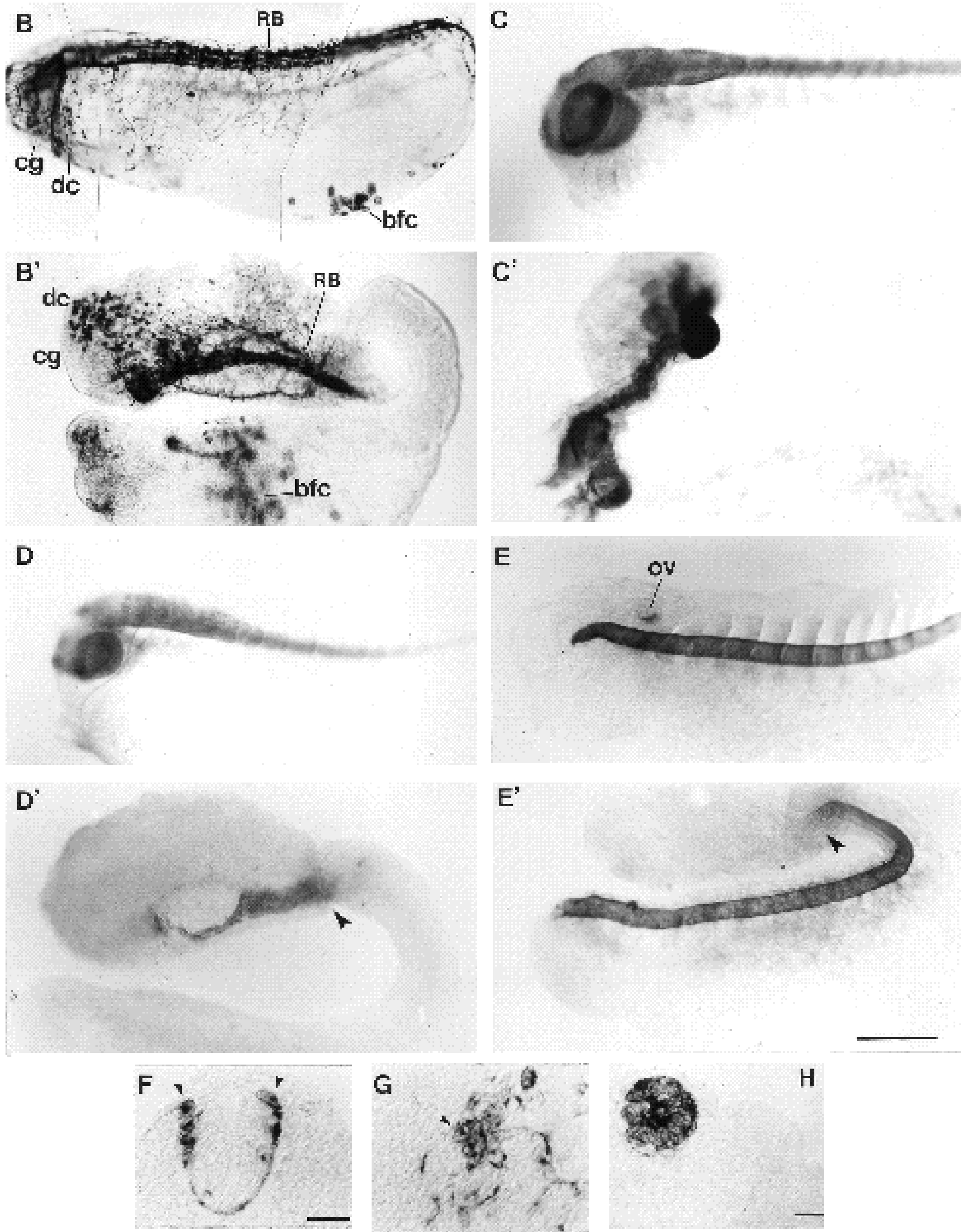
10^{-8} M at stage 13-14) did not result in the differentiation of Xit$^+$ neurons (*n*=62; not shown).

Extent of neural differentiation in extreme dorsalized lithium-treated embryos

The formation of an elongated neural tube in the dorsal side of exogastrulae could be due to the action of long-range planar signals or to the progressive transmission of short-range planar signals along the future A-P axis in the dorsal side. To test for the range of action of neural-inducing signals, the extension movements that occur normally during gastrulation were prevented such that most of the dorsal ectoderm would not be in contact with involuted axial mesoderm. To this end, early blastula-stage embryos were treated with high doses of lithium (Fig. 7; Kao et al., 1986; Kao and Elinson, 1988; Cooke and Smith, 1988). This treatment results in the acquisition of dorsal character by all mesodermal cells so that the entire marginal zone behaves as Spemann's organizer or dorsal lip in transplantation assays (Kao and Elinson, 1988) and differentiates exclusively into axial mesoderm. These embryos appeared to be deficient in convergence and extension movements. Indeed, labelling such embryos at the early tadpole stage with mAb MZ15 shows that notochordal tissue is detected as a ring around the still open blastopore (*n*=5; Fig. 7B, D). The changes in shape of the embryo are likely to be due mainly to epiboly by ectodermal cells. The extent of neural differentiation in embryos treated similarly was assesed by labelling with mAb Xen1. Neural tissue was detected only in a narrow band of ectoderm near the mesoderm with most of the ectoderm not expressing the Xen1 antigen (*n*=5; Fig. 7A, C), suggesting that neural-inducing signals operate only at short distances.

