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Neural induction and regionalisation in the chick embryo

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Summary

Induction and regionalisation of the chick nervous system were investigated by transplanting Hensen's node into the extra-embryonic region (area opaca margin) of a host embryo. Chick/quail chimaeras were used to determine the contributions of host and donor tissue to the supernumerary axis, and three molecular markers, Engrailed, neurofilaments (antibody 3A10) and XlHbox1/Hox3.3 were used to aid the identification of particular regions of the ectopic axis.

We find that the age of the node determines the regions of the nervous system that form: young nodes (stages 2-4) induce both anterior and posterior nervous system, while older nodes (stages 5-6) have reduced inducing ability and generate only posterior nervous system. By varying the age of the host embryo, we show that the competence of the epiblast to respond to neural induction declines after stage 4.

We conclude that during normal development, the initial steps of neural induction take place before stage 4 and that anteroposterior regionalisation of the nervous system may be a later process, perhaps associated with the differentiating notochord. We also speculate that the mechanisms responsible for induction of head CNS differ from those that generate the spinal cord: the trunk CNS could arise by homeogenetic induction by anterior CNS or by elongation of neural primordia that are induced very early.

Key words: neural induction, Hensen's node, primitive streak, regionalization, anteroposterior axis, engrailed, HlXbox1.

Introduction

Embryonic induction has been defined as "an interaction between one (inducing) tissue and another (responding) tissue as a result of which the responding tissue undergoes a change in its direction of differentiation" (Gurdon, 1987). Spemann and Mangold (1924) were the first to discover a region with inducing ability, the 'organiser', by transplanting the dorsal lip of the blastopore of an amphibian embryo to the ventral side of a host. They found that new host-derived neural structures are induced from regions of ectoderm that would otherwise give rise to epidermis. In amniotes, Waddington (1932, 1933) was the first to show that transplantation of Hensen's node, the anterior (cranial) tip of the primitive streak, to an appropriate ectopic site induces the formation of a new embryonic axis. For this reason Hensen's node has been identified as the 'organiser' of the amniote embryo.

But the embryonic axis induced by the transplanted Hensen's node consists of more than a collection of differentiated neurons; what forms is a coherent Central Nervous System (CNS), which is regionally organised. This property is known as regionalisation. In the chick embryo, transplantation experiments (reviewed by Gallera, 1971; Nieuwkoop et al., 1985) suggest that the developmental age and orientation of the grafted Hensen's node, the age of the host epiblast and the position of the graft all influence whether and to what extent new axes are induced, as well as their regional characteristics. However, most of the experiments on which these findings are based were done long before good cellular markers were available. For this reason, along with the impossibility of ascertaining the size of the graft in each experiment, and, in many cases, of distinguishing cells derived from the host embryo from those of the graft, it is difficult to compare one study with another.

We have therefore undertaken a detailed study of the induction and regionalisation of the CNS. By transplanting Hensen's nodes of different ages into competent host embryos we examined the stage-dependence of inducing signals: does the inducing ability of the node change with time and do nodes of different ages induce specific regions of the CNS? By transplanting an inducing node into host embryos of different ages

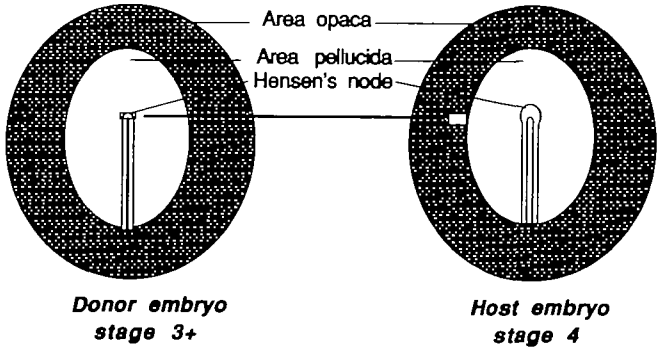


Fig. 1. Hensen's node (or the tip of the primitive streak in younger embryos) was dissected from a donor embryo and transplanted to the inner margin of the area opaca of a host embryo. After 24-36 hours of further incubation, the host embryo and surrounding membranes were processed as required.

we examined the stage-dependence of epiblast response: what is the 'window' during which the epiblast is able to respond to inducing signals (competence) and do changes in epiblast competence have a role in the regionalisation of the CNS?

We have combined examination of the morphology of secondary axes with the use of region-specific molecular markers. One of these is an antibody (4D9; Patel et al., 1989) to a protein (En2) with homology to the *Drosophila* engrailed/invected gene product, which is expressed in the cells of the midbrain-hindbrain region of chick and other vertebrate embryos. We also used an antibody (3A10; Furley et al., 1990; Yamada et al., 1991), which recognises a phosphorylated, neurofilament-associated antigen. This antigen is first expressed in the chick embryo in cells of the diencephalon and hindbrain. A third marker, 3N4, is an antiserum raised against a peptide common to the long and short homeoproteins of the *Xenopus laevis* XlHbox1/human and mouse Hox3.3 (Carrasco et al., 1984; Oliver et al., 1988). In mouse and zebrafish embryos, this gene product is expressed in the cervical spinal cord and somites (Oliver et al., 1988; Molven et al., 1990). In some experiments we used the chick/quail chimaera technique (Le Douarin, 1973) to distinguish graft and host cells, while in others the carbocyanine dye, DiI, was used to label the graft.

We also controlled the position and orientation of the graft. The grafted Hensen's node was placed just inside the area opaca (Fig. 1), a region which only gives rise to extra-embryonic tissues. In comparison with the area pellucida, this region constitutes a more neutral environment in which to test neural induction because it does not contribute to the embryo and because transplants to the margin of the area opaca give rise to secondary structures that remain separate from the host embryo.

We find that the age of the inducing node determines the regions of CNS that form: young nodes (stage 2 to 4; Hamburger and Hamilton, 1951) induce both anterior and posterior CNS, while old nodes have reduced inducing ability and generate only posterior CNS. The competence of the epiblast to respond to neural inducing signals declines after stage 4 (definitive streak stage).

Materials and methods

Fertile hens' eggs (Light Sussex × Rhode Island Red) were incubated at 38°C for 8-24 hours to give embryos at stages 2-6 (Hamburger and Hamilton, 1951).

Grafting technique

Blastoderms were explanted in Pannett-Compton saline (Pannett and Compton, 1924); host embryos still attached to their vitelline membrane were placed (membrane downwards) on a watch glass, stretched around a glass ring (New, 1955; Stern and Ireland, 1981). The region to be grafted was removed from the donor embryos whilst these were submerged in saline. Hensen's node (or the tip of the primitive streak at stages 2, 3 and 3+) was measured using an eyepiece graticule. Each graft was 150-200 μm in length, and its width equalled that of the primitive streak. Grafts were excised using a sharpened insect pin by cutting along the anterior and lateral contours of the node. The anterior cut edge was marked with carmine powder before final excision. Grafts were placed just inside the area opaca and positioned at the same level as the node of the host embryo, with the epiblast facing the basal surface of the host epiblast and orientated along the rostrocaudal (=anteroposterior; a/p) axis of the host embryo (Fig. 1). In some cases two grafts were placed in each host embryo, in the left and right area opaca. We found induction to be equally successful on both sides of the embryo. Following transplantation, the host embryo was incubated in New (1955) culture as modified by Stern and Ireland (1981) for 24-36 hours.

Immunocytochemistry

En2 is a homeobox-containing transcription factor (Joyner and Martin, 1987; Hemmati Brivanlou and Harland, 1989). Expression of the En2 protein was revealed with a monoclonal antibody, 4D9, using immunocytochemical methods essentially as described by Patel et al. (1989). Supernatant was obtained from hybridoma cells kindly provided by Dr Corey Goodman. The monoclonal antibody 3A10 labels cells containing a phosphorylated neurofilament associated protein. 3A10 supernatant was collected from hybridoma cells obtained from the Developmental Studies Hybridoma Bank, Iowa, USA. 3N4 is purified IgG from a rabbit antiserum raised against a fusion protein derived from cDNA for the human *Hox 3.3* gene, which recognises both long and short forms of this homeoprotein (Oliver et al., 1988 and De Robertis, unpublished).

Immunostaining was performed in whole mounts. Embryos to be stained with antibody 4D9 were fixed in phosphate-buffered, 4% formol saline, pH 7.0, for 1 hour and incubated in 0.25% hydrogen peroxide in phosphate-buffered saline (PBS), pH 7.4, for 2-3 hours to block endogenous peroxidase activity. They were then washed in PBS, then in PBT (PBS containing 0.2% bovine serum albumin (BSA), 0.5-1% Triton X-100 and 0.01% thimerosal) and incubated for 30 minutes in PBT containing 5% heat-inactivated goat serum. Supernatant was added (1:1) and embryos incubated overnight at 4°C. Following extensive washing with PBT and incubation with PBT/goat serum for 30 minutes, embryos were placed in a 1:200 dilution of peroxidase-conjugated goat anti-mouse IgG

(Jackson Immuno-research) overnight. After further washes in PBS and 0.1 M Tris, pH 7.4, peroxidase activity was revealed by placing the embryo in 0.5 ml of 1 mg ml^{-1} 3,3'-diaminobenzidine tetrahydrochloride (DAB; Aldrich) in 0.1 M Tris, pH 7.4, and H_2O_2 added to a final concentration of 0.001%. In most cases labelled embryos were cleared in glycerol. A few were later processed for wax histology.

