ANALYSIS OF NEURAL INDUCTION AND PATTERNING IN AMNIOTE EMBRYOS

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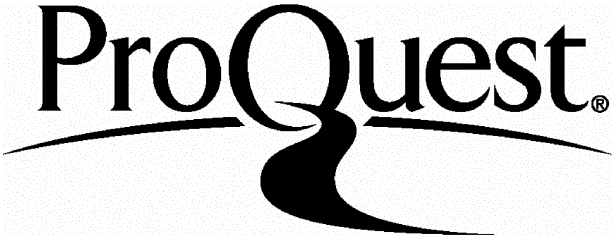

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ABSTRACT

When grafted into a host embryo, Hensen's node, the chick gastrular organiser, is able to induce an ectopic second neural axis. The use of pan-neural and regionally specific neural genes, particularly forebrain markers such as *BF-1* and *GANF*, shows that the chick node is able to induce a nervous system with complete anterior pattern, expressing all the markers tested. Evidence in mouse suggests that additional signalling information, established separately from the node and its derivatives, is required to generate complete anterior pattern. The ability of mouse node grafts to induce the same range of neural markers in chick hosts has therefore also been examined. In this assay, the mouse node is not able to induce expression of chick forebrain markers, and an anteriorly truncated second axis is formed.

This work has also investigated the role of the foregut endoderm in patterning the anterior brain of the chick. This tissue is formed by cells that move through the node into the lower layer during gastrulation. Removal of the lower layer at stage 4 has no apparent effect. However, removal of foregut endoderm during the early head process stages (4+ to 5) causes reduction in forebrain pattern. By the 12 somite stage, most neuraxes lack telencephalon and eyes, and *BF-1* and *GANF* expression domains are absent or severely reduced. This syndrome is preceded by a failure to establish normal *FGF 8* expression in the anterior neural ridge signalling centre, at early somite stages. However, gene expression throughout axial mesoderm (*BMP 7*, *chordin* and *shh*) appears unaffected in all embryos. The homeobox gene *hex* and the chick *Frzb* homologue *crescent* are both expressed in the anterior definitive endoderm at the time when removal of this tissue results in forebrain defects. These results suggest that the definitive foregut endoderm contains information crucial for forebrain patterning in the chick embryo.

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ABBREVIATIONS

AP	antero-posterior
ANR	anterior neural ridge
AVE	anterior visceral endoderm
BSS	balanced salt solution
BMP	bone morphogenetic protein
cDNA	complementary DNA
CNS	central nervous system
DEPC	diethylpyrocarbonate
DiI	1,1dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate
DNA	deoxyribonucleic acid
dpc	days post coitum
DV	dorso-ventral
EGO	early gastrula organiser
EDTA	diaminoethanetatraacetic acid
Fig.	Figure
FGF	fibroblast growth factor
HH	Hamburger and Hamilton
kb	kilobase
LB	Luria-Bertani
M	molar
ml	millilitre
mM	millimolar

mRNA	messenger RNA
OD	optical density
PBS	phosphate buffered saline
PBT	phosphate buffered saline/ Triton-X-100
PC saline	Pannett and Compton bird embryo saline
PFA	paraformaldehyde
RDVM	rostral diencephalic ventral midline
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
st.	stage
TAE	Tris-acetate-EDTA buffer
TE	Tris-EDTA buffer
TGF	transforming growth factor
Tris	Tris(hydroxymethyl)aminomethane
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
Xgal	5-Bromo-4-chloro-3-indolyl-β-D-galactosidase
µg	microgram
µl	microlitre
µM	micromolar