Notoplate extension in exogastrula ectoderm

The results discussed above raise the possibility that the formation of the neural tube in exogastrula ectoderm requires the spread of short-range planar inducing signals along the A-P axis. This could be achieved by a signalling cascade or by the movement of the signalling cells. In normal embryos, the development of the neural plate is accompanied by the A-P extension of a group of midline ectodermal cells termed the notoplate (Jacobson, 1981; Keller and Danilchik, 1988; Jacobson and Sater, 1988). Notoplate convergent extension movements and the appearance of a characteristic dorsal midline groove also occur in Keller sandwiches, in vitro explants of dorsal tissue (Keller and Danilchik, 1988). Exogastrula ectoderm also exhibited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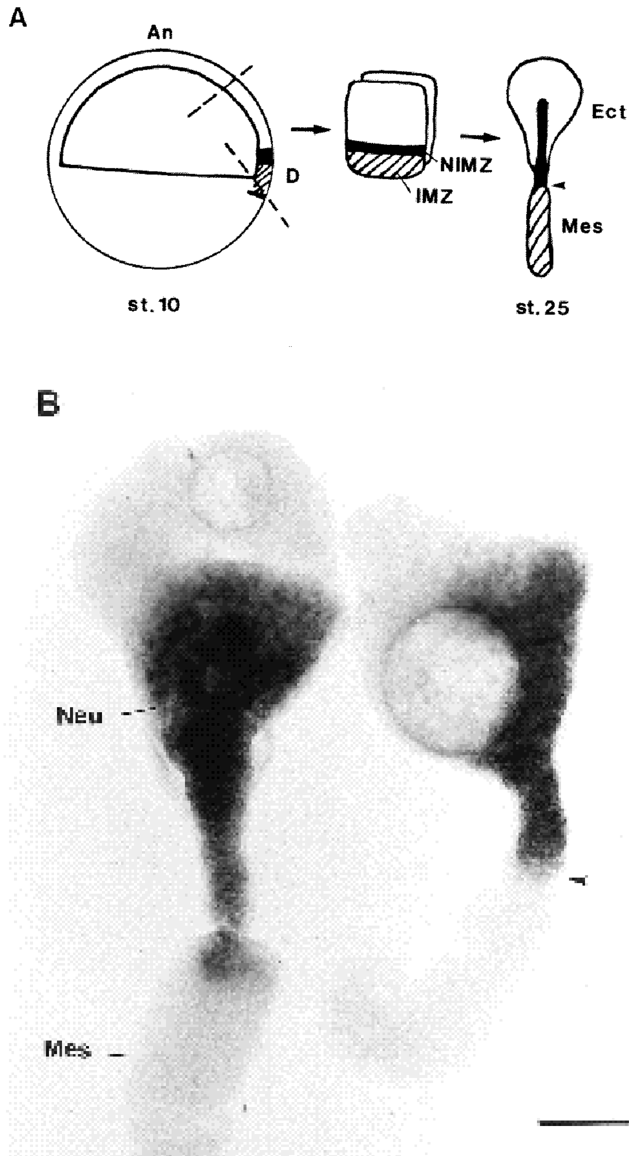


Fig. 4. Neural differentiation in Keller sandwiches. (A) Schematic diagram showing the procedure used to prepare Keller sandwiches and the location of the dorsal (D) NIMZ (non-involuting marginal zone) and IMZ (involuting marginal zone) cell groups. Sandwich explants were prepared from stage 10+ embryos. (B) Expression of the Xen1 antigen in the ectoderm of Keller sandwiches visualized by whole-mount immunoperoxidase labelling. Note the extent of neural tissue, the elongation of both the NIMZ and IMZ and the vesicle that normally forms in the non-neural portion of the ectoderm. Arrowheads point to the limit of involution. An, animal pole; Ect, ectoderm; Mes, mesoderm; Neu, neural ectoderm. Scale bar = 100 μm.

a groove in its dorsal midline (Fig. 8B) suggesting that the notoplate may extend into the ectoderm of exogastrulae. The formation of an extended neural tube could therefore result, in part, from the progressive induction of surrounding ectoderm to become neural tissue by planar signals originating from the extending notoplate.

