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Reconciling different models of forebrain induction and patterning: a dual role for the hypoblast

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SUMMARY

Several models have been proposed for the generation of the rostral nervous system. Among them, Nieuwkoop's activation/transformation hypothesis and Spemann's idea of separate head and trunk/tail organizers have been particularly favoured recently. In the mouse, the finding that the visceral endoderm (VE) is required for forebrain development has been interpreted as support for the latter model. Here we argue that the chick hypoblast is equivalent to the mouse VE, based on fate, expression of molecular markers and characteristic anterior movements around the time of gastrulation. We show that the hypoblast does not fit the criteria for a head organizer because it does not induce neural tissue from naïve epiblast, nor can it change the regional identity of neural tissue. However, the hypoblast does induce transient expression of the early markers *Sox3* and *Otx2*. The spreading of the hypoblast also directs cell movements in the adjacent epiblast, such that the prospective forebrain is kept at a distance from the organizer at the tip of the primitive streak. We propose that this movement is important to protect the forebrain from the caudalizing influence of the organizer. This dual role of the hypoblast is more consistent with the Nieuwkoop model than with the notion of separate organizers, and accommodates the available data from mouse and other vertebrates.

Movies available on-line:
http://www.biologists.com/Development/movies/dev4408.html

Key words: Forebrain, Prosencephalon, Head organizer, Trunk/tail organizer, Caudalization, Patterning, Activation-transformation, Hypoblast, Anterior visceral endoderm

INTRODUCTION

Recent evidence in mammals (reviewed in Beddington and Robertson, 1998, 1999; Knoetgen et al., 1999a) has been taken to support the idea that separate organizing centres exist for the vertebrate trunk and head (reviewed by Saxén and Toivonen, 1962), and that a 'head-organizing activity' resides in the anterior visceral endoderm (AVE), a tissue with an extraembryonic fate (see for example Knoetgen et al., 1999a). Evidence for this in other vertebrates is less clear. Amphibians and fish do not have an obvious extraembryonic endoderm, but various tissues with an embryonic fate have been ascribed this role (e.g. Glinka et al., 1998; Houart et al., 1998; Piccolo et al., 1999). In the chick, an early endodermal layer is present before gastrulation, which has an extraembryonic fate, but it is composed of at least two cell types: the hypoblast proper ('endophyll' of Vakaet, 1970 and Callebaut et al., 1999; 'primary hypoblast' of Stern, 1990) and the endoblast ('sickle endoblast' of Vakaet, 1970; 'secondary hypoblast' of Stern, 1990; see also Stern and Ireland, 1981; Bachvarova et al., 1998; Arendt and Nübler-Jung, 1999) (Fig. 1). It is unclear which of these cell populations is the direct equivalent of the AVE, since a detailed analysis of the expression of molecular markers has not yet been carried out in the chick.

Waddington's hypoblast rotation experiments (Waddington, 1930, 1932, 1933b), subsequently extended by Eyal-Giladi and co-workers (Azar and Eyal-Giladi, 1979, 1981; Mitrani et al., 1983, 1990b), first suggested a role for the early chick lower layer in regulating embryonic polarity. Eyal-Giladi and Wolk (1970) even reported that the chick early lower layer can induce a prosencephalon directly in the epiblast, based on morphological changes in trans-filter co-cultures. In contrast, Khaner (1995) reported that rotation of the lower layer does not alter embryonic polarity, and Knoetgen et al. (1999b) showed that the chick lower layer cannot induce neural or forebrain markers, while the rabbit equivalent can. Based on these results, Kessel and co-workers (Knoetgen et al., 1999a,b) have suggested that mammals may have evolved a new mechanism for patterning the head.

Because of these apparently contradictory results, we have undertaken a detailed investigation of the role of the chick lower layer in regulating axial polarity and in neural and head induction. We find that the hypoblast proper (but not the endoblast) expresses several markers that characterize the mouse AVE. However, grafts of the hypoblast induce neither mature neural tissue nor a patterned forebrain. They do, however, induce transient expression of the early markers *Sox3* and *Otx2*. By repeating Waddington's hypoblast rotation

experiments together with marking techniques and time-lapse filming, we show that the effect of the hypoblast on axial polarity is due to an ability of this tissue to direct cell movements in the overlying epiblast. These results therefore reveal a dual role for the hypoblast: an initial, transient, induction of *Sox3/Otx2*, and the coordination of cell movements of the prospective forebrain anteriorly. Finally we discuss these findings in the context of two alternative models for forebrain patterning. We conclude that, with some modification, Nieuwkoop's activation/transformation hypothesis (Nieuwkoop et al., 1952; Nieuwkoop and Nigtevecht, 1954) provides the best explanation for results from both chick and mouse.

MATERIALS AND METHODS

Quail eggs were obtained from Strickland Quail Farm, GA; hens' eggs were obtained from Spafas, CT (White Leghorn) or AA Labs, Ramona Duck Farm, CA (Rhode Island Red). Both chick and quail embryos were staged according to Hamburger and Hamilton (1951) for primitive streak and later stages (HH; Arabic numerals) and Eyal-Giladi and Kochav (1976) for prestreak stages (E-G&K; Roman numerals), and incubated at 38°C for 1-22 hours to give embryos at stages appropriate to each experiment.

Grafting techniques

Host embryos were explanted at HH3$^+$-4 and placed in modified New culture (New, 1955; Stern and Ireland, 1981). The region to be grafted was removed from the quail donor using the tip of a fine glass needle. Some of the grafts were placed in direct contact with the epiblast of the area pellucida by making a small hole in the lower layer of the lateral germinal crescent and placing the graft in the space between epiblast and lower layer. In other cases, the graft was apposed to the extraembryonic epiblast of the inner third of the lateral area opaca; in these cases, a flap of germ wall was used wherever possible to cover the graft and anchor it in place. Following transplantation, embryos were cultured for 4-24 hours at 38°C.

Immunocytochemistry

Embryos to be stained with QCPN antibody (to identify quail cells; obtained from the Developmental Studies Hybridoma Bank maintained by the department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205 and the Department of Biological Sciences, University of Iowa, Iowa City 52242, Under contract N01-HD23144 from the NICHD) were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) containing 0.2 M EGTA overnight at 4°C. Following in situ hybridization (see below) embryos were rinsed for at least 1 hour in several changes of PBS and then blocked for 30 minutes in blocking buffer (PBS containing 0.2% BSA, 0.5% Triton X-100 and 1% heat-inactivated goat serum). QCPN supernatant was added at a dilution of 1:5 in blocking buffer and incubated overnight at 4°C. After extensive rinsing in PBS, embryos were incubated in goat anti-mouse IgG-HRP (Jackson) diluted 1:2500 in blocking buffer overnight at 4°C. Finally, embryos were washed extensively in PBS and rinsed twice in 0.1 M Tris-HCl (pH 7.4), immersed for a few minutes in DAB (500 μg/ml in Tris-HCl), and H_2O_2 then added to a final dilution of 1:10,000 from a 30% stock. Staining was stopped by rinsing several times in tap water.

In situ hybridization

The expression of regional neural markers was assessed by whole-mount in situ hybridization using the protocol of Théry et al. (1995). Following in situ hybridization, embryos were postfixed in 4% formol saline, dehydrated for 5 minutes in methanol and 10 minutes in isopropanol, cleared for 30 minutes in tetrahydronaphthalene prior to embedding in paraffin wax and sectioned at 10 μm. The probes used are summarized in Table 1.

Sox3 and *Sox2* probes were transcribed from *Pst*I 3′ fragments of the cDNAs, to generate probes that recognized the appropriate transcripts in chick but not quail embryos.

Hypoblast rotation

Hypoblast rotation was performed in embryos at stage XI (E-G&K) to stage 3 (HH) in New culture (New, 1955; Stern and Ireland, 1981), while submerged in Pannett and Compton (1924) saline. After marking the posterior area opaca with powdered carmine, the hypoblast layer (including Koller's sickle in some cases) was detached using 27G hypodermic needles and rotated by 90° to the left (i.e., anticlockwise when the embryo is viewed from the ventral side) before removing the saline from above the embryo. The posterior edge of the rotated hypoblast was made to overlap the lateral marginal zone

Table 1

Probe	Marker for	Reference	Kind gift of
Otx2	hypoblast forebrain/midbrain	(Bally-Cuif et al., 1995)	L. Bally-Cuif, E. Boncinelli
Six3	anterior forebrain	(Bovolenta et al., 1996, 1998)	P. Bovolenta
Sox3	early general neural marker (chick-specific)	(Uwanogho et al., 1995; Collignon et al., 1996; Rex et al., 1997)	R. Lovell-Badge, P. Scotting
Sox2	general neural marker (chick-specific)	(Uwanogho et al., 1995; Rex et al., 1997; Streit et al., 1997)	R. Lovell-Badge, P. Scotting
Hex/PRH	hypoblast and foregut	(Yatskievych et al., 1999)	G. Goodwin
Crescent	hypoblast and endoblast	(Pfeffer et al., 1997)	P. Pfeffer
HNF3β	hypoblast and axial mesoderm	(Ruiz i Altaba et al., 1995)	A. Ruiz i Altaba
goosecoid	prestreak middle layer cells, hypoblast* and prechordal mesendoderm	(Izpisúa-Belmonte et al., 1993)	N/A
cCer/Caronte	hypoblast later left somites and lateral plate mesoderm	(King and Brown, 1999; Rodríguez Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999)	A. Lassar, M. Marvin
Nodal/*cNR1*	primitive streak later left lateral plate mesoderm	(Jones et al., 1995; Levin et al., 1995)	M. Kuehn
Dkk-1	hypoblast, posterior streak, other tissues later	(E. Laufer, unpublished)	E. Laufer
GANF/Rpx/Hesx1	anterior epiblast and anterior neural folds	(Hermesz et al., 1996)	S. Mackem
chordin	node, notochord	(Streit et al., 1998)	T. Jessell, K. Lee

*NB: *goosecoid* is expressed in the hypoblast of White Leghorn, but not Rhode Island Red strain.

of the host. The operated embryo was then transferred to a 35 mm Petri dish over a pool of albumen for culture and time-lapse videomicroscopy.