Embryos to be stained with 3A10 were processed using the protocol described above for 4D9, except that the initial PBT washes were replaced by overnight incubation in 2% BSA in PBS.

The protocol for staining whole embryos with 3N4 antiserum was adapted from Oliver et al. (1988). Embryos were fixed in Bouin's fixative for 30-60 minutes, washed in 70% ethanol until all yellow colour was removed, hydrated and washed in TBS (50 mM Tris, 0.9% NaCl, pH 7.4) for 2 hours. They were then blocked in TBST (10 mM Tris, pH 7.4, 100 mM $MgCl_2$, 0.5% Triton X-100, 1% BSA and 5% fetal calf serum) for two days at 4°C, after which they were placed in 1:100 dilution of primary antibody in TBST for 3 days. Embryos were washed over a period of 2-3 days with TBST containing 0.5% Tween-20 in place of Triton X-100 and with the addition of 5% heat-inactivated goat serum. After numerous washes, embryos were incubated for 2-3 days at 4°C in alkaline-phosphatase-coupled goat anti-rabbit IgG (BioRad) (1:1000). After further washes in TBST, embryos were washed briefly in buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5, 20°C) and placed in 45 μl Nitro Blue tetrazolium (Sigma; 75 mg ml^{-1} in 70% dimethylformamide) and 35 μl X-phosphate solution (5-Bromo-4-chloro-3-indolyl phosphate, Sigma; 50 mg ml^{-1} in dimethylformamide) in 10 ml of the above buffer in the dark for 10-15 minutes. The reaction was then stopped by rinsing in tap water. Embryos were cleared in glycerol.

Quail nucleolar marker

Chick embryos grafted with quail nodes were fixed in Zenker's fixative (Drury and Wallington, 1967) for 30 minutes, washed in tap water for 1-2 hours, dehydrated and cleared before embedding in Paraplast. Wax sections were cut at 10 μm and stained with haematoxylin following the method of Hutson and Donahoe (1984; see Stern and Keynes, 1987). Secondary axes were sectioned transversely except for a few which were cut longitudinally. Every fifth section was scored for chick/quail cell contribution in transverse sections. In structures cut longitudinally all sections were scored. As the fixatives for the quail nucleolar marker and the antibodies are not compatible, these markers cannot be revealed simultaneously. However, in some cases the secondary structures were bisected along the midline and each half fixed and processed separately.

DiI injection and photooxidation

DiI (1,1'-dioctadecyl-3,3,3',3' tetramethyl indocarbocyanine perchlorate, Molecular Probes), is a lipophilic carbocyanine dye which inserts into cell membranes close to the site of injection (see review by Honig and Hume, 1989). It was used in some experiments to label nodes prior to transplantation. DiI is used routinely in our laboratory and the details of its application have already been described (Stern, 1990; Selleck and Stern, 1991). Briefly, the dye is dissolved at 0.5% in absolute ethanol and diluted 1:9 with 0.3 M sucrose in distilled water at 40°C. Microelectrodes were made using 50 μl Yankee disposable Micropet capillary tubes (Clay Adams) pulled with an Ealing vertical microelectrode puller. Electrodes were back-filled with DiI and the dye injected by applying gentle air pressure. Dye was applied repeatedly to each node in order to label as many of its cells as possible.

Labelled nodes were excised and transplanted to host embryos. Following incubation, specimens were fixed in 0.25% glutaraldehyde in 4% buffered formol saline, pH 7.0. Prior to wax embedding and sectioning, the DiI label was converted by photo-oxidation of DAB (Maranto, 1982; Buhl and Lübke, 1989; Stern, 1990; Selleck and Stern, 1991). Embryos were rinsed in 0.1 M Tris, pH 7.4, placed in DAB in 0.1 M Tris, pH 7.4, at 500 μg ml^{-1} and incubated at 4°C for 30 minutes. Specimens were then illuminated at the excitation wavelength (484 nm) by epifluorescence using a Olympus Vanox AHBT microscope until all fluorescence had been quenched. Photo-oxidised embryos were then rinsed in 0.1 M Tris, pH 7.4, dehydrated and embedded in paraplast and sectioned at 10 μm.

Criteria used to identify different regions of the CNS

Normal pattern of immunoreactivity with antibodies 4D9, 3A10 and 3N4

In agreement with previous investigators (Gardner et al., 1988; Hemmati Brivanlou and Harland, 1989; Patel et al., 1989; Davis et al., 1991), we find that strong 4D9 immunoreactivity is seen by stage 11/12 (14-18 somites), restricted to the posterior midbrain/anterior hindbrain region of the CNS (Fig. 2A). At stage 12, antibody 3A10 reveals labelled cells in the diencephalon and hindbrain; the midbrain/hindbrain border region where the En2 protein is expressed does not contain 3A10-positive cells (Fig. 2B). Immunoreactivity with antibody 3N4 at stages 9-11 is strongest in posterior regions of the spinal cord and adjacent mesoderm. From here it decreases in intensity anteriorly, until it disappears completely at about the level of the otic vesicle. At stage 12, 3N4 immunoreactivity in the mesoderm is out of register with that in the CNS. The anterior boundary of expression in the mesoderm lies at the level of somites 6 and 7 (Fig. 2C).

The patterns of 4D9, 3A10 and 3N4 immunoreactivity were combined with characteristic morphological features (see Dias and Schoenwolf, 1990) to identify specific regions of the CNS.

(1) Forebrain is distinguished by the presence of optic vesicles and, in sections, the absence of neural crest cells and notochord. The telencephalon is not stained with any of the antibodies. The diencephalon is distinguished by the presence of a characteristic group of 3A10-positive neurons on each side of the midline.

(2) Midbrain is defined by its oval cross-section and the expression of En2 in its posterior half.

(3) Hindbrain is strikingly segmented, narrower than the midbrain and flanked by the otic placodes. The hindbrain also has a characteristic pattern of 3A10 staining which reveals its segmented organisation and a low level of 3N4 immunoreactivity posterior to the otic placodes.

(4) Spinal cord is characterised in sections by its tall, narrow shape and small slit-like central canal. It has 3N4 immunoreactivity and is often accompanied by somites in the neighbouring mesoderm. Unlike the

trunk somites, the occipital somites (somites 1-5) do not stain with 3N4.

Results

1. Stage dependence of the inducing signal

(a) Young Hensen's nodes induce new neural tissue

Quail nodes of stages 3, 3+ and 4 were grafted into host embryos at stage 3+. The results are summarised in Table 1. Sections of the structures generated show that the neural tube, including the midbrain/hindbrain region where En2 is expressed, is composed of chick cells (Fig. 3A,B), although quail cells from stage 3+ to 4 nodes are sometimes found in the floor plate (Fig. 3B). Mesodermal tissues (notochord and somites), on the other hand, are always derived from the quail donor (Fig. 3A). This pattern of contribution is consistent until, at the posterior end of the secondary axis, these tissues merge into a knob of apparently undifferentiated quail cells. The same pattern of contribution is also observed when DiI is used to label donor chick stage 3 to 3+ nodes (Fig. 4A).

(b) Young nodes induce anterior and posterior CNS

The ability of young nodes to induce En2 expression in these secondary axes was assessed by transplanting the node (or the anterior tip of the primitive streak) from