To examine the fate of notoplate cells in exogastrulae, rhodamine-lysine-dextran (RLD) was injected into a single B1 blastomere of 32-cell-stage *Xenopus* embryos (Fig. 8A). As shown previously (Jacobson and Hirose, 1981; Dale and Slack, 1987), in normal tadpole-stage embryos labelled cells were found in the axial mesoderm, predominantly in the notochord and in the notoplate, which later gives rise to the ventral midline of the central nervous system (CNS), primarily the floor plate (n=10; Fig. 8F). Injection of RLD into blastomere B1 of embryos which were then induced to exogastrulate resulted in the presence of RLD-labelled cells in the dorsal region of the ectoderm (n=30; Fig. 8C-E), coincident with the area of ectoderm in which neural structures appeared (Fig. 2), and in the axial mesoderm. These observations provide evidence that the notoplate undergoes convergent extension movements in exogastrula ectoderm.

Involvement of the notoplate in neural induction

To examine the possibility that the elongating notoplate induces neural differentiation in exogastrula ectoderm, the distribution and lineage of the notoplate and of HNK-1^+ neurons was compared in the neural tube of normal embryos and exogastrulae. In normal embryos, HNK-1^+ Rohon-Beard neurons are located in the dorsal region of the neural tube (Nordlander, 1989) whereas notoplate/floor plate cells occupy the ventral midline (Fig. 8F). Rohon-Beard neurons were never labelled after injection of blastomere B1 with RLD. Thus, these two cell groups derive from different lineages (Jacobson and Hirose, 1981; Dale and Slack, 1987). Similarly, in exogastrulae in which blastomere B1 had been injected, large HNK-1^+ neurons were not RLD-labelled even though they appeared in the proximity of RLD-labelled cells in the dorsal ectoderm (n=15; Fig. 8G, H).

To determine more directly whether the notoplate can induce neural differentiation, the midline of the neural plate of normal pigmented early neurula-stage embryos that had been labelled completely with RLD was combined in vitro with unlabelled albino animal cap ectoderm from early gastrulae. In 23/29 recombinants, HNK-1^+ cells, most of which had the characteristic morphology of Rohon-Beard neurons were detected in recombinates but were not labelled with RLD, indicating that these neurons derived from the animal cap ectoderm, which also expressed Xen1 (not shown), and not from the notoplate (Fig. 8J, K). Neural differentiation was not detected in ectoderm cultured in the absence of the notoplate (Fig. 8I) or when combined with ventral ectoderm (not shown). Recombinates of animal cap ectoderm and lateral neural plate at stage 16 developed few HNK-1^+ cells (not shown). The number of HNK-1^+ neurons detected in animal cap/notoplate recombinates was about 10 times lower than the number of these neurons induced in exogastrulae (compare Fig. 8G, H and J, K; and not shown). This may reflect the difficulty in establishing an equivalent degree of contact between the notoplate and ectoderm in recombinates. Similarly, late gastrula-stage notochord was also able to induce HNK-1^+ Rohon-Beard neurons (7/13 recombinates; not shown) and Xen2^+ neural tissue (13/13 recombinates; not shown) in ectodermal recombinates although, again, the number of induced neurons was much lower than observed in exogastrula ectoderm.