In some experiments, quail donors and chick hosts were used. The quail donor was placed ventral side up on the same vitelline membrane as the chick host. The posterior area opaca of the chick host was marked with carmine and the hypoblast of the host peeled away. The hypoblast layer of the quail donor was then excised and slid over to the host, whilst rotating it by 90°. After filming or culture, the chimaeras were fixed and processed with QCPN antibody and whole-mount in situ hybridization as described above.

In other experiments, hypoblast rotation was coupled with marking of cells that should normally contribute to the organizer (coordinates {0,20} at stage XII and {0,40} at stage XIII) or to the forebrain (coordinates {0,20} at stage XI, {0,35} at stage XII and {0,60} at stage XIII) based on the fate maps of Hatada and Stern (1994), using the carbocyanine dyes DiI and DiO (Molecular Probes). In a few cases, one of the dyes was placed as described above and the other dye was placed in an equivalent position but rotating the coordinates by 90° (as if the polarity of the embryo was that of the rotated hypoblast). Time-lapse filming was used to follow the movements of cells in embryos labelled with only one dye (DiI), because we observed decreased survival in embryos labelled with DiO that were exposed to epifluorescence illumination throughout their development.

Time-lapse video analysis

Time-lapse video filming was done using a Zeiss Axioplan microscope fitted with epifluorescence and transmitted light optics and mechanical shutters (UniBlitz) for both light sources, and a cooled, integrating CCD camera (Princeton Instruments, model TEA/CCD-1317-K/1). The shutters and image acquisition were controlled by a Digital computer running MetaMorph v2.5 software (Universal Imaging Corp.), and individual frames stored using an optical-magnetic disk recorder (OMDR; Panasonic LQ-3031). Unlabelled embryos were filmed by collecting a single monochrome frame under transmitted light optics, at 4 minute intervals. DiI-labelled embryos were filmed by collecting one transmitted light image (stored in the green and blue channels of a RGB pseudo-colour pallette) and one fluorescence image (stored in the red channel); in these cases, one frame was taken every 10 minutes.

RESULTS

Molecular analysis of the chick lower layer

The visceral endoderm (VE) of the mouse is a layer of tissue that surrounds the epiblast before gastrulation. Marking experiments have shown that, during gastrulation, its anterior part (the AVE) gradually moves towards the anterior half of the egg cylinder, probably propelled by differential growth (Lawson and Pedersen, 1987; Thomas and Beddington, 1996; Weber et al., 1999). The VE is eventually displaced by streak-derived definitive endoderm, and most of it becomes extraembryonic (Lawson and Pedersen, 1987). The AVE expresses characteristic molecular markers: *Otx2* (Simeone et al., 1993; Ang et al., 1994), *HNF3β* (Sasaki and Hogan, 1993; Weinstein et al., 1994), *Hex* (Thomas et al., 1998), *gsc* (Blum et al., 1992), *cerberus* (Belo et al., 1997; Shawlot et al., 1998), *Hesx1/Rpx* (Hermesz et al., 1996; Thomas and Beddington, 1996) and *nodal* (Conlon et al., 1994; Varlet et al., 1997). Of these, *Hex* is restricted to the AVE from 5.5 dpc. The remaining markers either start more broadly throughout the VE and become restricted to the AVE during gastrulation (*Otx2, nodal, gsc, cerberus*), or start to be expressed during gastrulation, specifically in the AVE (*HNF3β and Hesx1/Rpx*). These observations suggest that the VE is divided into at least two distinct regions prior to the onset of gastrulation.

As in the mouse, the chick early lower layer is subdivided into two regions (Fig. 1). The first, or hypoblast, forms a complete layer covering the epiblast by stage XIII (Vakaet, 1970; Eyal-Giladi and Kochav, 1976; Stern and Ireland, 1981; Stern, 1990). Soon afterwards, cells are added to this lower layer from the posterior edge, forming the endoblast, which displaces the hypoblast anteriorly (the 'sickle endoblast' of Vakaet, 1970; see also Stern and Ireland, 1981; Bachvarova et al., 1998). Both components have an extraembryonic fate (Modak, 1966; Nicolet, 1970, 1971; Vakaet, 1970; Rosenquist, 1971, 1972; Fontaine and Le Douarin, 1977; Wolk and Eyal-Giladi, 1977; Stern and Ireland, 1981) and can be distinguished by cell morphology (Stern and Ireland, 1981). Despite the superficial similarity between the chick hypoblast and the mouse AVE, a detailed comparison of the appropriate markers has not yet been undertaken in the chick.

Fig. 2 shows that, like the mouse VE, the hypoblast expresses *Otx2* (Bally-Cuif et al., 1995), *HNF3β* (Ruiz i Altaba et al., 1995; up to stage 3$^+$), *gsc* (as previously reported; Hume and Dodd, 1993; Bachvarova et al., 1998), and *cCer/caronte*

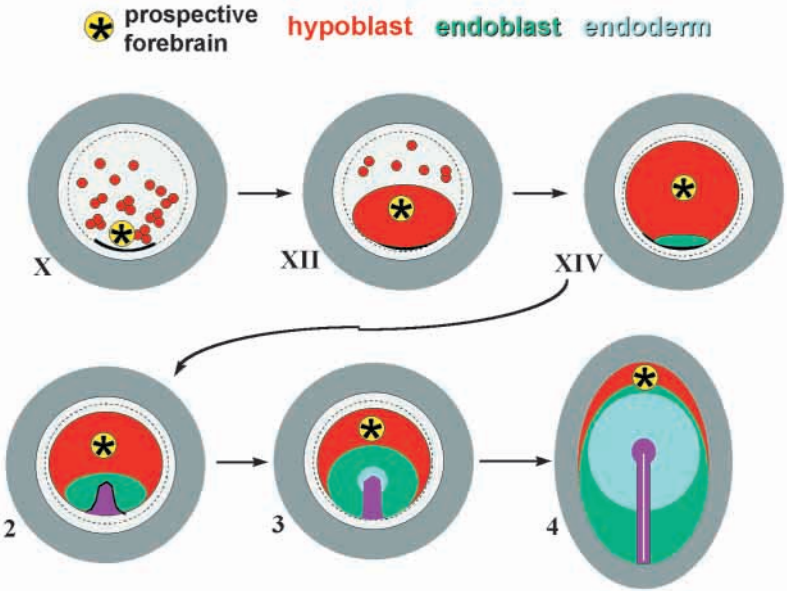

Fig. 1. Diagram illustrating the movements of the various components of the lower layer in the chick embryo. The hypoblast (red) arises from islands of cells present at the time of laying (stage X), and gradually coalesces into a layer in a posterior-to-anterior direction. At stage XIV, shortly before the appearance of the primitive streak (purple), a second component, the endoblast (green) starts to spread from the posterior margin of the germ wall, displacing the hypoblast further anteriorly. At stages 3-3$^+$, a third component, the definitive endoderm (light blue) starts to insert into the primitive lower layer at the tip of the streak. These are streak-derived cells which, unlike the previous two, have an embryonic fate. The diagram also shows the position of the centre of the territory that will contribute to the forebrain (star), based on the fate maps of Hatada and Stern (1994).

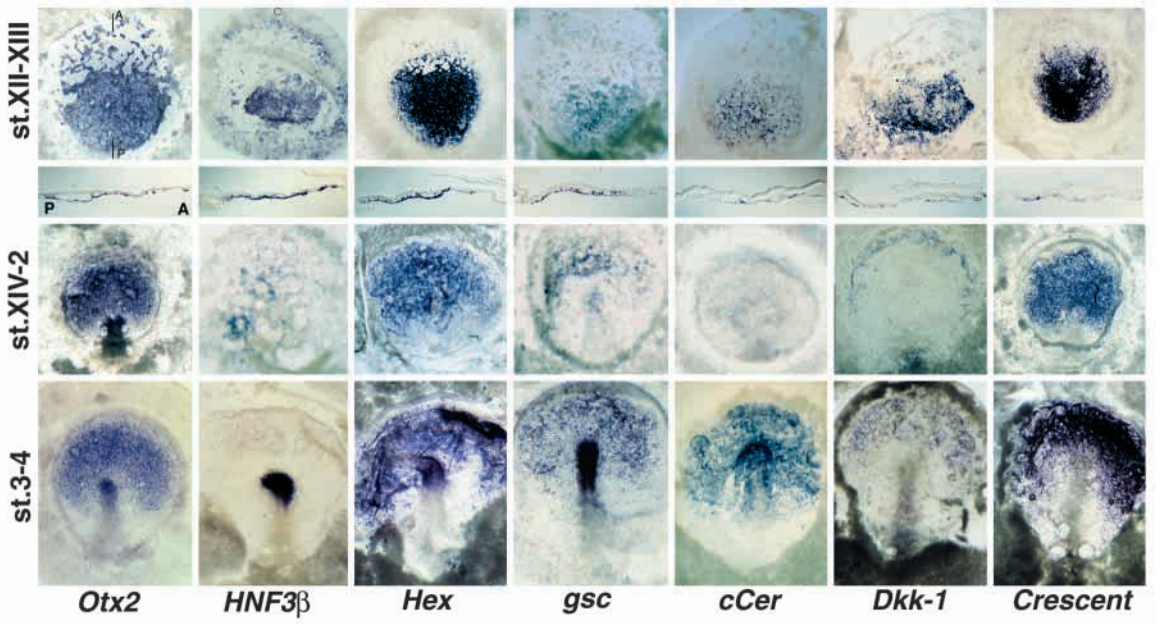

Fig. 2. Expression of the chick homologues of genes that mark the AVE of the mouse. In addition to *Otx2*, *HNF3β*, *Hex*, *goosecoid* and *cCer*, we also show the expression of *Dkk-1* and *Crescent*, which has not yet been described in the mouse. Most of these genes are markers for the chick hypoblast. Note that *cCer* is downregulated at about stage XIV, but reappears in the hypoblast later. Histological sections of stage XII-XIII embryos are shown.