CHAPTER I

INTRODUCTION

1.1 Foreword

The formation of a structured embryo from a single cell is an amazing feat of development. The combined use of multiple model systems has enabled scientists to make enormous advances in elucidating the developmental processes and mechanisms involved at each step. Each model organism has particular advantages and offers different embryological, genetic and molecular approaches, allowing a more comprehensive understanding of early developmental processes to be achieved. The *Xenopus* embryo is particularly amenable to molecular studies, to analyse the consequences of extensive over-expression of a gene. The chick embryo, due to its large size, planar organisation and ease of subsequent culture, allows position-specific manipulations and tissue graftings to be made. The mouse and zebrafish embryos are very important genetic models, with the ability to generate targeted mutations a very powerful tool for studying the effects of loss of a gene function. As will become clear in the following introduction, such a combinatorial approach has highlighted how conserved many gene functions and expression patterns have been throughout evolution. However, important differences in the timing of developmental processes, and in the use of varying gene combinations at particular stages in the different species, have become apparent as more information has been obtained. These variations contribute to the formation of very different organisms from a relatively common beginning.

1.2 Cell movements in amniote embryos during gastrulation

Gastrulation is a fundamental process in vertebrate embryonic development, whereby cells of the single-layered embryo are displaced or reshuffled and become rearranged in a system of three concentric germ layers. Areas destined to form the internal body structures are brought into the interior of the embryo. Tissue movements are highly organised during gastrulation, and result in cell populations becoming correctly positioned for future interactions. The physical proximity of precursor tissues facilitates cell communication and inductive interactions that are critical for tissue patterning and subsequent morphogenesis (see Balinsky, 1970 for a general comparative review).

1.2.1 The chick embryo

The chicken blastoderm develops as a flat circular disc consisting of two parts; the inner *area pellucida* surrounded by the *area opaca* (Fig1.1A). All tissues of the embryo are formed from the majority of the *area pellucida* (as defined at primitive streak stage), with the remaining *area pellucida* and *area opaca* forming only extra-embryonic structures. Before the onset of gastrulation, the chick blastoderm has two layers; the epiblast and the hypoblast below it. The hypoblast will later be swept to the periphery and does not contribute to the embryo proper, but it may well have an important patterning role.

The first sign of asymmetry in the circular *area pellucida* is a thickening of the epiblast at the future posterior end, known as Koller's sickle (Hamburger and

Hamilton stage2). This initiates a sweeping of epiblast cells, from lateral and anterolateral positions, towards the midline in the posterior half of the blastoderm (Fig1.1B). As cells converge and become concentrated at the midline, they form the primitive streak, which begins to elongate towards the anterior of the embryo. As the streak lengthens, the cells in its thickened walls change shape. They acquire a bottle shape, with the neck of the bottle keeping the cells in touch with the surface, causing the formation of a narrow furrow along the length of the streak. As the streak approaches full length (HH stage 4; Fig1.1C), a specialised region, known as Hensen's node, forms at its anterior tip.