To estimate the percentage of cells in the exogastrula

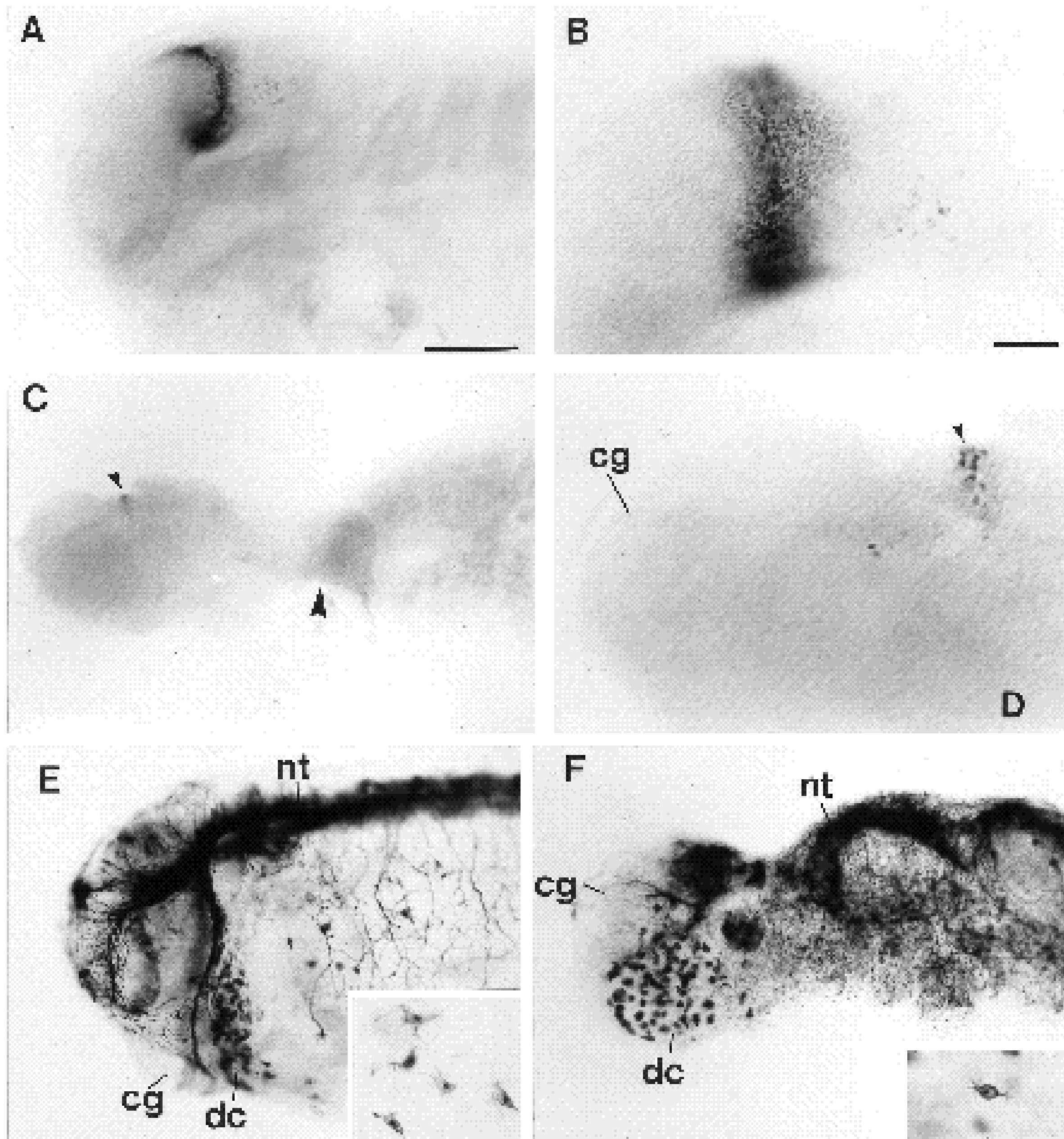
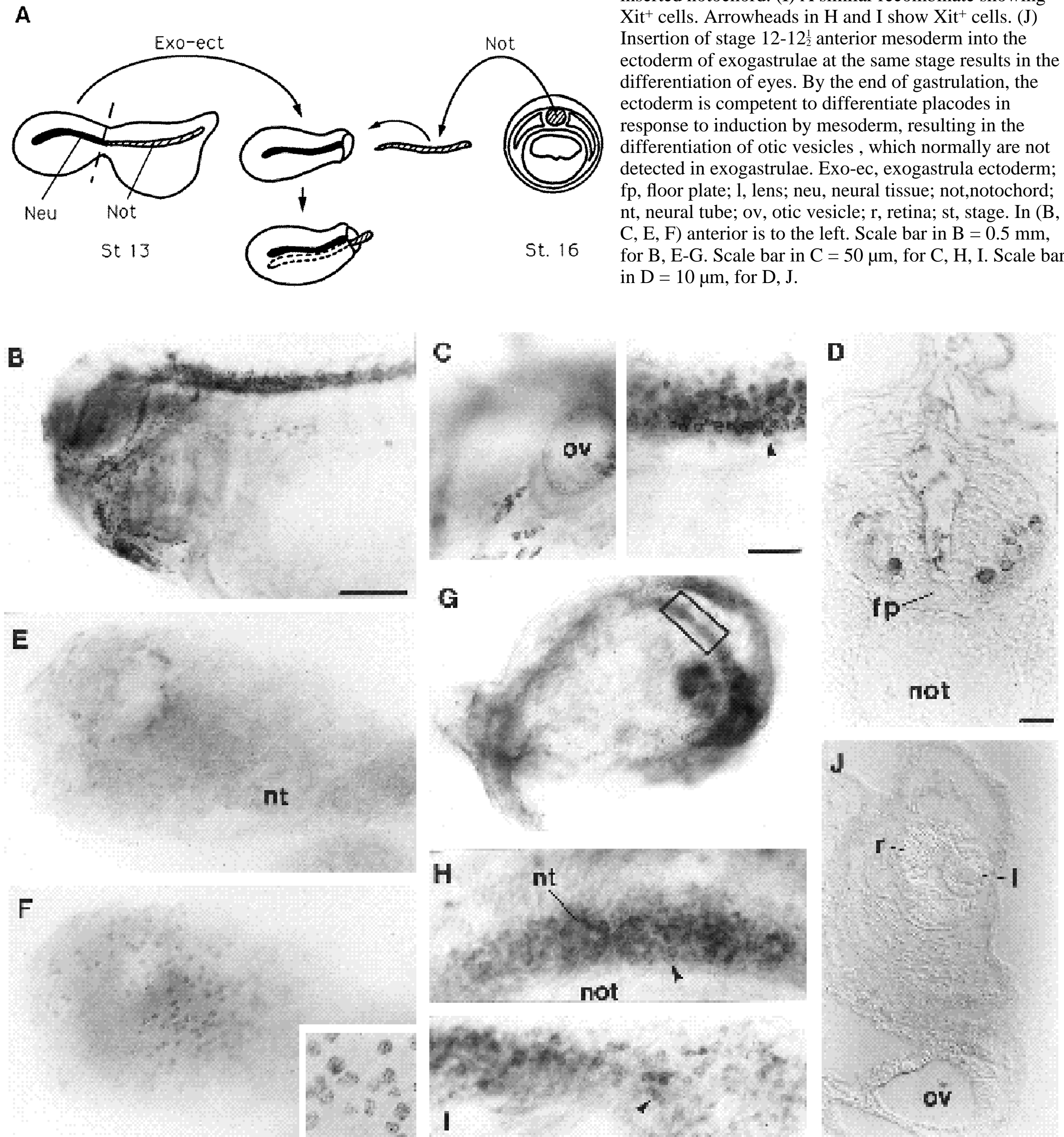