(Rodríguez Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999; up to stage XIII, then again at stages 3^+-4). As the endoblast forms, all of these markers become restricted to the anterior part of the embryo (Fig. 2). Despite these similarities, there are also some differences. In the chick (unlike the mouse), *Rpx/Hesx1/GANF* (Kazanskaya et al., 1997) only starts to be expressed at the late primitive streak stage (stage 4^+), in the remnants of the hypoblast located in the germinal crescent as well as in prechordal mesoderm and the epiblast overlying it. In mouse, *Hex* demarcates a unique domain prior to gastrulation, whereas, in chick, *Hex* (Yatskievych et al., 1999) expression is identical to the other hypoblast markers until late streak stages. The only known chick homologue of *nodal* (Jones et al., 1995) is not expressed in the hypoblast (data not shown). In addition, *Dkk-1*, which does not appear to be expressed in the VE of the mouse (Glinka et al., 1998), and *Crescent* (Pfeffer et al., 1997), whose mouse homolog has not yet been described, both mark the hypoblast of chick (Fig. 2). In conclusion, despite some differences in the timing of expression of some markers, our study indicates that the chick early lower layer (hypoblast and endoblast) is equivalent to the mouse VE. Within this layer, the chick hypoblast is the closest equivalent to the mouse AVE.

The hypoblast does not induce neural tissue or forebrain in competent epiblast

To test whether the hypoblast acts as a true 'head organizer', we tested its ability to induce forebrain directly in a region of epiblast that can form a complete neural axis (including forebrain) in response to grafts of Hensen's node (Gallera and Ivanov, 1964; Gallera and Nicolet, 1969; Dias and Schoenwolf, 1990; Storey et al., 1992). We define 'forebrain' as tissue that expresses both general neural and regional prosencephalic markers. We selected markers which, from stage 6-7, are specifically expressed in the entire neural plate (*Sox3*, *Sox2*) and throughout the forebrain and midbrain (*Otx2*). The hypoblast was removed from stage XII and XIII quail embryos and grafted into the inner third of the lateral area opaca of stage 3^+ chick hosts (Fig. 3A, upper diagram). After 18-24 hours' incubation (the hosts had reached stage 7-10), embryos were fixed and processed by in situ hybridization with *Sox3*, *Sox2* and *Otx2* and by immunostaining with the quail marker QCPN. None of the embryos showed induced expression of any of these markers (0/9, *Sox3*; 0/8, *Sox2*; 0/8, *Otx2*; Fig. 3B,C and not shown).

One possible reason for the failure of hypoblast grafts to induce these markers is that, in normal development, the competence of the epiblast to respond to signals from the hypoblast may be confined to pre-primitive streak stages (when the hypoblast is adjacent to it). To address this possibility, quail hypoblasts from stage XII and XIII embryos were grafted to the lateral marginal zone of prestreak chick hosts, grown overnight and assessed by in situ hybridization for *Sox2* and *Otx2*. None of the embryos showed induced expression of Sox2 (0/7; Fig. 3D) or *Otx2* (0/6; not shown). These results confirm and extend those of Knoetgen et al. (1999b) and suggest that the hypoblast does not act as a 'head-organizer': it neither induces neural tissue nor does it emit signals sufficient for the formation of an ectopic forebrain in regions that are competent to do this in response to a graft of Hensen's node.

The hypoblast does not alter the regional identity of prospective hindbrain

Although the hypoblast lacks the ability to induce anterior neural tissue directly, it may have a patterning function similar to the prechordal mesoderm, which can rostralize the prospective hindbrain in the chick (Dale et al., 1997, 1999; Foley et al., 1997; Muhr et al., 1997; Pera and Kessel, 1997)

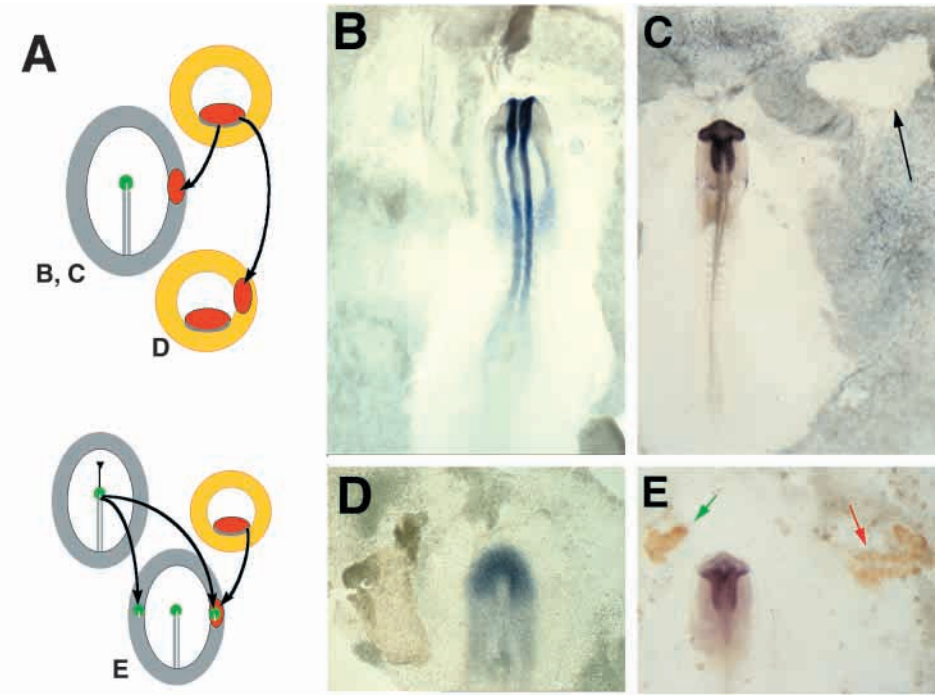

Fig. 3. The hypoblast does not induce neural tissue or forebrain, and does not rostralize posterior CNS. (A) Diagrams of the operations performed. The upper diagram shows a graft of a quail stage XII hypoblast to the lateral area opaca of a pre-primitive streak or primitive streak stage chick host. (B-D) The operated embryos were incubated overnight and the results shown. The grafted hypoblast does not induce (B) the early neural marker *Sox3*, or (C) the forebrain marker *Otx2* (arrow) in stage 3^+ hosts, or (D) the expression of *Sox2* in prestreak stage hosts. The lower diagram in A shows the design of an experiment to test whether the hypoblast can rescue the ability of older Hensen's nodes (stage 5-6) to induce a forebrain. On the left, an old node is grafted by itself as a negative control; on the right, the node is grafted together with a quail stage XII hypoblast. (E) Results of this experiment showing that neither the old node by itself (green arrow) nor an old node with a hypoblast (red arrow) induces expression of *Otx2* in the chick host.

and in the mouse (Ang and Rossant, 1993). We used a similar assay as that previously employed to reveal this activity in prechordal mesoderm (Foley et al., 1997): grafts of quail hypoblast were placed adjacent to the presumptive hindbrain region in stage 3^+-6 embryos. After 24 hours incubation, embryos were assessed for ectopic expression of the anterior neural markers *Six3* and *Otx2*. No ectopic expression was found (0/3, *Six3*; 0/6, *Otx2*; not shown).

One possible reason for the failure of the hypoblast to rostralize the hindbrain in this experiment could be that it was performed at a stage when prospective hindbrain cells may already have lost their competence to respond to hypoblast signals. To overcome this, we took advantage of the fact that Hensen's node loses its ability to induce forebrain between stages 4 and 5 (Dias and Schoenwolf, 1990; Storey et al., 1992); however, such older nodes can induce a forebrain when grafted together with prechordal mesendoderm (Foley et al., 1997). To test whether the hypoblast can similarly rescue the ability of old nodes to induce anterior markers, stage 5 quail nodes were grafted together with stage XII/XIII quail hypoblast into the area opaca of a stage 3^+ chick host (Fig. 3A, lower diagram). After 24 hours, no ectopic expression of *Otx2* (0/15) was observed in the host (Fig. 3E). Taken together, these results suggest that the hypoblast does not provide signals that can generate forebrain from more caudal prospective nervous system.

The hypoblast regulates an early phase of *Otx2* and *Sox3* expression in the epiblast

The territory of epiblast covered by the spreading hypoblast at stages XI-XIII transiently expresses *Otx2* (Bally-Cuif et al., 1995), raising the possibility that the hypoblast may regulate this expression. To test this, we grafted stage XII/XIII quail hypoblast to the inner third of the area opaca of a stage 3^+-4 chick host (Fig. 4A) and assessed the expression of *Otx2* after 4-6 hours. 12/18 embryos showed ectopic expression of *Otx2*; in four of these, the ectopic expression was continuous with the endogenous domain of *Otx2*. However, in the remaining eight (Fig. 4C), the ectopic and normal domains of expression were well separated. Histological analysis confirmed that *Otx2* expression had been induced in the host epiblast overlying the graft (Fig. 4E; 4/6 cases analyzed).