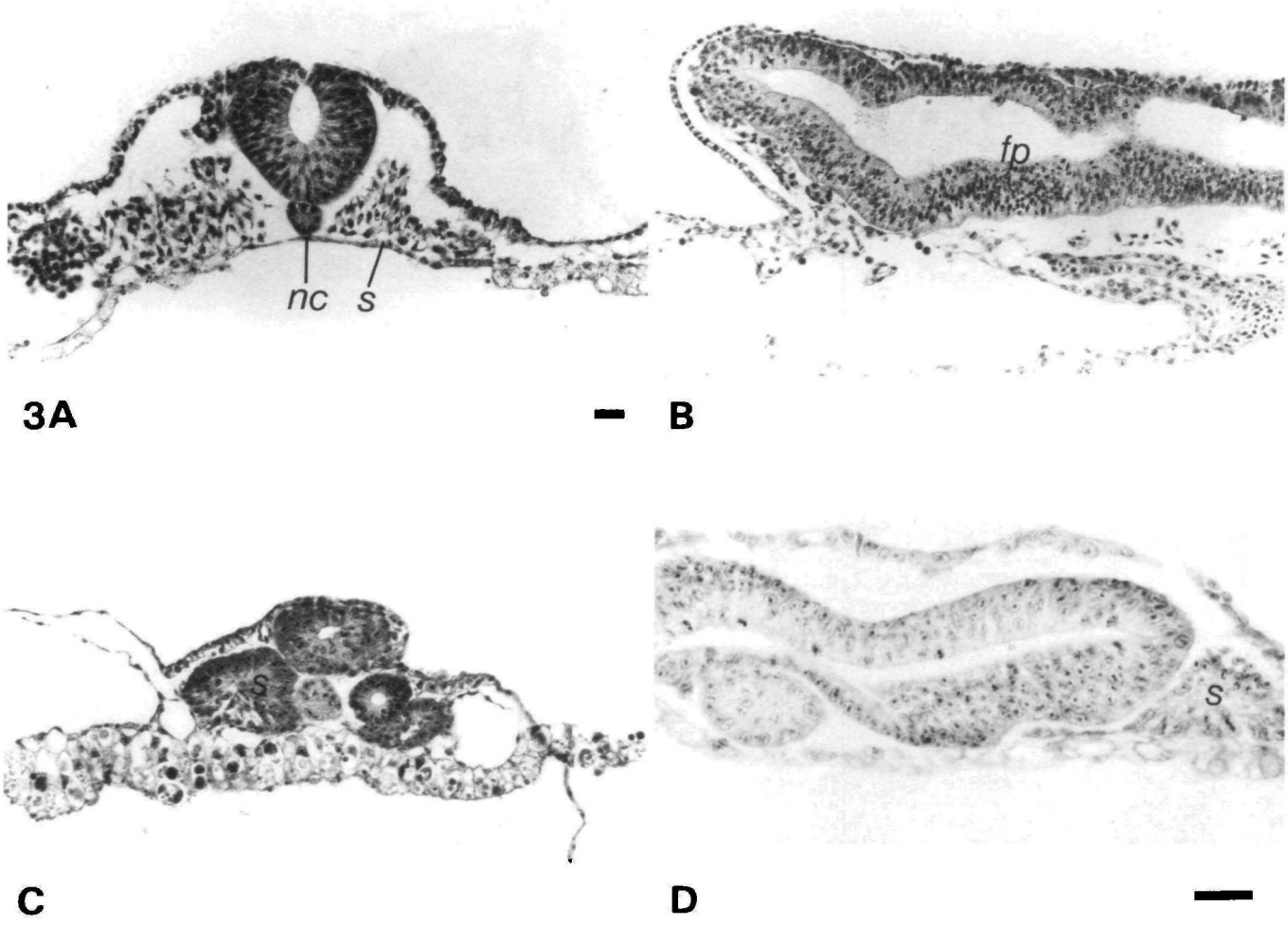


Fig. 3. Chick/quail chimaeras generated by transplanting quail nodes of different stages into competent hosts, processed for the quail nucleolar marker. (A) and (B) are sections through chimaeras made with young nodes, and (C) and (D) are sections through chimaeras with older quail nodes. (A) Transverse section through a secondary embryo induced by transplanting a stage 3 Hensen's node into stage 3+ host embryo. Notochord (nc) and somitic cells (s) are quail-derived, while the neural tube is composed of chick cells. (B) Sagittal section through a secondary embryo induced by a stage 3+ quail node in a stage 3+ chick host. The section passes through the floor plate (fp) region of the midbrain/hindbrain, which contains quail cells. (C) Transverse section through secondary structures generated by transplanting a stage 6 quail node into a stage 3+ host. Somites (s) and notochord are quail-derived; the small neural tube also contains mainly quail cells. (D) Sagittal section through the secondary axis in a chimaera made with a stage 6 quail node and a stage 3+ host chick. The neural tube contains a mixture of chick and quail cells. The somite (s) is mainly quail donor-derived. Bars, 25 μm.

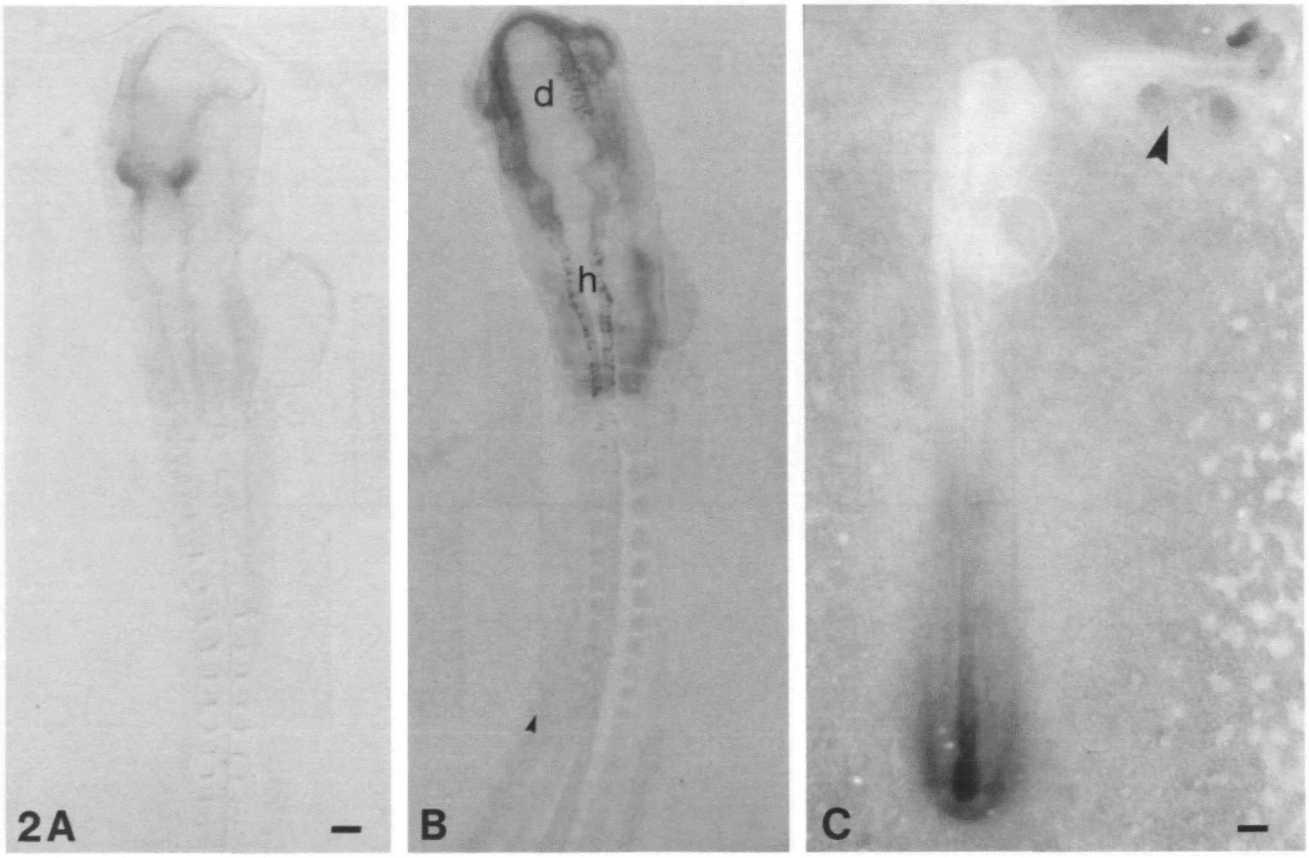


Fig. 2. Normal pattern of expression of three region specific molecular markers. (A) En2, a nuclear protein expressed in the posterior midbrain and first rhombomere in stage 12 chick embryos. En2 protein is revealed using monoclonal antibody 4D9 and a peroxidase-conjugated secondary antibody. (B) Monoclonal antibody 3A10 labels neurons in the diencephalon (d) and hindbrain (h) but not in the En2-expressing region at stage 12. Revealed by immunoperoxidase. (C) XlHbox1/Hox3.3 protein is detected with a rabbit antiserum (3N4) and alkaline-phosphatase-conjugated secondary antibody. Immunoreactivity is present in the hindbrain and spinal cord, in the somites of the cervical region and in the tail at stage 12. An ectopic neural axis has been induced by a graft of Hensen's node (arrowhead); the posterior part of this axis is labelled. Bars, 120 μm (A,B), 250 μm (C).