Fig. 5. Anteroposterior pattern in exogastrula ectoderm. (A-D) Engrailed (en-2) homeobox protein expression detected by whole-mount immunoperoxidase labelling with mAb 4D9 in normal (A, B) and exogastrulated (C, D) tadpole-stage (stage ~36) embryos. En-2 expression in normal embryos is detected in a band at the midbrain/hindbrain junction and in a few cells in the lateral hindbrain (A, B). In total exogastrulae (C, D), en-2 expression is localized in the anterior region of the neural tube. The small arrowheads in C, D point to the region of en-2 expression. The large arrowhead in C points to the limit of involution. D shows a high magnification picture of the anterior ectoderm of the embryo shown in C. (E, F) HNK-1 antigen expression in tadpole-stage (stage ~38) normal embryos (E) and exogastrulae (F). At this stage, many neuronal cells are labelled by the mAb HNK-1 in the neural tube (nt). Note the expression of HNK-1 by a group of distal cells (dc) outside of the neural tube in an anteroventral position, posterior to the cement gland (cg) in a normal embryo (E) and an exogastrula (F). The high magnification insets in E, F show the morphology and HNK-1 labelling pattern of distal cells. In all panels anterior is to the left. Labelling with each mAb was done on 50-100 exogastrulae derived from different batches of embryos. Scale bar in A = 0.5 mm, for A, C, E, F. Scale bar in B = 50 μm, for B, D.

neural tube which are derived from blastomeres other than B1, this blastomere was labelled with RLD and the resulting exogastrulae were subsequently labelled with Xen1 (*n*=5; not shown). B1-derived cells comprised only 20-30% of neural cells. As in the case of HNK-1$^+$ neurons, blastomere A1 gave rise to 10-20% of Xen-1-labelled neural cells (*n*=10; not shown) while blastomere B2, which normally populates most of the spinal cord (Dale and Slack,

Fig. 6. Effects of axial mesoderm on neural pattern. (A) Scheme showing the method of insertion of mesoderm into the ectodermal sac of exogastrulae. The ectoderm of stage 13 exogastrulae was dissected, avoiding the ectodermal region close to the junction with mesoderm and combined with dissected notochord (as shown) or anterior mesoderm isolated from neurula-stage or late gastrula-stage embryos, respectively. (B-D) Whole-mount labelling of a tadpole-stage (stage 36) embryo with mAb Xit. (B, C) Xit-immunoreactivity is expressed by different cell types including ventral neurons with labelling in the region of the nuclear envelope and superficial ectodermal cells with diffuse labelling in the head (arrowhead in C). (D) Cross section through the anterior spinal cord of a normal stage 36 tadpole embryo showing that within the neural tube, most cells expressing Xit are found in the basal plate and occupy a ventral position characteristic of motoneurons. Xit immunoreactivity in the neural tube first appears at the late tailbud stage (stage ~26-28, not shown). (E-F) Xit antigen expression in exogastrulae. In tadpole-stage (stage 32-34) exogastrulae, Xit-immunoreactivity is absent from the neural tube (E) but present in some superficial ectodermal cells (F). Inset in F shows labelled ectodermal cells at high magnification. (G-I) Recombinates of exogastrula ectoderm and notochord show Xit expression in cells in the neural tube. (G) Low-magnification photograph of a recombinate labelled in whole-mount with mAb Xit. (H) A high magnification of the region of the neural tube boxed in G shows the presence of Xit^+ cells inside the neural tube near the inserted notochord. (I) A similar recombinate showing Xit^+ cells. Arrowheads in H and I show Xit^+ cells. (J) Insertion of stage 12-12½ anterior mesoderm into the ectoderm of exogastrulae at the same stage results in the differentiation of eyes. By the end of gastrulation, the ectoderm is competent to differentiate placodes in response to induction by mesoderm, resulting in the differentiation of otic vesicles , which normally are not detected in exogastrulae. Exo-ec, exogastrula ectoderm; fp, floor plate; l, lens; neu, neural tissue; not,notochord; nt, neural tube; ov, otic vesicle; r, retina; st, stage. In (B, C, E, F) anterior is to the left. Scale bar in B = 0.5 mm, for B, E-G. Scale bar in C = 50 µm, for C, H, I. Scale bar in D = 10 µm, for D, J.