At stages XI-XIII, the expression of *Sox3* resembles that of *Otx2*: an expanding domain of expression in the epiblast

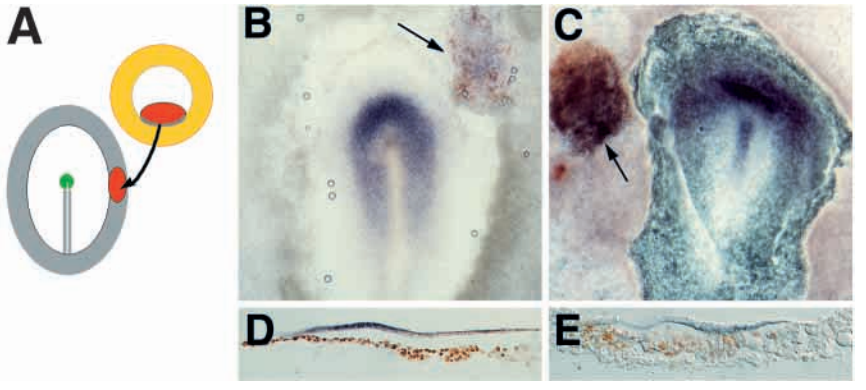

Fig. 4. The hypoblast transiently induces expression of *Sox3* and *Otx2*. (A) Diagram of the operation. After 4-8 hours, the grafted hypoblast has induced expression of (B) *Sox3* (arrow) and (C) *Otx2* (arrow). Histological sections confirm that the expression of both *Sox3* (D) and *Otx2* (E) has been induced in the epiblast of the host embryo. QCPN (quail) nuclear staining, brown; in situ hybridization signal, purple.

overlies the spreading hypoblast (Uwanogho et al., 1995). We therefore tested for induction of *Sox3* by the hypoblast in a similar experiment. To ensure that the ectopic *Sox3* expression is in the chick host epiblast, we used a 3′ probe that hybridizes with chick but not with quail *Sox3*, and the results confirmed by sectioning. *Sox3* was not induced by grafts of hypoblast after 4 hours (0/14). However, after 8 hours incubation, *Sox3* induction was observed (8/14, of which four had expression well-separated from the host domain; Fig. 4B,D).

By definitive streak stages, the hypoblast has been displaced into the anterior portion of the embryo (germinal crescent) by the endoblast and definitive endoderm (Modak, 1966; Nicolet, 1970, 1971; Vakaet, 1970; Rosenquist, 1971, 1972; Fontaine and Le Douarin, 1977; Wolk and Eyal-Giladi, 1977; Stern and Ireland, 1981; Stern, 1990). As shown above (and Fig. 2), the hypoblast at this stage still expresses most of the markers described (but not *HNF3β*; three other genes, *cCer/caronte*, *goosecoid* and *Dkk-1*, are downregulated shortly afterwards). To test whether this older hypoblast retains its ability to induce *Otx2* expression, hypoblast from the germinal crescent of stage 3^+/4 quail embryos was grafted to the inner third of the area opaca of stage 3^+/4 chick hosts and analysed for ectopic expression of *Otx2* after 4-6 hours. In no case (0/6) was ectopic expression observed, suggesting that, by stage 3^+, the hypoblast of the germinal crescent has lost its ability to induce *Otx2*.

Together, these experiments suggest that the pre-primitive streak stage hypoblast has the ability to induce *Sox3* and *Otx2*. However, this induction is only transient and is not followed by expression of more definitive markers of either neural tissue or forebrain.

The hypoblast controls cell movements but not cell fates

(i) Hypoblast rotation causes the primitive streak to bend

After rotating the hypoblast layer by 90° in pre-primitive streak stage embryos (stage XI-XIII; *n*=74), we observed that the primitive streak arose from the normal location but, in almost all embryos, subsequently developed a bend, with the tip of the primitive streak pointing in the direction of spreading of the rotated hypoblast (Fig. 5E,F). When embryos were allowed to develop beyond the primitive streak stage (to stages 5-9), the axes gradually became straight, but their orientation was a compromise between the original polarity of the embryo and that of the rotated hypoblast (Fig. 5G). Comparable results were obtained when the hypoblast layer was rotated after primitive streak formation (stages 2-3; *n*=12). Our results therefore confirm those of Waddington (1932, 1933b).

In 4 of the 74 cases where the rotation was performed before primitive streak formation, a second primitive streak appeared, from the left side of the embryo (where the hypoblast had been placed at the time of the operation). These secondary primitive streaks progressed more slowly and ultimately disappeared, despite the fact that two embryos analyzed showed expression of *goosecoid* at the tip (Fig. 5K,L).

Identical results were obtained whether Koller's sickle had been included with the rotated hypoblast or not. To ensure that the effects seen are due to the hypoblast itself, and not to contaminating middle layer cells, the same operation was done using a quail lower layers and chick hosts (*n*=10). The results were identical to those described above, and QCPN staining and histological analysis revealed that no quail cells were present in the primitive streak, in its derivatives, or in the embryonic or extraembryonic mesoderm (Fig. 5J).

As described above, the lower layer comprises two different cell populations: the hypoblast anteriorly (which expresses *goosecoid, HNF3β, Otx2, Hex* and *Dkk-1*), and the endoblast posteriorly, which does not express any of these markers (see above and Bachvarova et al., 1998). To test whether this intrinsic polarity of the lower layer is important in orienting the axis, we performed 90° hypoblast rotation coupled with 180° reversal of the axis of polarity of this tissue (i.e., the original anterior edge of the hypoblast placed over the left marginal zone of the host embryo, and its original posterior edge towards the centre of the area pellucida). Identical results were obtained as those described above for simple 90° rotation, suggesting that the direction of physical spreading of the hypoblast layer may be more important in directing cell movements than any intrinsic polarity of the lower layer.

(ii) Hypoblast rotation distorts the fate map by redirecting cell movements

Waddington (1932, 1933b) already recognized that the rotated hypoblast could alter the orientation of the primitive streak either by induction (i.e., changing the fates of prospective axial cells in the epiblast) or by affecting cell movements. Despite some attempts (Waddington, 1933b), he was unable to distinguish between these two alternatives. Subsequent authors (e.g. Azar and Eyal-Giladi, 1979, 1981) interpreted the experiments as implying induction, but neither cell fates nor cell movements were analyzed. Here we examined both, using a combination of time-lapse filming to visualize cell movements and DiI/DiO labelling to trace cell fates.

We studied the influence of the hypoblast on cell fates in embryos labelled with DiI/DiO to mark prospective organizer cells in the epiblast and an equivalent population 90° away (Fig. 5E-I), followed by hypoblast rotation (*n*=42). We also traced the movements and fates of prospective forebrain cells (Figs 5A-D, 6A-D) (*n*=19). In all cases we found that both the organizer and the forebrain were made up of cells derived from the territories that normally contribute to these structures, and were devoid of any cells from the equivalent site at 90°.

Time-lapse filming of both labelled (Fig. 6) and unlabelled (not shown) embryos revealed a dramatic influence of the hypoblast on epiblast cell movements. In embryos where the hypoblast had been rotated by 90° before primitive streak formation (*n*=10), the endogenous 'Polonaise' movements (Gräper, 1929) of the host continued normally, but a second centre of similar movements arose on the left side, where the hypoblast had been placed. The two simultaneous sets of movements appeared to compete with each other, resulting in a rightwards distortion of the normal movement pattern (e.g. Fig. 6C, 0-13.8 h). When rotation was performed after the appearance of the primitive streak (stage 2-3; *n*=2), contact of the leading edge of the expanding, rotated hypoblast with the primitive streak appeared to encourage both elongation of the streak and emergence of mesoderm laterally, causing the primitive streak to bend in the same direction as the spreading

hypoblast (not shown). This effect occurred very quickly, within about 20 minutes of the leading edge of the hypoblast contacting the primitive streak. These results suggest that the bending of the primitive streak elicited by the rotated hypoblast has different causes in pre-primitive streak and primitive streak stages. Before streak formation, the hypoblast generates competing epiblast movements that distort those of the host. After streak formation, the hypoblast appears to influence both streak elongation and the emergence of mesoderm cells from it, which together cause the streak to bend.

Time-lapse analysis of DiI-labelled embryos (*n*=5; Fig. 6) also confirmed that rotation of the hypoblast distorts the fate map by displacing all axial territories in the direction in which it spreads. Our findings support the conclusions reached by Callebaut et al. (1999), although they did not use molecular markers or trace cell movements directly.

Taken together, these experiments reveal that the hypoblast influences the axial polarity of the embryo and the position of the future organizer and forebrain territories due to an effect on cell movements, without affecting cell fates.