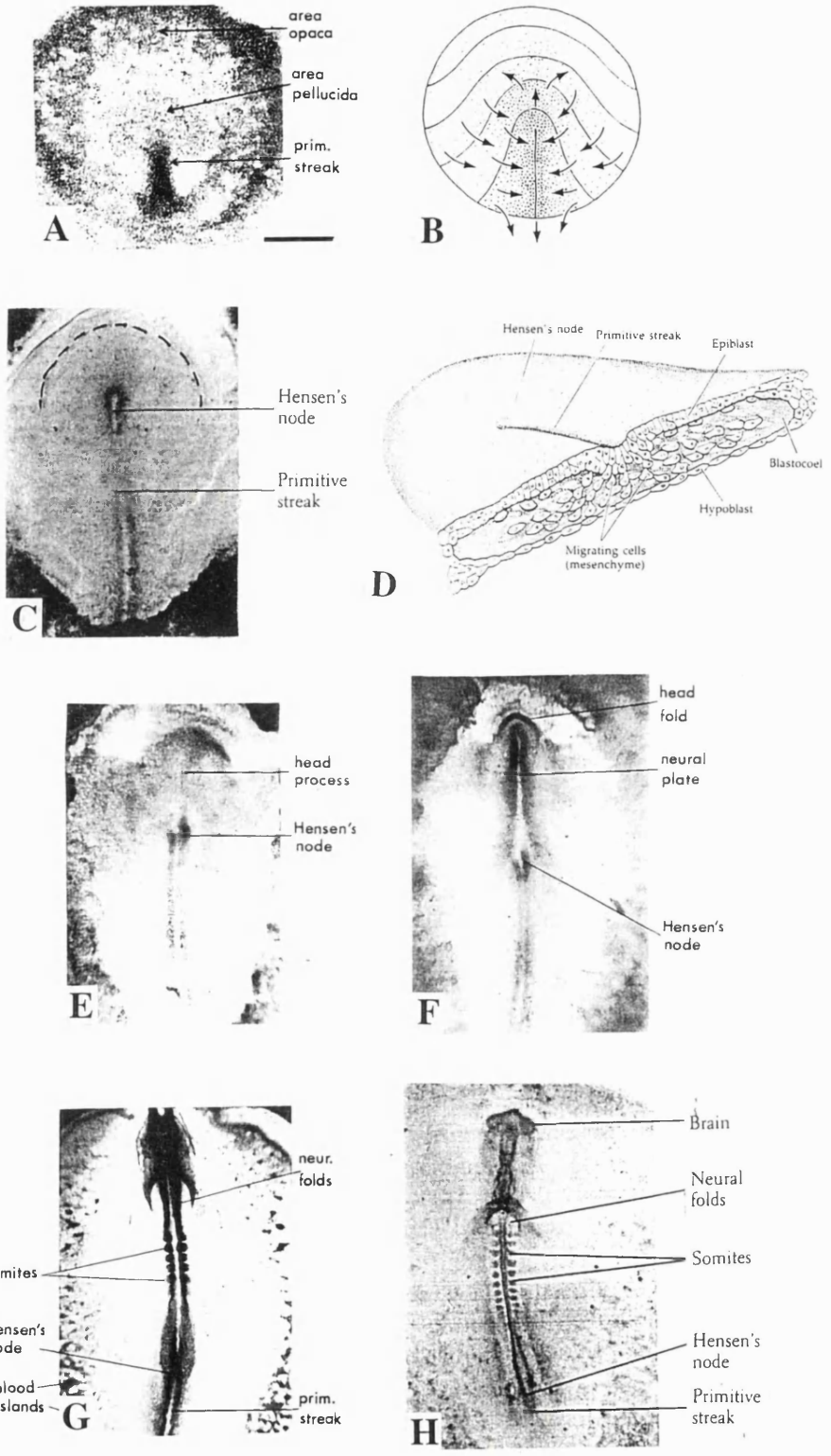
Gastrulation constitutes a coordinated, mass immigration of individual cells down through the furrow of the streak, then spreading out laterally and anteriorly (Fig1.1D). The first cells to ingress are the embryonic endoderm cells. These insert themselves into the hypoblast beneath the anterior part of the streak, gradually pushing the hypoblast out towards the edge of the *area pellucida*. Mesodermal cells then migrate between the epiblast from which they came and the newly-formed endoderm. As these cells delaminate, they are replaced by the adjoining areas of epiblast moving toward the midline, and in turn ingressing through the streak. Therefore, although the primitive streak persists, the cells which make it up do not stay in the same place and are continually replaced.

After the full-length streak stage, the node regresses back towards the posterior of the embryo (HH stage4+ onwards). As it does so, cells leave the front of the node to form the axial mesoderm; first prechordal tissue and then notochord

Figure 1.1 Normal stages of Chick development

Wholemount embryos viewed from the dorsal aspect, anterior to the top of the page. **A** Short primitive streak (HH st.3). **B** Diagram of movements in the epiblast during formation of the primitive streak. **C** Full-length primitive streak (HH st.4). Dotted line shows believed boundary of the prospective neural plate. **D** Diagram of the anterior half of the *area pellucida* cut transversely to show the migration of mesodermal and endodermal cells from the primitive streak. **E** Head process stage (HH st.5). **F** Formation of the headfold and regression of Hensen's node (HH st.6+). **G** Closure of the neural tube and formation of somites (HH st.9-). **H** Formation of the optic vesicles and three clear primary brain vesicles. Bending of fused heart tube (HH st.11-). Scale bar = 600μm for **A-C**; 350μm for **D**; 700μm for **E-G** and 1mm for **H**.