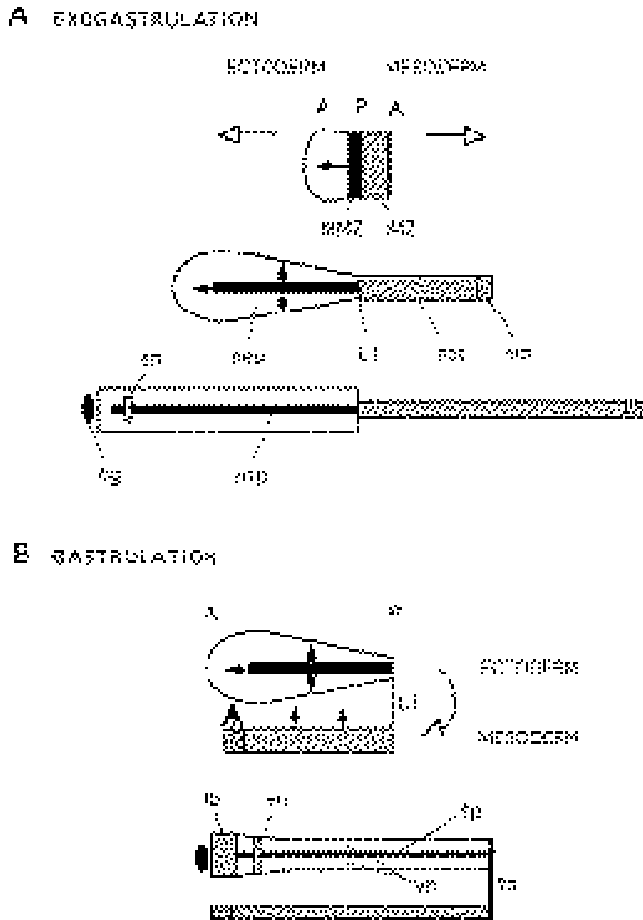


Fig. 9. Schematic model showing cell movements and inductive interactions of midline cells involved in the formation of the nervous system. (A) Diagram depicting the convergent extension movements and inductive signalling of midline ectodermal and mesodermal cells in exogastrulae. The dorsal non-involuting marginal zone (NIMZ) cells which give rise to the notoplate, and the involuting marginal zone (IMZ) cells which give rise to the head mesoderm and notochord, are found in the dorsal lip region. For simplicity, all diagrams represent these axial cell groups and the induced tissue at different stages of development. NIMZ and IMZ cells elongate in opposite directions forming the notoplate (ntp) and notochord (not), respectively. Open arrows represent cell movements. The axial mesoderm and ectoderm are connected only at the posterior pole (P), marked by the limit of involution (LI). The proposed involvement of the notoplate in the induction of the exogastrula neural tube is indicated by filled arrows which represent neural-inducing signals. The A-P location of the induced cement gland (cg) and the band of engrailed-2-immunoreactive neurons (en-2) is also shown. hm, head mesoderm; neu, induced neural ectoderm; A, anterior.(B) Diagram depicting the convergent extension movements and inductive signalling of midline cells in normal embryos. During gastrulation, the axial mesoderm involutes under the overlying dorsal ectoderm. The notoplate and notochord are therefore coextensive, unlike in exogastrulae (A). This juxtaposition of midline mesodermal and ectodermal cells allows the head mesoderm to induce the forebrain (fb) in anterior ectoderm and the notochord to induce a floor plate (fp) and ventral neuronal types (vn) in the posterior CNS, completing the formation of the embryonic nervous system. tb, tail bud.

1987) gave rise to about 60% of cells in the exogastrula neural tube (n=2; not shown). Taken together, these results suggest that the notoplate can induce neural tissue and that planar signals derived from the notoplate may be involved in neural induction.

Discussion

The experiments reported here provide evidence that *Xenopus* exogastrulae develop organized neural tissue of posterior character with A-P polarity. Patterned neural tissue develops in response to planar signals in the apparent absence of involuted axial mesoderm. These results extend previous studies showing the expression of neural genes in exogastrula ectoderm and Keller sandwiches (Kintner and Melton, 1987; Dixon and Kintner, 1989; Ruiz i Altaba, 1990).