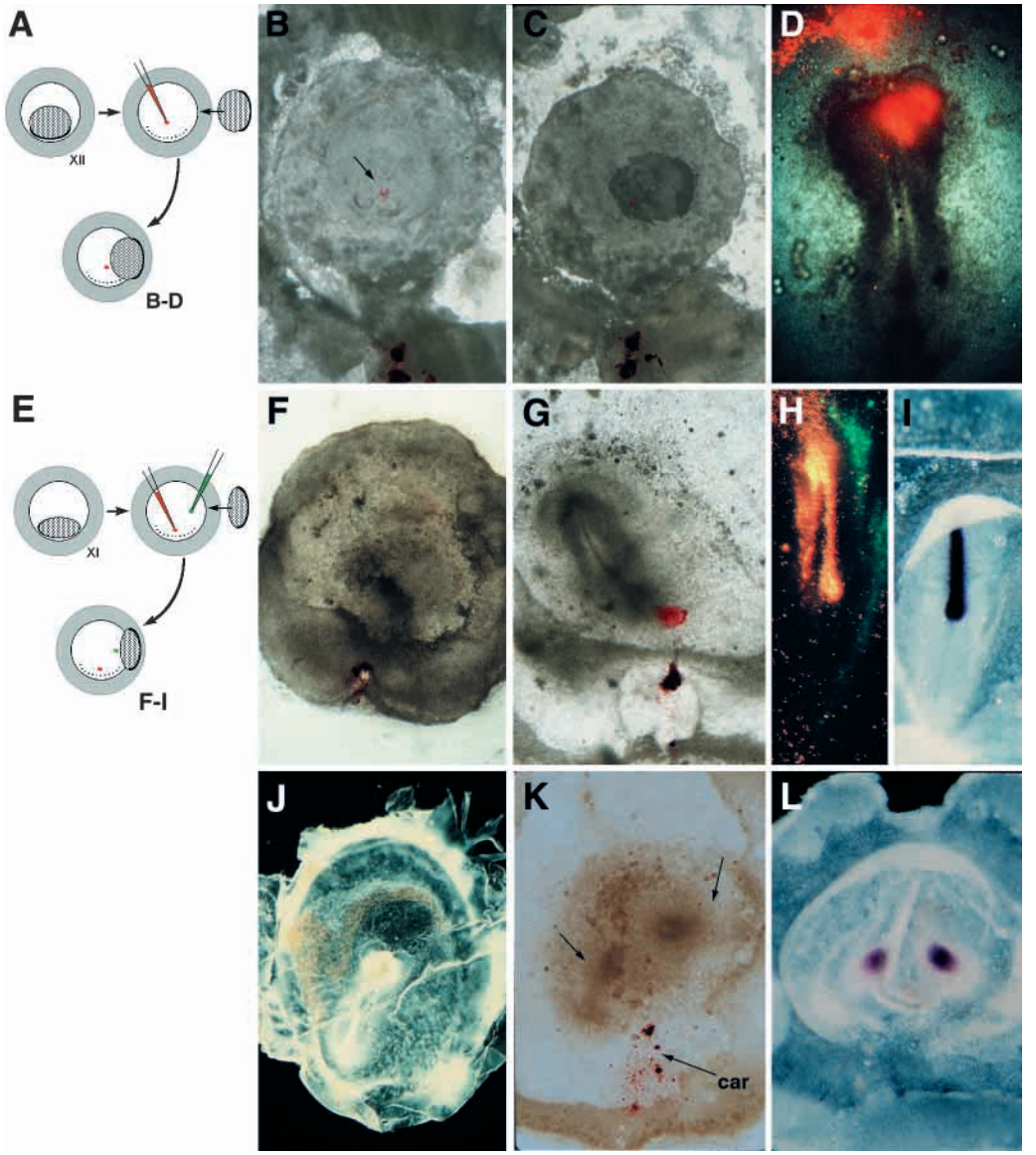

Fig. 5. Hypoblast rotation does not change cell fates. (A) Diagram of the experiment performed. At stage XII, the hypoblast is removed and the centre of the prospective forebrain territory marked with DiI, before replacing the hypoblast, rotated by 90° to the left (note that embryos are viewed from the ventral side). (B) Embryo with its hypoblast removed, immediately after placing the DiI mark (arrow). (C) The same embryo after regrafting of the (rotated) hypoblast, together with Koller's sickle. (D) The same embryo after 24 hours incubation – the DiI-labelled cells have contributed mostly to the forebrain (some label is also seen in the germinal crescent; this is derived from accidental labelling of the hypoblast overlying the marked region of epiblast). (E) Operation to test whether the hypoblast alters the fate of prospective organizer cells. A similar experiment to that in A above was performed, but labelling the prospective organizer territory with DiI (red) and a similar population but 90° away with DiO (green). This second population should normally contribute to more lateral tissues. The hypoblast is then replaced, rotated by 90°. (F) About 12 hours after the operation, the primitive streak arises from the original posterior end (carmine-marked), but displays a sharp bend to the right. (G) Some 23 hours after the operation, the axis has straightened somewhat but lies obliquely to the original posterior end. The embryo was fixed 1 hour later. (H) Under fluorescence, the axial tissues are labelled with DiI and more lateral tissues with DiO, as would be expected from the normal fate map; although the rotated hypoblast has distorted the orientation of the axis (not seen in the photograph), it has not altered cell fates. (I) The same embryo as in F-H, following in situ hybridization with the axial marker *chordin*, confirming that DiI-labelled cells are indeed in the head process and axial endoderm. (J) Embryo in which the hypoblast rotation had been performed using a quail donor for the hypoblast and a chick host. Quail cells are revealed by immunostaining with QCPN (brown stippling), and appear around (mostly to the right) but not within the embryonic axis. (K) One of the few cases in which hypoblast rotation generated two primitive streaks (arrows). 'car' indicates the position of the carmine mark used at the time of the operation to label the posterior pole of the embryo. (L) The same embryo following in situ hybridization with *goosecoid*, showing that both primitive streaks are terminated by a node.

DISCUSSION

A number of different theories have been put forward to explain how the vertebrate nervous system is patterned during development. Among them, two have been particularly favoured by recent investigators: the idea of separate organizers for the head and trunk/tail proposed by Spemann and his followers (Spemann, 1931, 1938; Mangold, 1933) and the 'activation-transformation' model of Nieuwkoop (Nieuwkoop et al., 1952; Nieuwkoop and Nigtevecht, 1954). We begin this discussion by considering recent evidence both in favour and against these two models, primarily in mouse and chick. Then, based on the present results, we propose a modification of Nieuwkoop's hypothesis that reconciles the two models and accommodates the available data from other systems (Fig. 7).

Models for induction and patterning of the vertebrate CNS

(i) Separate organizers for the head and trunk/tail

Spemann (1931, 1938), Mangold (1933) and Holtfreter (1936, 1938) proposed that separate organizing centres are responsible for inducing different regions of the CNS directly. This was based primarily on the initial finding of Spemann's that grafts of a young organizer induce a complete CNS, while older organizers induce only posterior structures. Soon afterwards, it was found (Holtfreter, 1936, 1938) that different regions dissected from the gastrula-stage organizer, as well as heterologous tissues such as liver and kidney (Holtfreter, 1933, 1934) and various chemical inducers (Chuang, 1938) can induce either forebrain or more posterior structures (reviewed in Toivonen, 1978). It is this set of findings that led away from the original idea that a single organizer region gradually changes its ability to induce different parts of the CNS, to the more extreme idea that head, trunk and tail organizers may be independent entities.

Some recent evidence has been interpreted as supporting the notion of separate organizing centres for the trunk and head in amphibian and mammalian embryos (see for example, Glinka et al., 1997, 1998; Knoetgen et al., 1999a,b; Dosch and Niehrs, 2000). Unlike the 'organizers' of chick and amphibian, which induce complete neural axes when grafted to an ectopic site, the mouse node appears to lack the ability to direct the formation of head structures (Beddington, 1994; Tam et al., 1997). Grafts of the Early Gastrula Organizer (EGO) from early streak stage embryos induce posterior, but not anterior, neural structures (Tam et al., 1997). Older nodes, from 0B-stage (Downs and Davies, 1993) donor embryos, also induce ectopic neural axes lacking head structures (Beddington, 1994). Furthermore, mice