Numbers of stages after Hamburger and Hamilton, 1951.
Diagrams reproduced from Balinsky, 1970 and Hamburger and Hamilton, 1992.


area opaca
area pellucida
prim. streak
A
B
Hensen's node
Primitive streak
C
Hensen's node
Primitive streak
Epiblast
Blastocoel
Hypoblast
Migrating cells (mesenchyme)
D
head process
Hensen's node
E
head fold
neural plate
Hensen's node
F
neur. folds
somites
Hensen's node
blood islands
prim. streak
G
Brain
Neural folds
Somites
Hensen's node
Primitive streak
H

(the head process; Fig1.1E). Regression movements are a characteristic of amniote embryos, and they co-ordinate cell activities in laying down the initial organs of the embryonic axis in a patterned manner. Thus, anterior parts of the streak contribute to medial areas of the embryo (the somitic mesoderm), whereas the posterior half of the streak contributes to more lateral mesoderm (Schoenwolf *et al.*, 1992). The final residue of the streak becomes partially incorporated into the tailbud, at the very posterior of the embryo.

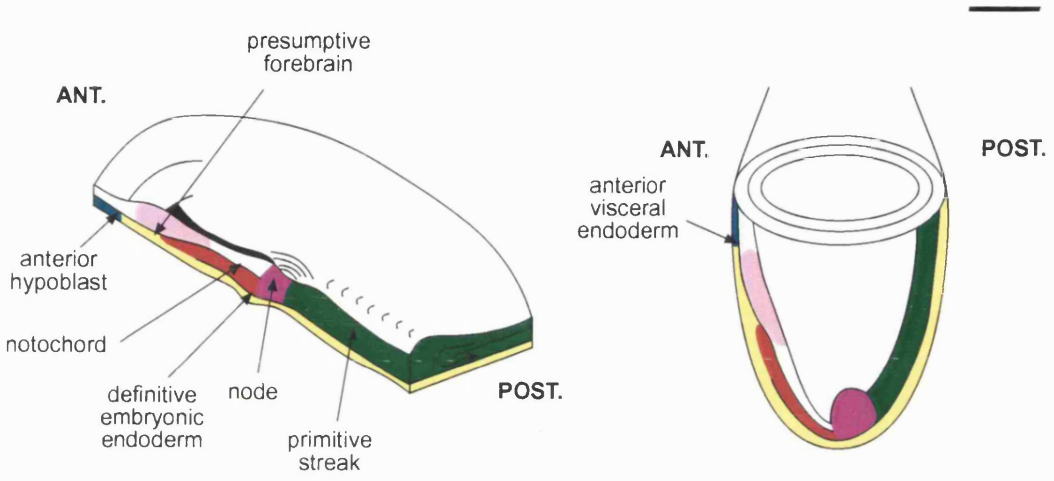
The rod-like structure of the notochord exerts tension on the *area pellucida*, stretching the embryo along its antero-posterior (AP) axis. The part of the ectoderm that will form the neural plate thickens and the anterior end bends ventrally to form the head-fold (HH stage 6; Fig1.1F). As development proceeds, the neural plate closes up to form the neural tube and begins to differentiate along its length into specialised regions of the brain and spinal cord (Fig1.1G,H). As the trunk region extends, the mesoderm lying on either side of the notochord becomes segmented into paired somites, that will form dermal, muscle and skeletal structures of the dorsal axis. Chick development is described in detail in (Bellairs and Osmond, 1998).