Neural differentiation by planar signals

The use of several neural markers shows the presence of neural tissue in the ectoderm of exogastrula embryos extending well beyond the border of the mesoderm and ectoderm and appearing as an elongated tubular mass. The Xen1 neural-specific marker was used to compare the degree of neural induction in apparently complete exogastrule and in Keller sandwiches in which mesodermal cells are never in vertical apposition with ectoderm (Keller and Danilchik, 1988). The extent of Xen1-labelled neural tissue was similar under both experimental conditions. This suggests that the differentiation of neural ectoderm in exogastrulae is independent of vertical signals from underlying mesoderm; even if invagination does occur transiently at the onset of exogastrulation.

The presence of neural tissue in the ectoderm of *Xenopus* exogastrulae differs from the conclusions reached from classical studies by Holtfreter and others in urodele amphibians such as *Ambystoma* (Holtfreter, 1933; see other references in Spemann, 1938; Hamburger, 1988). There are at least two possible reasons for these differences. First, the propensity for neural differentiation by ectodermal cells of different amphibian species may differ. *Xenopus* ectoderm fails to respond to many of the non-specific neural inducers described as effective by Holtfreter and colleagues in other species (Kintner and Melton, 1987; Kintner, personal communication). In addition, the fates of dorsal cell groups at early gastrula stages in *Xenopus* differ from those in the urodele *Ambystoma* in which some mesoderm derives from the superficial cell layer of the organizer (Smith and Malacinski, 1983). It is possible, therefore, that different amphibian species exhibit differing degrees of convergence and extension, with the consequence that in extreme urodele exogastrulae, the extension movements of the NIMZ may fail to occur, preventing the formation of the elongated neural structure detected in *Xenopus* exogastrulae.

Second, it remains possible that the studies of Holtfreter and others, which were performed in the absence of molecular markers of neural differentiation, failed to detect disorganized neural cells. Indeed, in the ectoderm of *Xenopus* exogastrulae (Fig. 3) or in Keller sandwiches (Keller and Danilchik, 1988), the presence of neural tissue is not

detectable by histology. It will therefore be important to use molecular markers and lineage tracing techniques in urodele exogastrulae to determine whether there are significant differences in the process of neural induction in different amphibian species.

Neural induction and the formation of a neural tube

The differentiation of most of the posterior neural tissue observed in *Xenopus* exogastrulae could result from a direct planar induction of adjacent ectoderm by dorsal lip mesoderm. However, with the exception of the induction of the notoplate (see below) dorsal lip mesoderm may induce relatively little neural tissue in a planar fashion before exogastrulation movements begin. The notoplate may then act to induce surrounding ectoderm to become neural, inducing first prospective brain cells. The posteriorly directed extension of the notoplate during gastrulation may allow the induction of neural properties in a large A-P area of the ectoderm, increasing the amount of neural tissue and leading to the formation of the prospective spinal cord (Figs 8, 9). A notoplate-derived planar neural signal may be propagated through the plane of the ectoderm by homeogenetic induction (Spemann, 1938; Leussink, 1970; Grunz, 1990; Servetnick and Grainger, 1991; Itoh and Kubota, 1991), with the limit of the neural plate controlled by the loss of competence of the ectoderm (Nieuwkoop and Albers, 1990). In cases where cell movements do not take place, neural differentiation would be predicted to occur to a more limited extent (Fig. 7; see also Jones and Woodland, 1989). However, direct experimental evidence to support the proposed role of the notoplate in neural development is difficult to obtain since the creation of notoplateless embryos with normal chordamesoderm has not been possible.

The signals required for the formation of the notoplate remain unclear. The notoplate arises from a region of ectoderm known as the dorsal non-involuting marginal zone (NIMZ), which lies just above the dorsal involuting marginal zone (IMZ), the dorsal lip mesoderm that is fated to form the notochord (Keller et al., 1985; Keller and Danilchik, 1988). Cells that give rise to the notoplate and the notochord undergo coextensive convergent extension movements during gastrulation resulting in the midline extension of these two tissues along the A-P axis forming the midline of the neural plate and axial mesoderm. The notoplate may be induced by neighboring dorsal lip mesoderm before the onset of gastrulation or by a graded signal from vegetal cells that is also responsible for inducing the notochord (Jacobson and Sater, 1988). In addition, dorsal ectodermal cells may be heterogeneous as a result of other signals (Sharpe et al., 1987; Savage and Phillips, 1989; Ruiz i Altaba and Jessell, 1991a; Sokol and Melton, 1991). This prepattern could contribute to the differential induction of notochord and notoplate cells.