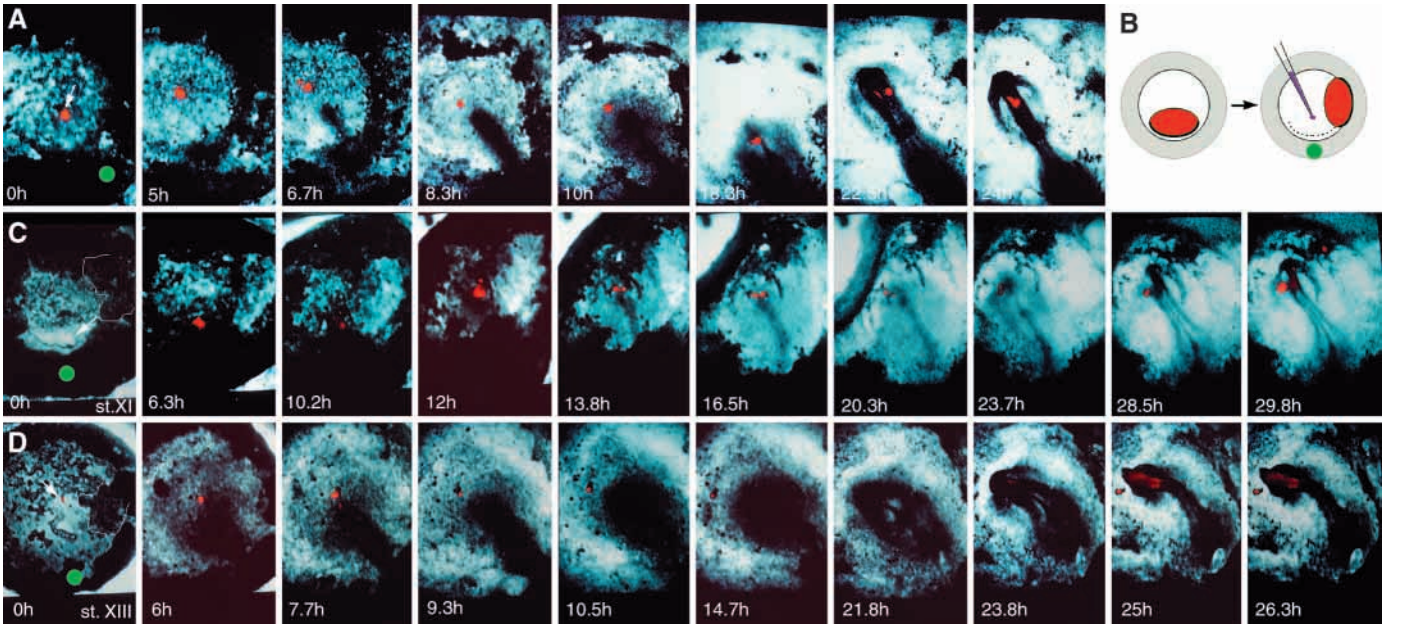

Fig. 6. The hypoblast directs cell movements, but does not alter cell fates. This figure is composed of frames from three time-lapse videos (which may be viewed as supplementary material at http://www.biologists.com/Development/movies/dev4408.html), illustrating our main findings. (A) Control embryo in which prospective forebrain cells were labelled with DiI (red) at stage XII. The green dot at the start of the film indicates the posterior pole of the embryo. Note the anterior movement of the red mark before the appearance of the primitive streak (0-6.7 hours); the tip of the primitive streak then almost catches up with the mark, but eventually this mark remains in the epiblast overlying the prechordal region. By 22-24 hours it is clear that the labelled cells are contained within the forebrain. (B) Diagram of the operation performed before filming the embryos illustrated in C,D: a DiI mark was placed, as described above, and the hypoblast then rotated by 90° (as in Fig. 5, all embryos shown from the ventral side). (C) Embryo in which the operation was performed at stage XI. Note that at this stage the prospective forebrain cells (white arrow) are located close to the posterior pole. The rotated hypoblast is highlighted with a thin white line. As before, the labelled cells move anteriorly before the primitive streak appears. The primitive streak then develops (13-20 hours), but is bent away from the side where the hypoblast was grafted. Eventually (23-30 hours) the labelled cells are found in the forebrain, as predicted by the normal fate map. (D) A similar experiment, but this time the operation was done at stage XIII. Note that, at this stage, the centre of the prospective forebrain territory is located much more anteriorly (white arrow). The result is identical to that shown in C. N.B. In the later panels of some of these sequences, the fluorescence appears more intense; this is because at the end of each film, the exposure time per frame was lengthened to compensate for dilution due to cell division, as well as to ensure that the majority of the labelled cells are made visible.

with a homozygous deletion of *HNF3β* express a full range of regional neural markers, despite the absence of a morphological node and its derivatives (Ang and Rossant, 1994; Weinstein et al., 1994; Klingensmith et al., 1999). Together, these data have been interpreted as indicating that signals from outside of the node may be required to induce forebrain structures.

Other recent research has implicated the anterior visceral endoderm (AVE) as the source of the forebrain-inducing signals that appear to be absent from the node. The expression of *Hex* (Thomas et al., 1998) and *Cerberus* (Belo et al., 1997; Shawlot et al., 1998) revealed that the mouse visceral endoderm (VE) possesses anteroposterior (AP) polarity before the appearance of the streak and ablation of the AVE leads to the development of embryos that lack head structures (Thomas and Beddington, 1996). It is interesting that several transcription factors expressed in the AVE (such as Otx2; Simeone et al., 1993; Ang et al., 1994, Goosecoid; Blum et al., 1992, and HNF3β*;* Sasaki and Hogan, 1993; Weinstein et al., 1994), are also markers of the node at later stages of development. Genetic evidence has been used to reinforce the notion of the AVE as a 'head organizer'. Mice lacking Lim1 (Shawlot and Behringer, 1995) or Otx2 (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996) function have a normal trunk and tail but lack head structures. In both cases, these anterior deficits are partially rescued in chimaeric mice with wild-type gene function in the visceral endoderm (*Otx2*, Rhinn et al., 1998, 1999, *Lim1;* Shawlot et al., 1999). Similarly, mice with a homozygous deletion of *nodal* have severe morphological defects (Conlon et al., 1991, 1994; Varlet et al., 1997), but overall AP pattern is rescued in chimaeric mice with wild-type Nodal function in the VE but defective Nodal signalling in the epiblast and its embryonic derivatives (Varlet et al., 1997). Taken together, these findings clearly demonstrate an important role for the VE in AP patterning of the embryo.

In contrast, embryological manipulations suggest that the AVE may not act as a direct 'inducer' or 'organizer' of the forebrain. Mouse AVE fails to induce neural tissue when grafted to a lateral region of a mouse egg cylinder (Tam, 1999), and has only been shown to pattern the anterior nervous system in conjunction with signals from both the EGO and the anterior epiblast (Tam, 1999). It is also important to consider that the mouse EGO and node transplantation experiments mentioned previously (Beddington, 1994; Tam, 1997, 1999) may not reveal the full inducing ability of the node. First, nodes from LS/0B stage embryos are likely to have lost the ability to induce anterior nervous system, as in amphibians (Spemann, 1931; reviewed in Spemann, 1938; Waddington, 1960) and chick (Dias and Schoenwolf, 1990; Storey et al., 1992) at the equivalent embryonic stages. Second, the EGO may be too young. Currently available mouse fate maps do not reveal the stage at which precursors of the prechordal mesoderm first become contained within the tip of the primitive streak (Lawson et al., 1991; reviewed in Tam et al., 1997). In the chick, at least some precursors of these tissues are located in the epiblast anterior to the primitive streak until the streak reaches its full length (Hatada and Stern, 1994). It is therefore possible that the failure of EGO grafts to induce anterior neural tissue is due to the absence of prechordal mesendoderm precursors from the graft, consistent with the finding that both chick (Dale et al., 1997; Foley et al., 1997; Muhr et al., 1997; Pera and Kessel, 1997) and mouse (Ang and Rossant, 1993; Shimamura and Rubenstein, 1997) prechordal mesendoderm can induce expression of anterior markers in more caudal CNS. Third, because of the small size of the mouse egg cylinder, it is possible that grafted nodes recruit cells from the host forebrain region into a second axis, which it then strongly caudalizes; similar results are obtained in the chick embryo when a node is grafted close to the prospective neural plate of the host: a second axis forms, but head structures are shared between the two axes (e.g. Waddington, 1932, 1933a).

So, while it is undeniable that the AVE plays an important role in AP patterning of the mouse embryo, it is still not clear in what capacity it functions. We would argue that the issue of whether mammalian embryos require separate 'organizers' for the head and trunk is still open to debate.

(ii) The activation-transformation model

Based on transplantation experiments in amphibians, Nieuwkoop proposed that induction and patterning of the anterior nervous system is a two-step process in which inductive signals first 'activate' ectodermal cells to an anterior neural state, and 'transforming' signals later caudalize some of these cells to specify more posterior regions (Nieuwkoop et al., 1952; Nieuwkoop and Nigtevecht, 1954). To date, evidence supporting this model has been obtained mainly in amphibians, and is less compelling for the 'activation' step than for the later 'transforming' signals. In *Xenopus*, the BMP antagonists Chordin, Noggin and Follistatin induce expression of anterior markers including cement gland (*XAG1*, *XCG1*) and forebrain (*Otx2*) genes (Noggin; Lamb et al., 1993, Follistatin; Hemmati-Brivanlou et al., 1994, Chordin; Sasai et al., 1995); however, other evidence suggests that simultaneous inhibition of both BMP and Wnt signalling is required to generate a head (Glinka et al., 1997, 1998; Piccolo et al., 1999). In the absence of other signals, no markers for more posterior regions of the CNS are expressed.

The evidence for posteriorizing ('transforming') signals is more substantial. Cox and Hemmati-Brivanlou (1995) have shown that recombination of *Xenopus* forebrain with the caudalmost regions of the embryo (which include the remnants of the organizer) restores *Krox20* expression, a marker for the ablated middle sections of the CNS. The reconstituted middle sections are derived primarily from the head portion of the explant, and FGF can mimic the caudalizing activity of the tail section. In addition to FGF (Cox and Hemmati-Brivanlou, 1995; Xu et al., 1997), Wnt3A (McGrew et al., 1995) and retinoids (Mavilio et al., 1988; Durston et al., 1989; Sive et al., 1990; Kessel and Gruss, 1991; Papalopulu et al., 1991; Ruiz i Altaba and Jessell, 1991; Marshall et al., 1992; Simeone et al., 1995; Bang et al., 1997; Kolm et al., 1997) can also act as caudalizing factors.

In amniotes, there is currently no direct evidence supporting an initial activation step. All transplantations and treatments that induce head structures also generate more caudal regions of the CNS. More problematic for the model are the findings that older nodes can induce caudal CNS without any sign of forebrain (e.g. Dias and Schoenwolf, 1990; Storey et al., 1992; Beddington, 1994), and that grafts of prechordal mesoderm can 'rostralize' the prospective hindbrain in both chick (Dale et al., 1997; Foley et al., 1997) and mouse (Ang and Rossant, 1993; Shimamura and Rubenstein, 1997). One can reconcile these findings with Nieuwkoop's theory by proposing that the

prechordal mesoderm plays a protective role against caudalizing signals. This provides a simple explanation for the finding that young nodes induce a full CNS, while old nodes only induce more caudal regions: the prechordal cells emerging from grafts of young nodes protect the portion of the induced neural plate against caudalizing signals from the aging node. Older nodes, which lack prechordal precursors, also induce nervous system (Knezevic et al., 1998; but see also Storey et al., 1992) but no region of this is protected against the strong caudalizing activity of the node.

A modified Nieuwkoop model for early patterning of the CNS

As discussed above, despite some evidence from different systems supporting either the head/trunk-tail or the activation/transformation hypotheses, neither model is entirely satisfactory. Our present results allow us to propose (Fig. 7) a modification of the Nieuwkoop model that accommodates the available data from both chick and mouse, and which is also consistent with data from zebrafish. The salient features of this model are discussed below.

(i) The hypoblast induces an early, 'preforebrain' but unstable state

We have identified the chick hypoblast as a likely equivalent of the mouse AVE. We show that it can induce the expression of *Sox3* and *Otx2* in competent epiblast, but only transiently. Over a longer period no definitive neural or forebrain markers are expressed and both *Sox3* and *Otx2* expression are lost.