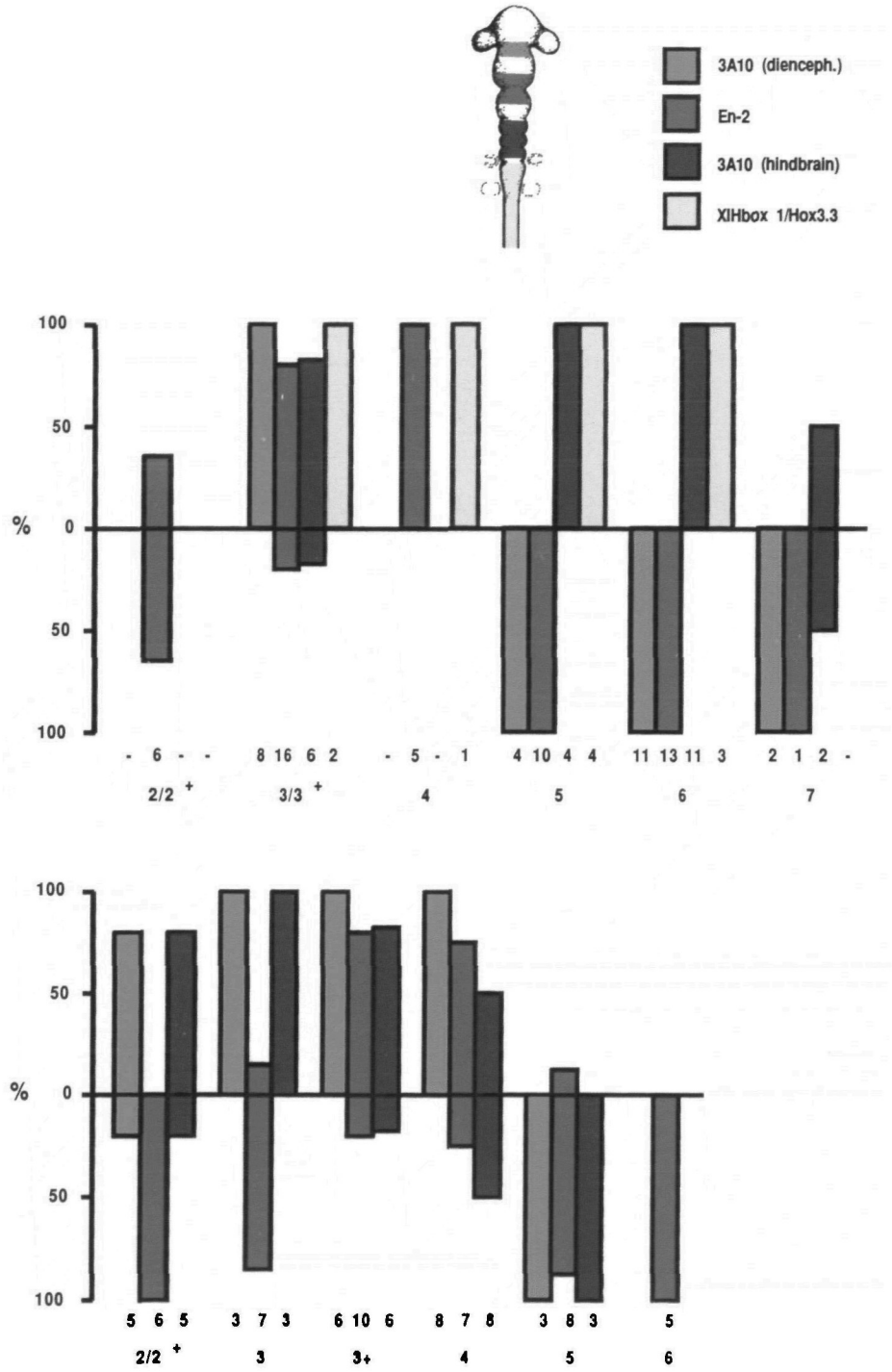


Fig. 6. Bar chart summarising the expression of the various regional markers (shown on the upper right) as a function of the age of the grafted node into stage 3+ host embryos (A) or as a function of the age of the host epiblast when grafted with a stage 3+ node (B). The number of antibody-positive secondary axes is shown as a percentage of the total number of secondary axes examined for the sample in question. The abscissa shows the percentage of embryos expressing (above 0) and not expressing (below the 0 line) each marker, and therefore all bars are equal in length. (A) Nodes of stages 2-4 induce secondary axes which express En2, both diencephalic and hindbrain patterns of 3A10 labelling and XlHbox1/Hox3.3 which marks the posterior hindbrain and spinal cord. Thus, young nodes are able to induce complete secondary axes. Older nodes (stage 5-6) generate axes which lack the diencephalic 3A10 labelling pattern and En2 expression, but which are positive for more posterior CNS markers, the hindbrain stripe of 3A10 labelling and XlHbox1/Hox3.3. Grafts of even older nodes (stage 7) show that the ability to express the hindbrain pattern of 3A10 labelling declines at stage 7.
(B) Anterior and posterior patterns of 3A10 labelling are present at stages 2/2+ to 4. While ectopic axes generated in stage 2/2+ hosts did not express En2, the host embryos did not express the marker either. The expression of the pattern of 3A10 labelling characteristic of the hindbrain is present in only half the axes generated in stage 4 hosts, and is diminished in all the axes examined; this may reflect the time available to generate a complete axis in stage 4 hosts (see discussion). Only one secondary axis generated in older (stage 5 and 6) hosts expressed a regional marker (En2). The non-expression of regional markers in all the other (18) secondary structures generated in older hosts reflects the end of the competent period as revealed by chick/quail chimaeras. The numbers below each bar represent the number (*n*) of ectopic axes studied for each combination.

Table 1. *Assessment of the extent of host- and donor-derived contribution to the ectopic nervous system in chick/quail chimaeras*

Host (chick) stage	Donor (quail) stage	Number of embryos	Mainly chick neural tissue	Quail neural tissue and small number of chick cells	Quail neural tissue, self-differentiated	Quail tissue, undifferentiated
3+	3-3+	8	8/8	–	–	–
3+	4	6	6/6	–	–	–
3+	5-6	10	5/10*	3/10	2/10	–
4	3+	5	5/5	–	–	–
5-6	3+	8	–	–	6/8	2/8

Hensen's nodes from quail donors at various stages were grafted into the area opaca margin of host chick embryos at different stages. In the column indicating mainly chick neural tissue, quail cells were present in some of the cases, particularly in the floor plate in all combinations and in other regions of the ectopic neural tubes that developed after grafts of older nodes (asterisk).

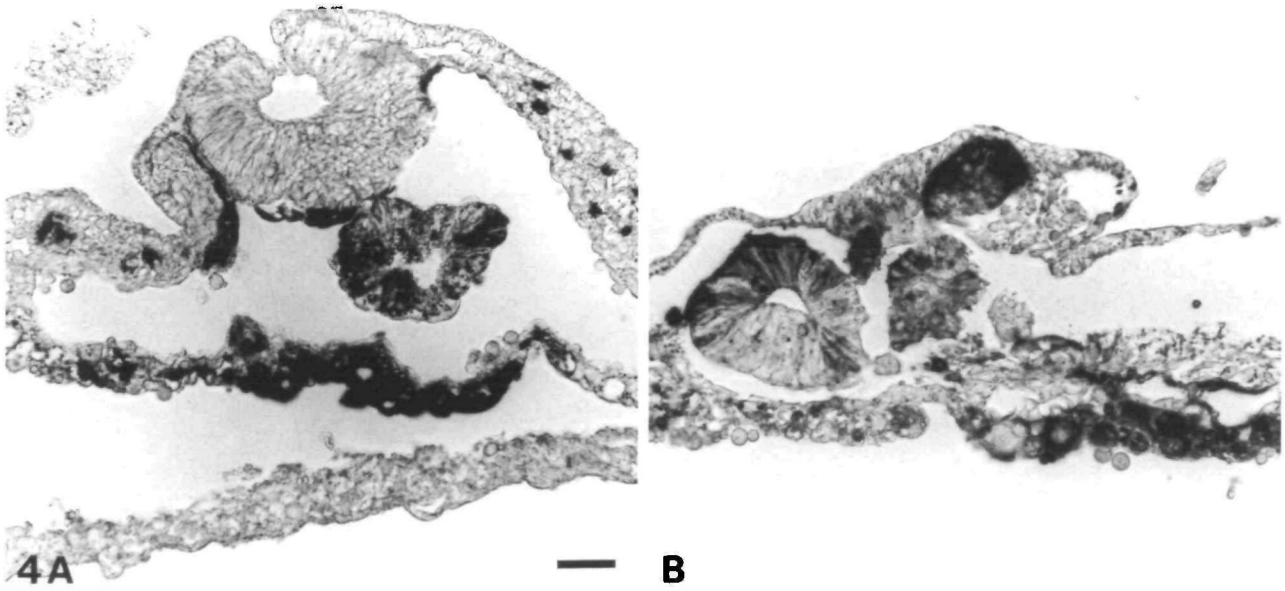


Fig. 4. Sections through supernumerary axes forming after transplantation of DiI labelled nodes. After photo-oxidation, DiI appears black. It is impossible to label every cell in the node. (A) Transverse section through secondary embryo induced by a DiI-labelled chick node from a stage 3 donor. Labelled cells are seen in the notochord, somite and endoderm, but not in the neural tube. (B) Transverse section through structure generated by a DiI-labelled stage 6 node. Note labelled cells in the neural tube. Bar, 25 μm.

donor embryos into host embryos of stage 3+. Stage 2, 3, 3+ and 4 nodes generate structures that express En2 (Figs 5A, 6). To confirm that the En2-expressing region of the secondary axes is derived from host cells, a few specimens were bisected along the midline prior to fixation and each half processed separately (Fig. 7). The results confirm that the En2-expressing cells are indeed derived from the host. Young nodes (stage 3 to 3+) induce secondary axes with the two 3A10-positive regions, corresponding to diencephalon and hindbrain (Figs 6, 8A). Secondary embryos generated in this way also express XlHbox1/Hox3.3 (Figs 6, 8C).

(c) Chick/quail chimaeras made with old nodes show reduced inducing ability

Grafts of stage 5 to 6 quail nodes induce host chick-derived neural tube in only 50% of cases (Table 1). Unlike grafts of younger nodes, which generate secondary axes containing mainly host-derived neural tube, these chimaeras show a much greater donor quail contribution to the CNS (Fig. 3C,D), as found by Dias and Schoenwolf (1990). To ascertain that this increased propensity to self-differentiate into neural plate is not peculiar to the chick/quail chimaera (see Chevallier et al., 1977), chick nodes labelled with DiI were transplanted into chick host embryos. The results obtained are comparable with those from chick/quail chimaeras (Fig. 4B).