Neural A-P patterning by planar signals

Classical transplantation experiments in amphibian embryos have suggested that the A-P pattern of the embryonic nervous system can be established by vertical signals from the underlying axial mesoderm (Mangold, 1933; Nieuwkoop et al., 1952). In contrast, the present results suggest that posterior neural tissue can be induced directly by planar signals. Moreover, the restricted expression of the Xhox3 (Ruiz i Altaba, 1990) and en-2 homeobox genes along the A-P axis of the exogastrula neural tube suggests that planar signals may induce A-P pattern of the neural plate during normal development. Indeed, the ability of both anterior and posterior chordamesoderm to induce en-2 expression in animal cap ectoderm (Hemmati-Brivanlou et al., 1990) suggests that the dorsal axial mesoderm does not define precise A-P values in the neural ectoderm. Thus, it is possible that the A-P pattern of the posterior neural tube is controlled primarily by planar signals derived from the notoplate or dorsal lip mesoderm cells. Gradation in such planar signals may underlie both the induction and patterning of the neural ectoderm. A predisposition of ectodermal cells to different fates could also contribute to the A-P patterning of the neural ectoderm (Ruiz i Altaba and Jessell, 1991a; Sokol and Melton, 1991).

It is possible that in normal embryos, A-P fates in both the mesoderm and overlying neural ectoderm are established concurrently but independently resulting in a congruency of the A-P pattern in both layers. The region where the prospective notochord (IMZ) and notoplate (NIMZ) cells are juxtaposed, the limit of involution (Fig. 9), defines the posterior pole of the developing embryo (see Keller and Danilchik, 1988). IMZ and NIMZ cells may both acquire their A-P values by using the limit of involution as posterior reference point. While A-P polarity may be initiated by planar signals prior to the onset of gastrulation, reciprocal vertical interactions between the dorsal mesoderm and neural ectoderm during gastrulation may refine A-P pattern (Suzuki et al., 1984).

Neuronal differentiation in response to planar and vertical signals

The analysis of neuronal differentiation indicates that only a subset of the cell types found in normal embryos develop in exogastrulae (Figs 2, 6). The development of these cell types, such as Rohon-Beard sensory neurons (Fig. 2) and possibly primary motoneuron (Lamborghini, 1980), may therefore be achieved by planar neural signals. Moreover, it is possible that the identity of these neurons depends on the position of neural precursors in the neural plate in relation to the notoplate at the midline.

The differentiation of neural cell types not present in exogastrulae may require additional vertical signals. For example, the development of ventral neuronal types which are determined at later stages, such as Xit^+ neurons, appears to depend on notochord-derived inductive signals (Fig. 6). Consistent with this, primary but not secondary motoneurons differentiate in the neural tube of notochordless *Xenopus* embryos (Clarke et al., 1991) and the differentiation of ventral neuronal classes in the chick hindbrain and spinal cord is dependent on signals from the notochord and the floor plate (Placzek et al., 1990; 1991; Yamada et al., 1991). At present, it is not known if all dorsal neuronal types can differentiate in response to early planar signals.

The differentiation of forebrain structures also requires vertical signals (Fig. 6). In previous studies (Dixon and Kintner, 1989; Sharpe and Gurdon, 1990), head mesoderm has been shown to be a poor anterior neural inducer when combined with blastula-stage animal cap ectoderm. How-

ever, head mesoderm can induce eye differentiation, indicative of anterior neural character, in the ectoderm of Keller sandwiches (Dixon and Kintner, 1989). Forebrain induction by head mesoderm may occur only when ectoderm has already received planar inductive signals, possibly from the notoplate.

Cooperation of planar and vertical neural signals

Collectively, the results presented here suggest that the development of the *Xenopus* CNS involves the actions of distinct planar and vertical signals (Fig. 9). Induction of the dorsal NIMZ is followed by the extension of the notoplate along the midline of the neural plate (Keller and Danilchik, 1988; Jacobson and Sater, 1988). The elongating notoplate may act as a source of planar signals involved in the induction and differentiation of the posterior CNS. Additional vertical signals from the notochord and head mesoderm may cooperate with planar signals to enhance and complete the pattern of differentiation of posterior neuronal classes and of the anterior CNS. Planar signals may also render the ectoderm competent to respond to vertical inductive signals from mesoderm and give rise to forebrain and ventral neurons. Local interactions within the neural tube are likely to refine further this initial pattern of cell differentiation (for example, see Nieuwkoop, 1989).

The coordinate actions of planar and vertical neural signals derived from midline ectodermal and mesodermal cells may also operate in other vertebrates. For example, in the chick embryo, the anterior tip of the primitive streak (Spratt and Haas, 1965; Nicolet, 1971; Schoenwolf and Smith, 1990) may induce neural differentiation in the surrounding epiblast, acting as a source of planar signals. As in *Xenopus*, vertical signals from head mesoderm may then be required to induce the forebrain and from the notochord to induce the floor plate and ventral neurons. Thus, many of the principles underlying early neural induction and patterning in *Xenopus* embryos may pertain to other vertebrates even though the precise cell movements of gastrulation differ in detail.

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