1.2.2 The mouse embryo

The mouse embryo develops initially from a blastocyst comprising two parts; the inner cells mass (ICM), most of which will mainly give rise to the embryo proper, and an outer shell of cells forming the trophectoderm, which forms extraembryonic tissues. The trophectoderm also encloses the blastocoel cavity. The primitive endoderm differentiates from the blastocoelic surface of the ICM, at about 4 days post coitum

(dpc). At this stage, the blastocyst must implant itself in the endometrial lining of the uterus, attaching via the trophectoderm cells that overlie the ICM. During the immediate post-implantation period (5-6dpc), the mouse embryo changes dramatically in size and shape. The ICM rapidly grows to fill the blastocoelic cavity and the whole embryo adopts a unique cylindrical shape (unlike most mammals). The ICM then epithelialises into a layer of epiblast cells and a new cavity, the proamniotic cavity, forms within the epiblast. The embryo thus acquires the shape of a cup made up of two layers, the inner epiblast and the outer visceral endoderm (part of the primitive endoderm). This is 'inside-out' in comparison to the arrangement of other mammalian embryos and to the formation of the body organs, and so requires an inversion of the germ layers after gastrulation. In relation to the site of implantation, the embryonic component of the egg cylinder lies distally, while extra-embryonic regions are proximal. Prior to gastrulation, visceral endoderm cells that are thought to establish an essential anterior patterning centre, are found at the distal tip of the egg cylinder. These cells then move anteriorly, just before the streak starts to form on the posterior side of the embryo (reviewed in Beddington and Robertson, 1998).

Gastrulation in the mouse, as in the chick, involves the recruitment of epiblast cells to a transient embryonic structure called the primitive streak. The streak arises on the posterior proximal side of the egg cylinder (6.5dpc), at the junction of embryonic and extra-embryonic tissue. As gastrulation progresses, the primitive streak elongates, ultimately extending to the distal tip of the egg cylinder. The node forms at the anterior end of the streak, but is only morphologically evident when gastrulation is well under way. At the primitive streak, the epiblast cells undergo an epithelial to



Chick (HH st.5)

Mouse (7 1/2 day egg cylinder)

Figure 1.2 Comparison of tissue organisation in mouse and chick gastrula stage embryos

Schematic diagrams showing tissue organisation in gastrula/ neurula stage chick and mouse embryos. Equivalent tissues are shaded the same colour in the two embryos. Blue = extra-embryonic hypoblast/ visceral endoderm. Yellow = definitive embryonic endoderm. Pale pink = forebrain neuroectoderm. Red = notochord. Purple = node. Green = movement of epiblast cells through streak, to form mesoderm. Scale bar = 450μm for chick embryo and 150μm for mouse embryo.

mesenchymal transition, then ingress and move laterally to form the new germ layers. As mentioned for the chick, cells move through the streak in a specific progression. Therefore, the first cells to ingress are extra-embryonic mesoderm, followed by embryonic mesoderm and definitive endoderm. The arrangement of endoderm and mesoderm cells is almost reversed as the new embryonic layers are formed, since the epiblast cells initially closest to the streak ingress through it first, and then migrate the furthest away. Definitive mesendoderm cells are inserted into the visceral endoderm, thus displacing it to overlie extra-embryonic regions (Fig1.2 shows tissue arrangement at this stage).

Midline cells leaving the node become organised into the prechordal and then notochordal plates, initially in the same layer as the endoderm, immediately anterior to the node. These cells then move to a mesodermal position and are re-organised into a cord (Poelmann, 1981; Sulik *et al.*, 1994). The neural plate becomes increasingly defined at the anterior end of the embryo, with the neural folds elevating to form the head folds (7.5dpc). Concomitant with development of the headfolds, the embryonic axis rostral to the node increases rapidly in length, leading to a posterior displacement of the node (Camus and Tam, 1999 and references therein). Development of the neural plate in more posterior regions is less advanced, and it gradually merges into the primitive streak region. The first somites are formed at 8dpc.