(d) Old nodes do not induce En2 expression

Secondary axes generated by transplants of old (stage 5 to 6) nodes into stage 3+ host embryos do not express En2 (Figs 5B,C, 6). To check that this finding is not peculiar to the epiblast of the area opaca, some

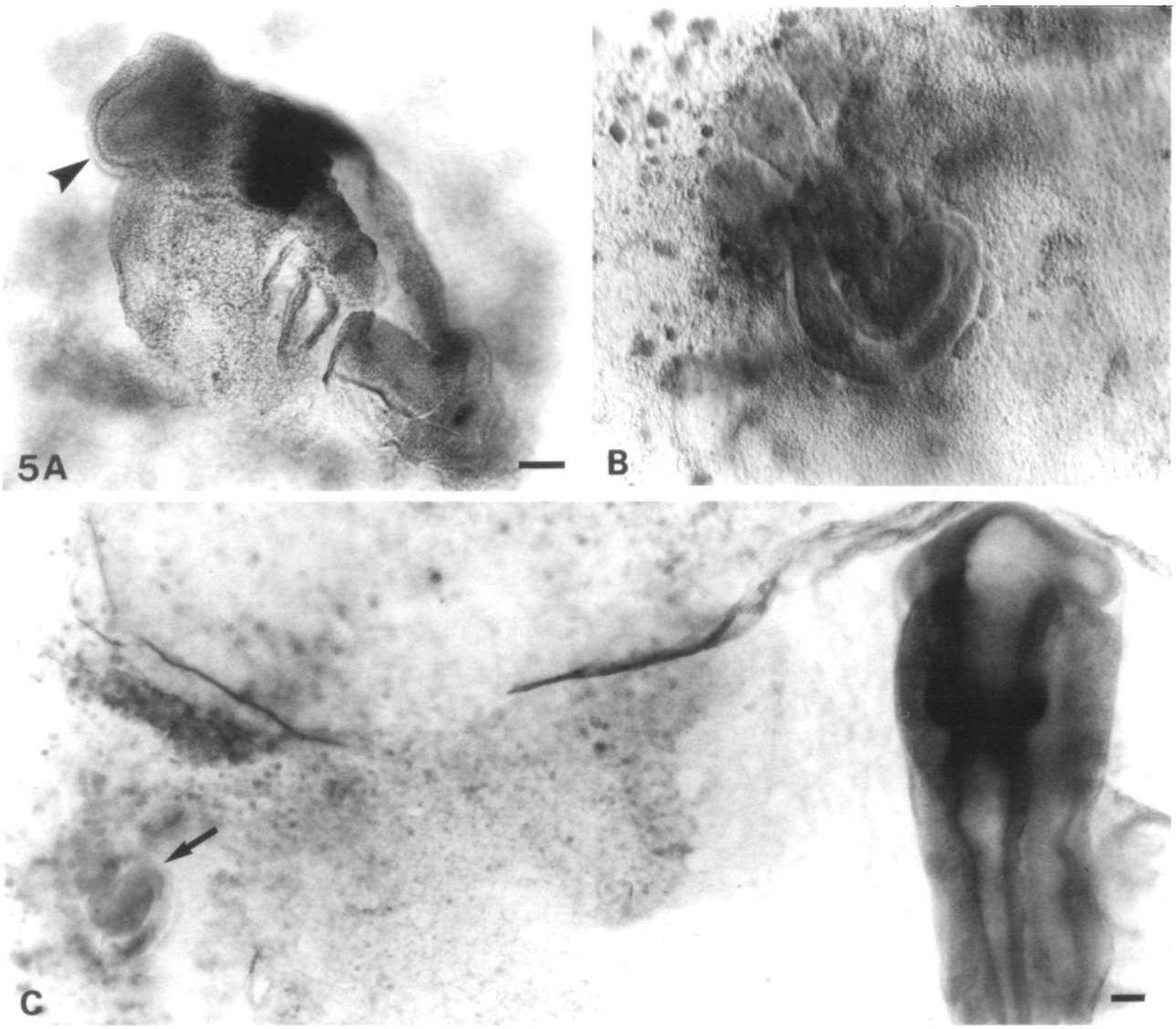


Fig. 5. (A) En2 expression in secondary embryo induced by transplanting a stage 3+ node into the area opaca of a stage 3+ host. Note optic vesicle (arrowhead) anterior to the En2-expressing region (mid-/hindbrain) and more posteriorly (lower right), the segmented hindbrain. (B, C) En2 is not expressed in secondary axes generated by transplanting a stage 6 Hensen's node into a stage 3+ host embryo. Both photographs are of the same embryo; B shows a higher magnification view of the ectopic axis seen at the far left of C (arrow). The host embryo, visible in the right portion of C, expresses En2. The ectopic neural tube and somites are visible in B. Bars, 80 μm (A,B), 150 μm (C).

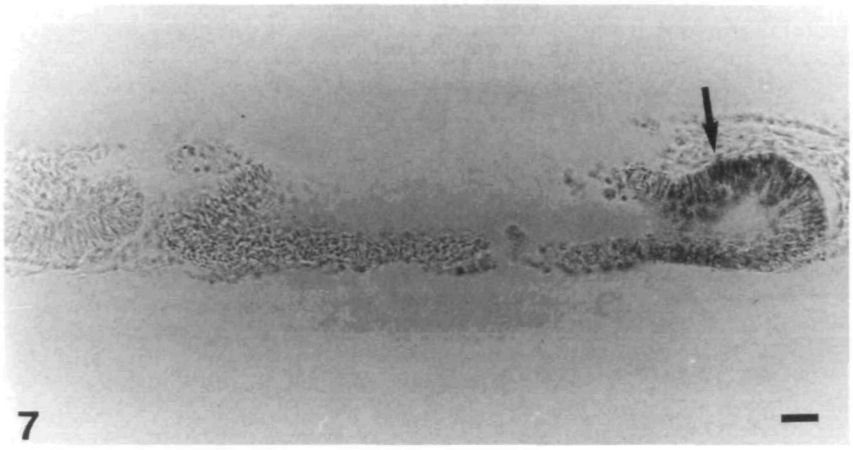
transplants were made into the area pellucida at the level of Hensen's node of the host embryo (n=10). Secondary axes in the area pellucida are more extensive than those in the area opaca and often fuse with the host embryo; however, none expresses the En2 protein.

(e) 3A10 immunoreactivity in structures generated by old nodes

Secondary axes generated by old nodes label with 3A10 (Figs 6, 8B), but the number of 3A10-positive cells varies. The morphology of these axes resembles hindbrain and spinal cord, and is often accompanied by somites. No optic vesicles or other morphological features of the forebrain are present. The pattern of 3A10 immunoreactivity is similar to that seen in the hindbrain of host embryos (Fig. 8B), and scattered labelled cells are confined to the anterior end of the ectopic neural tube.

(f) XlHbox1/Hox3.3 expression in structures generated by old nodes

Secondary axes generated by transplanting stage 6 nodes are 3N4-immunoreactive throughout the CNS (Figs 6, 8D). Stage 4+ to 5 nodes produced axes containing some 3N4-negative CNS, the extent of the unstained region varying between one third and one half of the neural tissue visible. Six out of the seven axes generated by old nodes included somites, all of which were 3N4-negative.



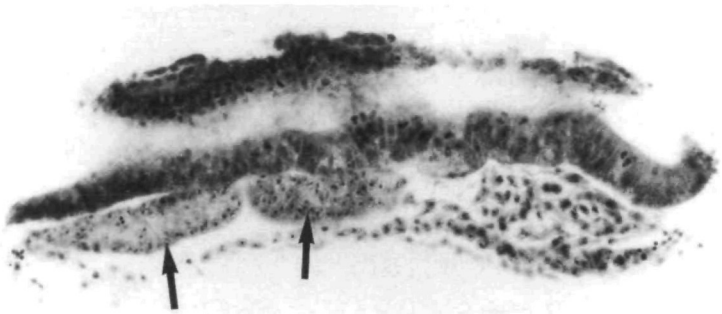


Fig. 7. En2 is expressed in induced (chick-derived) tissue in chimaeras. Sagittal sections through the same ectopic axis (generated by a stage 3+ quail node in a stage 3+ host embryo), which had been bisected prior to fixation. Each half was processed separately to reveal: (A) En2 expression (arrow) and (B) chick-derived CNS and quail-derived notochord (arrows). Bar, 30 μm.

2. Stage dependence of the epiblast response (competence)

Epiblast competence was assessed by transplanting stage 3+ quail nodes into young (stage 3+ and 4) and old (stage 5 to 6) hosts (Fig. 9; see Table 1). Sections through the secondary axes generated were examined for the presence of host chick-derived neural structures. All axes generated in stage 3+ and 4 hosts contained chick-derived CNS, while those produced in hosts at stage 5 to 6 either remained as an undifferentiated clump (2/8; 25%; Table 1) or self-differentiated (6/8; 75%). In all cases, the grafts into older hosts were surrounded by non-neural chick epithelium.