What is the biological significance of the transient expression of these markers? The normal expression of *Otx2* in the chick (Bally-Cuif et al., 1995) and mouse (reviewed in Simeone, 1998) has three distinct but overlapping phases: an early phase of expression in both the hypoblast/AVE and in the epiblast adjacent to this tissue, a second phase of expression in the organizer (node) proper, and a final phase of expression in the anterior neurectoderm. The first two phases are transient and, by the end of gastrulation (stage 6-7 in the chick), expression is only seen in the anterior neurectoderm, where it is maintained thereafter. *Sox3*, like another early preneural marker, *ERNI* (Streit et al., 2000), is initially (stage XI-XIII) expressed in a broad region that includes the entire prospective neural plate. Grafts of Hensen's node into the area opaca quickly induce both *Sox3* and *ERNI* but, if the node is removed some 5 hours after grafting, expression declines and is not followed by the later neural marker *Sox2* (Streit et al., 1998, 2000). These observations suggest that early neural inducing signals result in transient, unstable induction of *Sox3*, but that continued signalling from the organizer is required both to maintain its expression and to initiate expression of definitive

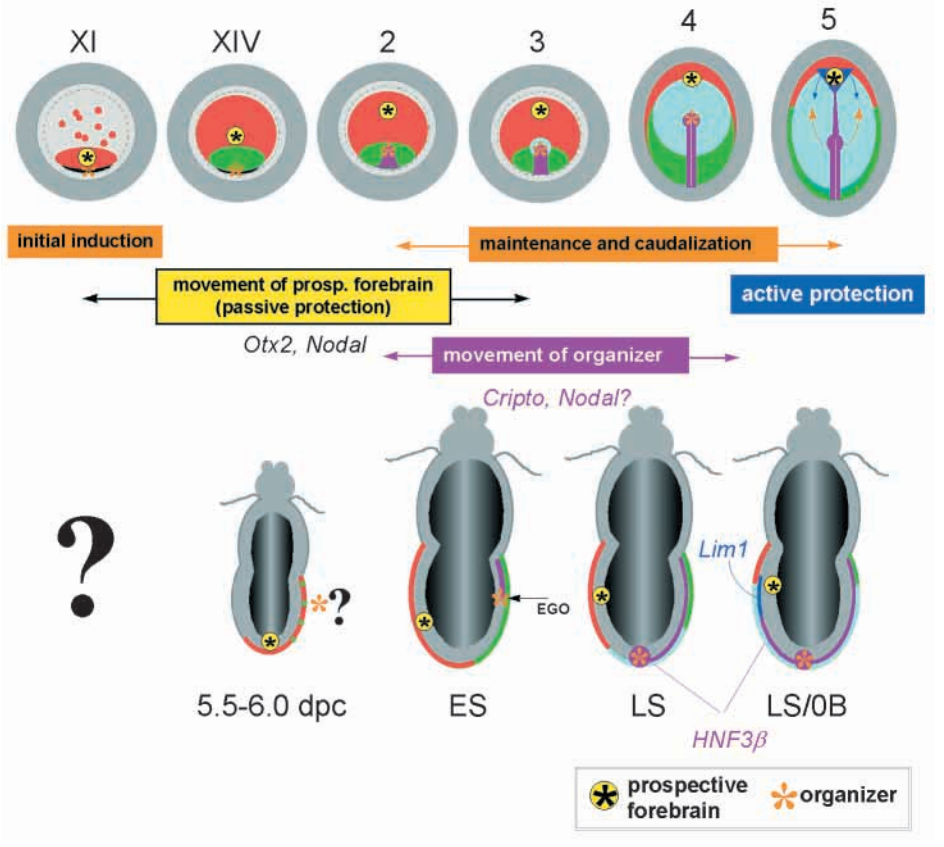

Fig. 7. A model for induction and patterning of the forebrain in chick and mouse. We describe a series of steps leading to the formation of the forebrain. The upper row of diagrams illustrates events at different stages in the chick embryo; the lowest row illustrates the equivalent stages in the mouse (the question mark has been included to signify that the positions of organizer and forebrain precursors have not been established for stages earlier than about E5.5, and there is therefore no equivalent to the first diagram of the chick). Between the two rows, the text boxes indicate the major events proposed by the model, and the mouse genes whose mutation appears to interfere with these events. An initial induction occurs early in development, when the prospective forebrain territory (yellow/black star) lies close to precursors of the organizer (orange star), but this induction is not sufficient to specify a forebrain state. Soon afterwards, the prospective forebrain territory moves anteriorly, while the organizer stays posterior. As the primitive streak appears, the organizer moves forward at its tip. By the early head-process stage, the prechordal mesendoderm (blue triangle) that has emerged from the node protects the forebrain territory against caudalizing signals from the node, and also reinforces the initial induction (blue arrows). In more caudal regions of the CNS, the reinforcement is due to signals from the node itself, which also caudalize (orange arrows). Colour code: red, hypoblast/AVE; green, endoblast/non-anterior VE; light blue, gut endoderm; purple, primitive streak/head process; dark blue, prechordal mesendoderm; yellow/black star, centre of future forebrain; orange star, organizer/node. Mouse stages (according to Downs and Davies, 1993): ES, early streak; LS, late streak; 0B, no allantoic bud; EGO, 'early gastrula organizer'. Neither mouse nor chick embryos are shown to scale.

neural markers. In this context, our finding that hypoblast grafts to a region of competent epiblast induce transient expression of *Sox3* and *Otx2* can be interpreted to mean that the hypoblast is a source of early inducing signals that are also present in the node, but that it lacks the later, maintenance functions of the organizer. The same conclusion was reached by Ang et al. (1994) and Shawlot et al. (1999), who showed that an interplay between positive and negative signals from the mesendoderm regulate the expression of *Otx2* and forebrain development, and that some of these signals act to maintain expression in regions that would otherwise lose it.

Also consistent with this interpretation is the recent finding that the rabbit hypoblast (a tissue similar to both the mouse VE and the chick hypoblast) can induce expression of the early preneural marker *Sox3* and the anterior marker *GANF* (Knoetgen et al., 1999b). Like the chick hypoblast, the rabbit hypoblast is unable to induce a recognizable forebrain and, moreover, the operated embryos were only allowed to develop for 6-10 hours in these experiments, which leaves open the possibility that induction by the rabbit hypoblast is as transient as that by the chick hypoblast.

(ii) The hypoblast directs cell movements to protect against caudalization by the organizer

Waddington (1930, 1932) showed that 90° rotation of the chick hypoblast can reposition the primitive streak and its derivatives. He noted that bending of the primitive streak was an early response to rotation, and even speculated on whether this was due to an inductive event or to altered cell movements: "... although it is clear that the endoderm has an important influence on the differentiation of the ectoderm, it is impossible to decide whether this influence affects only the form-building movements, or whether it affects also the actual qualitative developmental fate of the tissues" (Waddington, 1932, p. 203). A later study (Waddington, 1933b) attempted to discriminate between these two roles by rotating the hypoblast by 180°; in a small number of cases, he observed the appearance of a second primitive streak, and interpreted this finding as evidence for induction, albeit with some caution, suggesting that this is "probably an induction of form-building movements, rather than of a specific organ or tissue" (Waddington, 1933b, p. 503). However, later authors reproducing and extending Waddington's findings did not consider this distinction further, and the chick hypoblast has therefore often been considered as a source of signals that induce a primitive streak and subsequently an embryonic axis (Azar and Eyal-Giladi, 1979, 1981; Mitrani and Eyal-Giladi, 1981; Mitrani et al., 1990a). Our results give direct support for Waddington's initial conclusions, by showing that the hypoblast does indeed direct cell movements in the epiblast and that its effect on the orientation of the embryonic axis is not accompanied by changes of fate in the adjacent epiblast. Rather, rotation of the hypoblast distorts the fate map.

Fate maps of the prestreak stage chick embryo (Hatada and Stern, 1994) place the prospective forebrain territory at the posterior midline at stage X, adjacent to Koller's sickle (which contains some precursors of the organizer; Izpisúa-Belmonte et al., 1993; Streit et al., 2000). During stages XI-XIII, the prospective forebrain territory moves anteriorly so that, by the time the primitive streak appears, it lies in front of the tip of the streak. As the streak elongates to its full length, the node ends adjacent to prospective hindbrain; most, if not all of the forebrain territory lies well forward of Hensen's node (Spratt, 1952; Rosenquist, 1966; Schoenwolf and Sheard, 1990; Bortier and Vakaet, 1992). If neural induction is initiated by signals from the node, how is the forebrain ever induced? One possibility is that the initial signals emanate not from the node itself, but from some of its precursors at the posterior end of the embryo, before primitive streak formation. A recent finding strongly supports this possibility: middle layer cells associated with Koller's sickle can induce transient expression of the early preneural markers *ERNI* and *Sox3* (Streit et al., 2000) in competent epiblast of the area opaca. Our results suggest that the hypoblast could emit similar or cooperating signals, which initiate the process of neural induction, but are not sufficient to complete it.

We have reviewed above (under the heading 'activation/transformation model') evidence suggesting that the organizer is a source of strong caudalizing signals. We propose that the spreading hypoblast acts to direct cell movements in the epiblast to ensure that cells that received early inducing signals (the prospective forebrain territory) are kept separate from the developing node and thus protected from its caudalizing activity. We have also discussed above that the prechordal mesoderm may also protect the forebrain against caudalizing signals from the organizer. However, the activities of the hypoblast and of the prechordal mesoderm seem quite different. The prechordal mesoderm can alter the fate of hindbrain cells to forebrain at stages 3^{+}-4 (Foley et al., 1997), while the hypoblast cannot (this study). Therefore the hypoblast may protect prospective forebrain cells against caudalizing signals indirectly, by maintaining their distance from the organizer, whereas the prechordal mesoderm protects them directly, by antagonizing these signals. In addition, it is possible that the prechordal mesoderm (perhaps together with anterior head-process; see Rowan et al., 1999) also acts to reinforce the initial induction, since the early events are insufficient to lead to the formation of definitive forebrain structures.