When the embryo has about 6-8 pairs of somites, the embryo 'turns' so that the configuration of the germ layers is reversed. Here, the embryo effectively rolls through 180° about the midpoint of its body axis, to bring the dorsal ectodermal surface onto

the outer convex surface (of the U-shape) and the midgut onto the inner concave surface of the embryo. The embryo also wraps itself in its extra-embryonic membranes; the amnion and yolk sac, supporting further development of the embryo and allowing formation of the placenta. Mouse development is described in detail in (Kaufman and Bard, 1999).

1.3 Neural induction and the organiser

Hensen's node in the chick and the mouse node, both found at the anterior tip of the primitive streak, are very important signalling centres during gastrulation and have multiple effects on the organisation of the entire embryo. They are the functional equivalents of the amphibian dorsal blastopore lip which, as first shown by the pioneering experiments of Spemann and Mangold (1924), can induce a complete second axis when grafted to the ventral side of a host embryo. Most of the nervous system in this ectopic axis developed not from the transplanted tissue, but from the host ventral ectoderm, which in an undisturbed embryo forms epidermis. This small piece of tissue was therefore able to influence the host cells around it, changing their fate and arranging them into a complete second axis. Spemann named the dorsal blastopore lip the "organiser", and proposed that in normal development this region induces and organises a correctly patterned nervous system in neighbouring dorsal ectoderm.

In the 70 years since Spemann's discovery of the organiser, an enormous amount has been learnt about the cellular events associated with neural induction and patterning.

Equivalent organiser structures have been identified in other vertebrates; Hensen's node in chick (Waddington, 1933; Storey *et al.*, 1992), the node in mouse (Beddington, 1994) and the shield in zebrafish (Shih and Fraser, 1996). Conserved patterns of gene expression between these tissues, together with the observations that interspecies grafts in several combinations (Kintner and Dodd, 1991; Blum *et al.*, 1992; Hatta and Takahashi, 1996) lead to neural induction, suggest that at least some of the signalling mechanisms, by which the organiser recruits its surrounding cells to organise a basic body pattern, are highly conserved during vertebrate evolution. However, it is only in the last decade that the nature of these inducing signals released from the organiser has begun to be elucidated.

1.3.1 The 'Default model'; inhibition of BMP signalling

For a long time, investigators thought of neural inducers as substances that actively promote neural development, since a transplanted organiser dominates over the would-be fate of its neighbouring cells. Instead a more convoluted double-negative mechanism for neural induction is now favoured, based on experimental work in *Xenopus*. The default state of ectoderm is neural; this pathway is actively blocked on the ventral side of the embryo by neural inhibitors; neural inducers therefore act on the dorsal side of the embryo to provide a permissive condition for neurogenesis, by antagonising the neural inhibitors. This is supported by the observation that cellular dissociation of animal caps leads to the formation of neural tissue (Harland and Gerhart, 1997; Sasai and De Robertis, 1997; Wilson and Hemmati-Brivanlou, 1997), suggesting that cells within the ectoderm of the *Xenopus* gastrula have an autonomous tendency to differentiate into neural tissue.

The ventralising bone morphogenetic proteins (*BMP*s), particularly *BMP2* and *BMP4*, are proposed to function as epidermal inducers and neural inhibitors. The autoneuralisation of *Xenopus* animal caps can be suppressed by *BMP4* (Wilson and Hemmati-Brivanlou, 1995), or by effectors of BMP4 signalling (Suzuki *et al.*, 1997; Wilson *et al.*, 1997). Further support for the default model comes from experiments showing that not only do *BMP*s induce epidermal fate *in vitro*, but inhibition of endogenous BMP signalling neuralises ectodermal explants. Animal caps cut from embryos injected with RNA encoding dominant-negative *BMP* receptor (Suzuki, 1995; Xu *et al.*, 1995), dominant negative (non-cleavable) ligand (Hawley *et al.*, 1995) and antisense *BMP4* RNA (Sasai *et al.*, 1995) adopt a neural instead of an epidermal fate.