(a) Epiblast competence is mirrored by the ability to express regional markers

Hensen's nodes from stage 2 to 4 embryos induce expression of En2 and the neurofilament-associated protein labelled by 3A10. This finding allowed us to test the ability of the host epiblast at different stages to respond by expressing En2 or the 3A10 antigen. Stage 3+ nodes were transplanted into host embryos at stages 2 to 6 (Fig. 6B). Secondary axes generated from embryos grafted at stage 2 do not express En2 (however, the host embryos themselves also failed to express En2), but both the anterior (diencephalic) and posterior (rhombencephalic) patterns of 3A10 labelling are present. The frequency of En2 expression increases in embryos grafted from stage 3 and then declines when the transplants are performed after stage 4: only 1/8 embryos that had been grafted at stage 5 expressed En2 (Fig. 6B). The 3A10 antigen is also expressed in embryos grafted up to stage 4, although the pattern of expression changes in stage 4 hosts (see below).

(b) Grafts into young hosts induce more complete secondary axes

In our experiments, transplantation of a stage 3+ node into a stage 2 host embryo generates a more extensive secondary axis than when the same node is grafted into a stage 4 host, as found by Gallera and Ivanov (1964) and Gallera (1971). The secondary axes induced from young hosts include both anterior and posterior CNS, as judged by morphology ($n=10$) and by the pattern of 3A10 labelling (Figs 6B, 10A). This contrasts with the truncated secondary axes resulting from transplantation into stage 4 hosts, which show the diencephalic pattern of 3A10 expression but lack, or have a greatly reduced region of, the more posterior pattern of expression ($n=12$ by morphology) (see Figs 6, 10B).

Discussion

The results presented here identify the period during which neural inducing signals are present in the chick

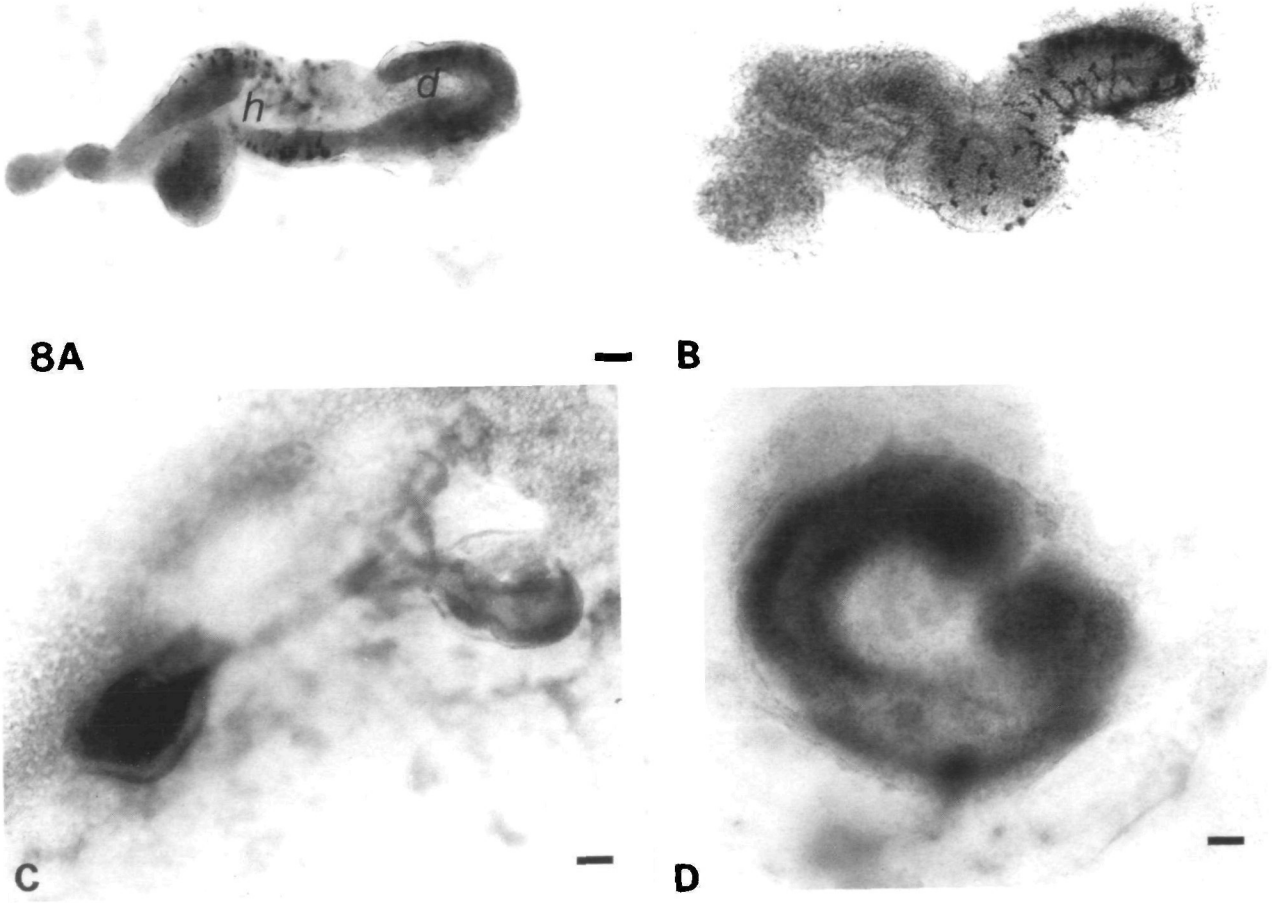


Fig. 8. Immunoreactivity with antibody 3A10 (A,B) and localisation of XlHbox1/Hox3.3 (C,D) in ectopic axes. (A) In this secondary axis induced by transplantation of a stage 3+ node into a stage 3 host, two stripes of 3A10 labelling are seen, corresponding to the diencephalon (d) and hindbrain (h). (B) In this secondary structure generated by a stage 6 node in a stage 3+ host embryo, only one 3A10-positive stripe is seen, and the labelled cells have the morphology of hindbrain neurons. These neurons are confined to one end of the ectopic neural tube. (C) Expression pattern of XlHbox1/Hox3.3 in secondary axis resulting from transplantation of a stage 3/3+ node into a stage 3+ host. 3N4 labelling is present at the posterior end of the secondary axis. (D) XlHbox1/Hox3.3 is expressed throughout this ectopic neural tube generated by transplantation of a stage 6 node into a stage 3 host. The neighbouring somites do not express this gene, suggesting that the region formed corresponds to posterior hindbrain. Bars, 50 μm (A,B), 100 μm (C), 25 μm (D).

embryo and the stage at which the competence of the epiblast to respond to neural inducing signals ceases. We show that the age of a transplanted Hensen's node determines the extent to which new neural structures are induced and the regions of the nervous system that form: young Hensen's nodes (stages 2 to 4) induce both anterior and posterior CNS, while older nodes (stages 5 to 6) have a reduced inducing ability and generate only posterior CNS. The age of the epiblast also affects the extent to which posterior CNS is formed: young epiblasts give rise to more complete secondary axes. The epiblast in the margin of the area opaca ceases to be competent to respond to neural inducing and regionalising signals at stage 4, which coincides with the time at which inducing signals begin to wane in the node.

Assessment of experimental design

We have chosen the epiblast at the inner margin of the area opaca for our grafts because it represents a comparatively neutral environment in which to study neural induction. First, the epiblast of this region does not contribute to any embryonic tissues and only gives rise to extra-embryonic membranes, and yet it is able to respond to neural inducing signals. Second, the secondary axes formed in this region remain separate from the host axis, so that a cellular contribution from the host can be ruled out (see Fig. 5C; see also Hornbruch et al., 1979). Third, the greater distance from the host axis makes it less likely that the graft will be influenced by diffusible signals emanating from the host (see, for example, Tsung et al., 1965; Dias and Schoenwolf, 1990). Finally, studies using an early marker for neural

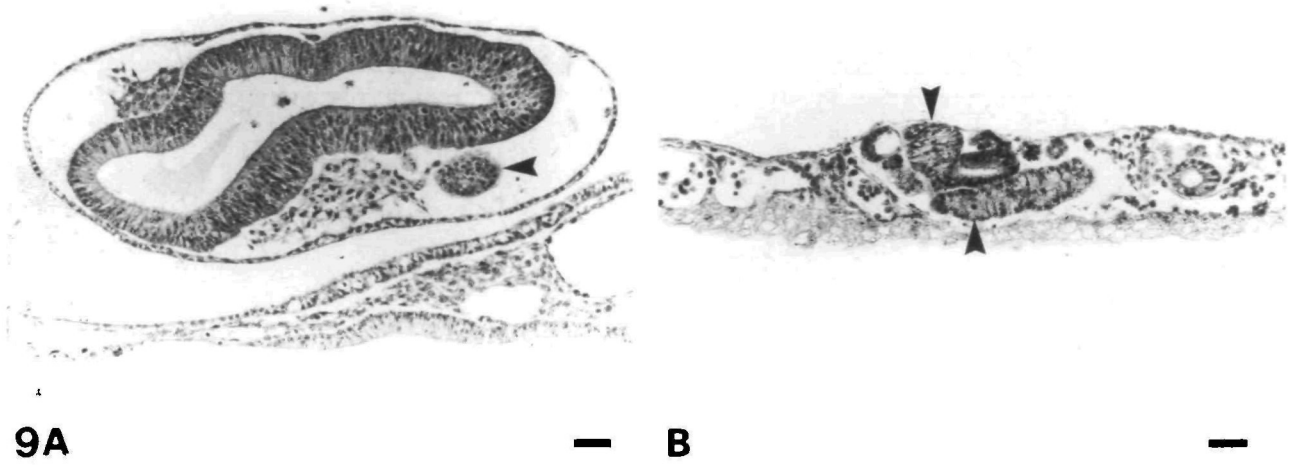


Fig. 9. Stage 3+ quail nodes transplanted into chick hosts at different stages. (A) Oblique section through structures induced in a stage 4 host. A host-derived (chick) CNS has been induced. Quail cells are present in the notochord (arrowhead) and in the paraxial mesoderm. (B) Section through structures generated in a stage 6 host. The grafts has self-differentiated largely into notochord (arrowheads) and somitic tissue; some neural tissue is also found. Scale bars: 50 μm (A), 100 μm (B).

induction, the L5 epitope (Roberts et al., 1991), suggest that the epiblast of the area pellucida may have a tendency to become neural as early as stage 3.