(iii) Extension to the mouse

Could this modification of the Nieuwkoop model account for the findings in mouse that have been interpreted by some (mostly working on other organisms) to favour the 'head/trunk-tail' model? Although the role of the mouse VE in directing cell movements in other cell layers has not been demonstrated directly, the phenotypes of several mouse mutants are consistent with the idea that the VE may have such a role. In mice with a homozygous deletion of *Otx2*, genes that are normally restricted anteriorly (such as *Hesx1*, *Lim1* and *cerberus*), remain abnormally located at the distal tip of the egg cylinder (Acampora et al., 1998; Rhinn et al., 1998). This defect can be rescued in transgenic mice with VE-restricted synthesis of Otx1 (Acampora et al., 1998). Although these studies did not include analysis of cell movements, these results could be explained by proposing that the Otx1-expressing VE rescues normal cell movements in the epiblast. Furthermore, one characteristic shared by all of the 'headless' mutants is an unusual constriction between the embryonic and extraembryonic regions of the egg cylinder at 6.5-7.5 d.p.c. This phenotype has generally been thought to result from aberrant cell movements during gastrulation and is also rescued

in chimaeric mice with a wild-type VE (*Lim1*, Shawlot and Behringer, 1995; Shawlot et al., 1999; *HNF3β*, Dufort et al., 1998; *Otx2*, Rhinn et al., 1998; *nodal*, Varlet et al., 1997; see Fig. 7). Together, these findings are consistent with the idea that one role of the VE is to facilitate, or even direct, cell movements in the adjacent epiblast.

Other results support the idea that elongation of the streak positions caudalizing signals at the distal tip. The *Cripto* mutant is characterized both by a failure of forebrain marker expression to move to the anterior part of the cylinder and by a failure of the primitive streak to elongate; despite the double-defect, forebrain markers nevertheless develop (Ding et al., 1998). This phenotype could be interpreted by proposing that the forebrain can develop in an abnormal, distal location because failure of the streak to elongate keeps the node and its caudalizing signals at a proximal location, and these signals therefore fail to act on the prospective forebrain, which is stuck at the distal tip. Even though the physical distance between these regions is small in the mouse, it is conceivable that the patterning molecules act over a distance of very few cell diameters.

Finally, several results show that elongation of the axial mesoderm (head process and prechordal mesendoderm) is important for proper forebrain development, perhaps consistent with a maintenance/protection role for these tissues. Interestingly, the VE (like the chick hypoblast, whose rotation bends the streak) may also play a role in regulating cell movements that facilitate the elongation of these structures. The partial rescue of a forebrain in *Lim1* chimaeric mice may be due in part to the rescue of normal gastrulation movements, allowing for the formation of head process/prechordal mesendoderm (Perea-Gómez et al., 1999; Shawlot et al., 1999). Also, expression of either Otx2 or Otx1 in the VE of *Otx2* mutant mice rescues the formation of anterior axial mesoderm (Acampora et al., 1998; Rhinn et al., 1998). Moreover, VE-restricted expression of Nodal in chimaeric mice that lack Nodal function in embryonic tissues rescues the severe morphological defects observed in homozygous mutant embryos, and one of the more striking features of these chimaeras is the proper elongation of the axial mesoderm (Varlet et al., 1997). Only one experimental finding is more difficult to explain with this model: the fact that relatively late ablation of the AVE causes a loss of expression of anterior epiblast markers (Thomas and Beddington, 1996). This finding can be accommodated by suggesting that, at least in the mouse, the AVE provides protective signals until the prechordal mesendoderm develops in the appropriate position.

Thus, all of the major elements of our model have been proposed separately in the mouse, but a critical comparison of how these individual ideas relate to each other or to different classical models of forebrain development has not yet been undertaken. We propose that all of these findings can be accounted for by a modification of the Nieuwkoop model, in which early morphogenetic movements directed by the VE contribute to protect the prospective forebrain against caudalizing signals from the organizer, and the prospective forebrain is maintained and further protected by signals first from the AVE and later from the head process/prechordal mesendoderm. This modification of the Nieuwkoop model fits all available mouse data better than the idea of separate head and trunk/tail organizers.

(iv) Extension to zebrafish embryos

In the zebrafish, there is no obvious equivalent of the hypoblast/VE; however, some data suggest that our model may also apply to this species. In the fish, induction and patterning of the nervous system does not appear to require signals from the axial mesoderm but rather requires signals from the non-axial mesoderm of the germ ring. Similar to the posteriorizing function that we have proposed for the node, signals from the germ ring can posteriorize prospective forebrain (Woo and Fraser, 1997). Furthermore, fate maps reveal that, at the start of gastrulation, the presumptive ventral forebrain is located posteriorly, in the epiblast adjacent to the embryonic shield. Subsequent movements carry these prospective forebrain cells to the centre of the blastoderm, far from the posteriorizing influence of the germ ring (Woo and Fraser, 1995, 1997). At the same time, gastrulation movements also contribute to distance the germ ring from the prospective forebrain.

(v) Amphibian embryos

As in teleosts, amphibian embryos do not have a region that is obviously homologous to the AVE/hypoblast. The yolky vegetal pole is generally considered to be endodermal, but its ultimate fate is mostly as gut contents, rather than gut lining, most of the latter being derived from the dorsal side of the embryo during gastrulation (Keller, 1975, 1976). In *Xenopus*, it has been suggested that ‘anterior endoderm’ acts as a head organizer because it co-expresses antagonists of Wnt and BMP and because misexpression of inhibitors of Wnt and BMP generates an ectopic head (Glinka et al., 1997, 1998). However, a more recent embryological study reveals that the precise domain that co-expresses these antagonists does not act as a head inducer, but rather as a heart-inducing region (Schneider and Mercola, 1999). Since most experiments involving misexpression are done by injection of RNA before the 4-cell stage, while most embryological experiments are generally conducted at gastrula stage, we feel that more work will be required to establish whether *Xenopus* embryos contain a region that shares the functional properties proposed here for the chick hypoblast, and if so, where it resides. However, it is interesting to note that one of the first proposals, i.e., that morphogenetic movements play an important role in prosencephalic specification and that this state requires reinforcing signals from prechordal tissue, was based on experiments in *Triturus* (Yamada, 1950).

Molecular nature of hypoblast-derived signals

Our results do not allow us to make definitive conclusions about the molecular nature of the signals emitted by the hypoblast that are responsible for either the transient induction of *Sox3/Otx2* or for its effects on cell movements. However, several recent results point to some likely candidates. FGFs, and specifically FGF8, are good candidates to mediate the transient induction of early neural markers: the hypoblast (as well as prospective organizer cells at the posterior edge of the prestreak embryo) expresses FGF8. Misexpression of FGF8 can also transiently induce *Sox3* and *ERNI*, and FGF antagonists abolish the induction both by the organizer and by posterior cells (Streit et al., 2000). It is conceivable that FGFs also contribute to the effects on cell movements, particularly because FGFs have been implicated in directing cell movements in other systems (reviewed by Montell, 1999).

In addition to FGFs, other likely candidates include components of the Wnt pathway or its antagonists. In zebrafish, a requirement for one member of this family, Silberblick (Wnt11), acting through a β-catenin-independent pathway, has been demonstrated to be essential for the cell movements of convergence and extension that drive major cell rearrangements during gastrulation (Heisenberg et al., 2000). *Silberblick* mutants are defective both in convergent extension of the mesoderm and in forebrain patterning (Heisenberg et al., 2000). Likewise, in *Xenopus*, a β-catenin-independent Wnt pathway has recently been shown to be important in regulating cell polarity and cell protrusions (Wallingford et al., 2000). The hypoblast expresses several secreted Wnt antagonists, including Cerberus/Caronte, Dkk-1 and Crescent (this study), and it is therefore possible that these antagonists contribute to its effects on extension of the primitive streak and/or the forward migration of epiblast territories.

Finally, the Nodal pathway may also be involved in the regulation of cell movements. Nodal is expressed transiently in the mouse VE (Conlon et al., 1994; Varlet et al., 1997). Both Nodal and Cripto, a modulator of Nodal signalling, are required for both extension of the primitive streak and normal forebrain development (see above and Ding et al., 1998; Schier and Shen, 2000). Although the chick hypoblast does not appear to express the known *nodal* gene, it may produce modulators of this pathway or as yet unidentified members of the Nodal family.

Conclusions

Our results are therefore most consistent with a model in which early inducing signals, starting before the onset of gastrulation, generate a region expressing early preneural and anterior neural markers (including *Sox3* and *Otx2*), but these signals are not sufficient to give rise to the definitive rostral CNS. Later in development, the organizer and/or its derivatives produce stabilizing signals that complete the process. The organizer also emits strong posteriorizing signals that can transform cells that have received neural inducing signals into more caudal regions of the CNS. The rostral CNS can only develop if it is protected from these caudalizing signals. This occurs in two stages: shortly before the start of gastrulation, the prospective forebrain territory moves anteriorly under the control of the spreading hypoblast, and this movement protects the territory from the organizer by maintaining its distance from it. Later, the prechordal mesendoderm (perhaps with the anterior head process) provides signals that actively protect the forebrain against caudalization. This model is closer to Nieuwkoop's activation/transformation hypothesis (Nieuwkoop et al., 1952; Nieuwkoop and Nigtevecht, 1954) than to the idea of separate organizers for different regions of the CNS, and accommodates data from fish, chick and mouse. We therefore propose that, unlike a previous suggestion that mammals have evolved a new way of patterning the rostral CNS (Knoetgen et al., 1999a,b), the mechanisms that establish this region are conserved among all vertebrate classes.

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