Gallera (1970a) has shown that the epiblast of the "area opaca" is slower to respond to the inducing stimulus than the epiblast of the area pellucida. However, Gallera does not specify the precise location of his grafts in the area opaca. In experiments conducted in our laboratory, only 2/10 grafts placed in the outer half of the area opaca generated axes expressing En2 (unpublished observations), while 8/10 nodes of the same stage induced En2 expression when placed in the inner area opaca (Fig. 6). We have therefore conducted all the present experiments in the area opaca/pellucida margin, where induction appears to be as efficient as in the area pellucida.

(1) Time course of induction

We find that Hensen's nodes from donor embryos at stages 2-6 are able to induce a secondary embryonic axis, but their neural inducing capacity decreases after stage 4. Beyond this stage, the node shows an increased tendency to self-differentiate, as shown by chick/quail chimaeras and DiI labelling. This finding confirms the conclusions of Gallera (1966; 1970b) and of Dias and Schoenwolf (1990). Veini and Hara (1975) have also reported an increase in the tendency of old nodes transplanted into the coelomic cavity to differentiate into neural structures.

Two events correlate with the loss of inducing ability from nodes older than about stage 4: the emergence of prospective head process cells from Hensen's node and the disappearance of presumptive definitive (gut) endoderm cells from the fate map of the node (Selleck and Stern, 1991). Whilst either cell type could be responsible for initiating neural induction, Gallera and Nicolet (1969) and Dias and Schoenwolf (1990) have favoured the latter. It is perhaps worth noting that Hornbruch and Wolpert (1986) found that the ability of Hensen's node to elicit extra digit formation when grafted into the anterior margin of the chick wing bud is still high at stage 9. This result may indicate that the neural inducing and limb polarising properties of the node are different; in any case, limb polarising activity cannot be related to the presence of either presumptive head process or presumptive gut endoderm cells in the node.

(2) Competence

The experiments described here identify the time at which the epiblast loses competence to respond to neural inducing signals. A graft of a stage 3+ Hensen's node will induce a secondary axis in stage 4 or younger epiblasts (Fig. 6B; Table 1), and this axis expresses all regional markers tested. After stage 4, the graft self-differentiates and the resulting ectopic axis does not express regional markers. This result suggests that the epiblast loses its competence to respond to inducing signals between stages 4 and 5.

It is more difficult to determine the beginning of the period of competence of the epiblast to respond to neural inducing signals. One problem is that the grafted node becomes older during the period of culture and it may lose its inducing ability before the young host epiblast acquires the ability to respond. However, our experiments do provide some evidence that the epiblast is already competent to respond to neuralising signals by stage 2. Stage 3+ nodes transplanted into a stage 2 host give rise to a secondary axis which is too young to express En2, but which does express both the diencephalic and the rhombencephalic stripes of 3A10 (which lie anterior and posterior, respectively, to the

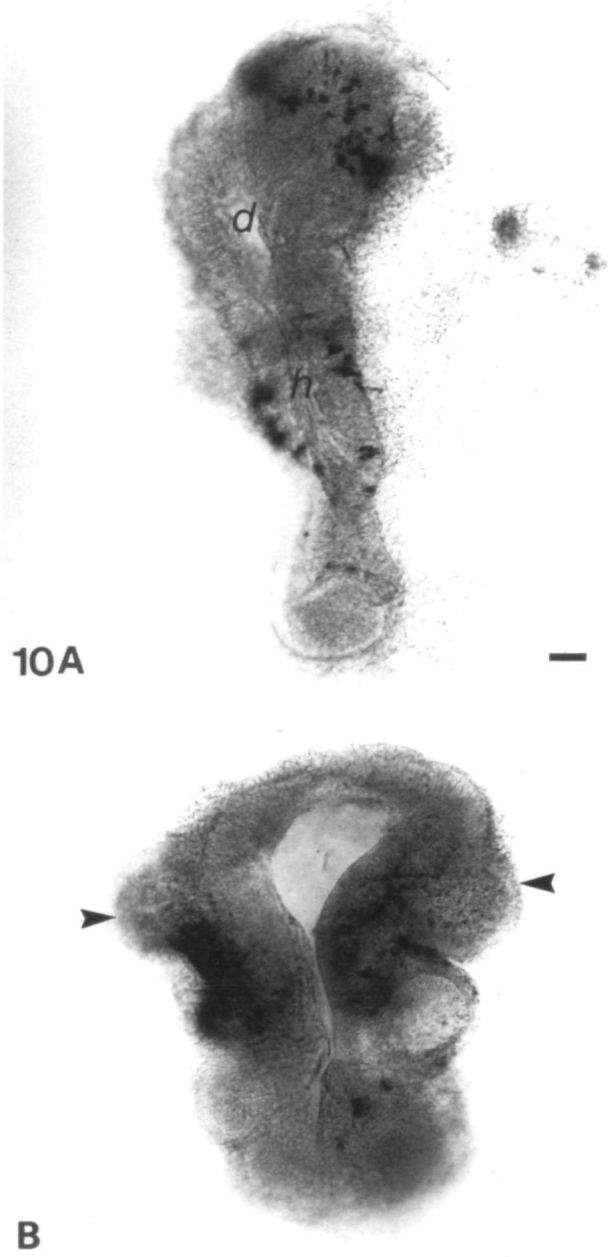


Fig. 10. Patterns of 3A10 labelling in ectopic axes arising in host embryos of different stages after grafting a 3+ node. (A) Axis induced in a stage 2 host. 3A10 immunoreactive neurons are present as two stripes, corresponding to diencephalon (d) and hindbrain (h). (B) Ectopic axis induced in a stage 4 host. Note the truncated appearance of the secondary structures; 3A10 labelling is confined to a single stripe. Optic evaginations can be seen (arrowheads), indicating that the forebrain is present. Bar, 50 μm.

mesencephalic En2 stripe). In these young embryos, however, we cannot rule out that the grafted node induced the ectopic nervous system indirectly, through a prior induction of primitive streak mesoderm in the host. In stage 2 hosts, even a graft of posterior primitive streak will induce the formation of host mesoderm (Vakaet and Haest-van Nueten, 1973). Competence for induction of a primitive streak, however, falls off with increasing host age (Woodside, 1937; Shieh et al., 1965; Gallera and Nicolet, 1969; Eyal-Giladi and Wolk, 1970). Thus, the epiblast at stage 2 may respond to a graft by forming a streak, which in turn induces new CNS, while at later stages neural competence may be tested directly (see Eyal-Giladi and Wolk, 1970). Our experiments show that at stages 3+ to 4, the mesoderm associated with the secondary axis is derived from the graft, suggesting that a new primitive streak had not been induced. This is consistent with the finding of Hornbrunch et al. (1979) that host-derived somites are only present in secondary axes when grafts are placed close to a stage 4 host embryo.

(3) Regionalisation

Nieuwkoop (1955) formulated a theory of 'activation-transformation', in which he proposed that 'activation' of the ectoderm results in differentiation of neural tissue with anterior (forebrain) characteristics. This tissue is then 'transformed' or posteriorised by further interaction with underlying mesoderm. Hara (1961; 1978) later adapted this theory to neural development in the chick. Both Hara's (1961) experiments, in which different regions of the head process induced different regions of the CNS and our results, discussed briefly below, suggest that the 'transforming' or regionalising step is carried out by the presumptive notochord.

The majority of young (stage 3+) nodes placed into older hosts self-differentiate (Table 1), and the structures generated do not express regional markers (Fig. 6B). The converse combination gives a different result: older (stage 5 to 6) nodes placed into young (stage 3+) hosts generate secondary axes that express 3A10 and XlHbox1/Hox3.3 (Fig. 6); 50% of these are composed largely of neural tissue derived from the graft (Table 1). This result is difficult to explain. One possibility is that self-differentiating older nodes give rise to more neural tissue than young nodes (see Veini and Hara, 1975); a certain amount of neural tissue may be required to set up regional differences. Further, it could be that even the amount of neural tissue generated by old nodes is too small for the establishment of regional differences and that regional identities have been specified in the epiblast of these older nodes prior to transplantation. As young nodes which have undergone self-differentiation do not express regional markers, this suggests that regionalisation takes place after stage 3+.

The mesoderm determines regional differentiation in the CNS

A number of previous studies (e.g. Tsung et al., 1965; Vakaet, 1965; Gallera, 1970b) have concluded that young nodes induce anterior structures while old nodes induce more posterior structures. Hara (1961) combined epiblast with different regions of the head process and concluded that different regions of the chordamesoderm induce different regions of the CNS. Results obtained in amphibian embryos support this conclusion (see Nieuwkoop et al., 1985). For example, anterior

mesoderm induces the expression of the anterior markers *XIF3* (Sharpe and Gurdon, 1990) and *En2* (Hemmati Brivanlou et al., 1990), while posterior mesoderm induces the expression of the posterior marker *XlHbox6* (Sharpe and Gurdon, 1990).

Our results show that Hensen's nodes that still contain presumptive head process cells (up to stage 4) induce CNS which includes the diencephalon, characterised by a particular pattern of expression of 3A10, and the midbrain/hindbrain region which expresses En2. Once the presumptive anterior notochord cells have left the node (stage 5), the CNS induced by such a node no longer expresses the diencephalic pattern of 3A10 or En2 but continues to express a different pattern of 3A10 labelling characteristic of the hindbrain, and XlHbox1/Hox3.3, which is expressed in the posterior hindbrain and in the spinal cord. This supports the conclusion that the information required to form the diencephalon and the midbrain/hindbrain region is conveyed by the presumptive chordamesoderm cells that underlie the presumptive anterior CNS, and suggests that the presumptive notochord cells remaining in older nodes may be responsible for regionalising more posterior CNS. Taken together, these findings suggest that the notochord may be the source of regionalising signals.

How many regionalising signals?

One question that emerges from these considerations is: are there as many different signals as there are regions in the CNS? Perhaps surprisingly, our results suggest that this might be the case. Stage 6 nodes generate occipital CNS (the neural tube expresses XlHbox1/Hox3.3 while the accompanying somites do not). By contrast, the CNS generated by stage 4+ to 5 nodes includes anterior as well as posterior hindbrain. Finally, stage 2 to 4 nodes uniquely induce anterior CNS including the diencephalon (3A10 labelled cells) and the midbrain/hindbrain, En2-expressing region.

De Robertis et al. (1989) found that the anterior and posterior boundaries of expression of XlHbox1 in the mesoderm of *Xenopus* embryos are initially in line with those in the overlying nervous system. Based on this finding, they suggested that the mesoderm imparts positional information to the nervous system in a manner that resembles homeogenetic induction because the same 'state' is transmitted between the inducing and responding tissues. So, if regionalisation does require as many signals as there are regions, how many such signals/regions are there in the embryo? Will the number of signals equal that of the genes whose expression is regionally restricted?

Does the age of the responding epiblast play a role in regionalisation?

When a stage 3+ node is grafted into hosts older than stage 4, the regional markers studied cease to be expressed at the same time (Fig. 6B). This result contrasts with that obtained when the age of the grafted node was varied (Fig. 6A): here, as discussed above, regional markers disappear in anteroposterior sequence with increasing age of the graft. These observations suggest that the age of the responding epiblast is not responsible for regionalisation of the induced CNS during normal development.

We observed a striking difference in the extent of the secondary structures induced by stage 3+ nodes in stage 2 host embryos as compared with those induced in stage 4 hosts: embryos grafted at stage 2 tend to yield a larger CNS that includes more posterior regions. Superficially, this appears to reflect a change in the ability of the epiblast to respond to particular regionalising/inducing signals. This finding has been described by Gallera and Ivanov (1964). They account for the phenomenon by suggesting that induction of the spinal cord takes longer than induction of the brain (see also Gallera, 1971). That is, a node transplanted into a stage 4 embryo is apposed to epiblast with a shorter period of competence ahead of it than that confronting a node transplanted into a stage 2 host. This idea, along with the finding that the frequency of primitive streak inductions decreases as a function of the age of the host epiblast (Gallera and Nicolet, 1969), could explain the reduced presence of posterior CNS structures generated from stage 4 hosts in our experiments.

One possibility that should not be overlooked is that the graft may move autonomously when placed into certain positions of a host of a certain age but not others, and that this movement affects the extent of the induced CNS. Thus, it may be that nodes grafted into stage 4 hosts remain stationary, whilst nodes grafted into younger hosts move, either autonomously or carried by the morphogenetic movements of the adjacent epiblast. Such lack of movement in older hosts might prevent the separation of cells that give rise to the head process from those destined for posterior notochord. The reduced presence of posterior CNS in structures induced from stage 4 hosts could therefore be accounted for in terms of the time available to generate a complete axis.

4. Homeogenetic induction

We have discussed the idea that nodes older than about stage 6 no longer have inducing ability, and that posterior notochord appears similarly unable to induce neural tissue. Examination of embryos at about stage 8, moreover, shows that the neural plate extends posteriorly a considerable distance behind the node (see for example, Hamburger and Hamilton, 1951). What, then, induces the posterior portions of the CNS during normal development? One possibility is that neural induction only concerns the formation of anterior neural structures, as suggested by Nieuwkoop (1955) and Hara (1961; 1978), and that the posterior CNS forms as a result of homeogenetic induction by this induced epiblast, propagating posteriorly in the plane of the epiblast. There is growing evidence that neural induction includes the horizontal passage of signals within the epithelium (Dixon and Kintner, 1989; see also Nieuwkoop et al., 1985), although this is not the only mechanism (Sharpe and Gurdon, 1990; for a review see Guthrie, 1991).

However, there is an alternative to homeogenetic induction to explain the formation of posterior CNS. It is possible that presumptive posterior neural plate is induced at the same time or only slightly later than anterior regions, and that this presumptive posterior neural plate then elongates as the primitive streak regresses. In support of this, Schoenwolf and colleagues (see Schoenwolf and Alvarez, 1989) have localised the presumptive spinal cord to a region on either side of the anterior tip of the primitive streak at stage 3+.

The above considerations suggest that the mechanisms that generate the head of amniotes could be fundamentally different from those that generate the trunk. In the trunk, the CNS appears to be produced either as a consequence of homeogenetic interactions with previously induced epiblast or as a result of the elongation of a small primordium of the spinal cord that is induced as early as portions of the head.

Conclusions

1. A number of findings suggest that induction and regionalisation of the CNS are separate processes. First, epiblast competence and neural-inducing ability of the node both decline after stage 4, suggesting that, during normal development, neural induction takes place early, before the end of stage 4. Second, young nodes (stage 3+) that self-differentiate (owing to the advanced stage of the host) give rise to neural tissue that does not express regional markers, while self-differentiatation of older nodes gives rise to neural tissue expressing both 3A10 and XlHbox1/Hox3.3. This could suggest that the epiblast contained within older nodes is regionalised prior to transplantation, while the epiblast in nodes from stage 3+ embryos has yet to receive regionalising signals. Third, recent elegant experiments by Martínez et al. (1991) demonstrate that signals that regionalise the CNS can act independently of neural inducing signals: as late as stage 10, a graft of En2-expressing midbrain can induce the ectopic expression of En2 in the diencephalon. Taken together, these observations suggest that the epiblast retains its capacity to be regionalised until late in development, while its ability to respond to neural inducing signals ceases much earlier, as the notochord begins to form.

2. Our results provide evidence for the view that the axial mesoderm, rather than the responding epiblast, determines the differentiation of regions within the CNS. The ability to induce expression of anterior markers (those expressed in diencephalon, posterior midbrain and anterior part of rhombomere 1) ceases in the node as the head process emerges (the head process extends anteriorly from rhombomeres 4/5). Nodes transplanted after this stage generate only posterior CNS, with nodes of increasing age giving rise to progessively more posterior regions. This suggests that the signals required to regionalise the CNS are conveyed by presumptive notochord cells and that there is a sequential, anteroposterior organisation within the mesoderm, which is conveyed to the overlying neural plate (see De Robertis et al., 1989; Sharpe, 1990).

3. The developmental mechanisms involved in generating the head region of the amniote embryo may be radically different from those that give rise to the trunk, because older nodes (and posterior notochord; Gallera, 1966) do not induce neural tissue. This contrasts with the neural inducing ability of young nodes (and anterior notochord; Hara, 1961) in the chick, and with the neural inducing abilities of both anterior and posterior notochord in *Xenopus* (e.g. Hemmati Brivanlou et al., 1990). We suggest two mechanisms by which the more posterior regions of the CNS could be generated: (i) homeogenetic induction by already induced, anterior epiblast and (ii) elongation of posterior neural primordia that are induced early.

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Note added in proof

Since this paper was accepted for publication, C. R. Kintner and J. Dodd (*Development* **113**, 1495-1506; 1991) have published a study of neural induction and regionalisation by chick Hensen's nodes on amphibian ectoderm. They also conclude that the ability of the node to induce *engrailed* expression is lost after stage 4 and that neural inducing signals are already present in young nodes.