Normal *BMP4* expression in *Xenopus* is also consistent with its proposed anti-neuralising function, being widespread in the entire ectoderm at the start of gastrulation and then cleared from the presumptive neural plate at the time when the organiser appears (Fainsod *et al.*, 1994; Hemmati-Brivanlou and Thomsen, 1995; Schmidt *et al.*, 1995).

Several candidate endogenous neural inducing molecules have been identified, that act by inhibiting BMPs. *Noggin* (Smith and Harland, 1992), *chordin* (Sasai *et al.*, 1994) and *follistatin* (Hemmati-Brivanlou *et al.*, 1994) are all expressed in the deep layers of the organiser and its derivatives. They can induce neural tissue directly in ectodermal explants, both as injected RNA and as soluble proteins (Lamb *et al.*, 1993; Hemmati-

Brivanlou *et al.*, 1994; Sasai *et al.*, 1995). Furthermore, all three molecules have been shown to bind directly to mature BMP2, 4 and 7 proteins and prevent them from reaching their receptor(s) (Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996; Fainsod *et al.*, 1997), suggesting that BMP inhibition plays a role in neural induction *in vivo*. This mechanism has been highly conserved during evolution, since in *Drosophila*, the product of the gene *short gastrulation* (sog; homologue of vertebrate *chordin*) antagonises the Decapentaplegic (Dpp; *Drosophila* homologue of BMP4) signalling pathway during early DV patterning of the embryo (Francois and Bier, 1995; Biehs et al., 1996; Nguyen *et al.*, 1998). In addition, analysis of double mutant *Drosophila* embryos has shown that both *dpp* and *screw*, a gene encoding another BMP ligand which acts to potentiate the Dpp signal, are epistatic to *sog*, suggesting that all functions of Sog involve the Dpp signalling pathway (Holley *et al.*, 1996; Nguyen *et al.*, 1998). Indeed, no receptors have yet been identified for Noggin, Chordin or Follistatin, suggesting that in vertebrates too, these ligands act solely to modulate BMP signalling.

More recently, other neural inducing molecules have been described. *Flik* (follistatin-like), the chick homologue of mammalian *TSC36*, encodes a secreted protein with a cysteine-rich domain strongly related to those found in Follistatin (Patel *et al.*, 1996). *Flik* is expressed in the node and node-derived axial mesoderm, as well as weaker expression in the neural plate. The mechanism of action of Flik protein *in vivo* is unknown at present. However, treatment of gastrular stage embryos with antisense oligodeoxynucleotides to the gene causes reduced allocation of neural tissue, together with a delay in clearance of *BMP4* mRNA from this region, suggesting that Flik

protein may somehow interact with BMP signalling pathways (Towers *et al.*, 1999). *Xnr3* (*Xenopus* nodal-related) is another gene expressed in the outer ectodermal layer of the organiser (Smith *et al.*, 1995) that can induce neural tissue directly in *Xenopus* when provided as RNA (Hansen *et al.*, 1997). *Xnr3* and *BMP4* have mutually antagonistic activities (Hansen *et al.*, 1997), suggesting that *Xnr3*, itself a diverged TGFβ superfamily member, may competitively bind to BMP receptor(s) and act in a dominant negative way to inhibit BMP signalling (Thomsen, 1997).

In *Xenopus*, therefore, the evidence that the actions of the organiser in neural induction rely on modulation of BMPs by endogenous inhibitors, is compelling. However, results from other organisms suggest that this interpretation is too simplistic, and that neural induction is likely to involve co-operation of different classes of signal (reviewed in Streit and Stern, 1999). BMP inhibition is not sufficient for neural induction in the chick, since mis-expression of either *noggin* (A.Streit and C.D.Stern, unpublished) or *chordin* (Streit *et al.*, 1998) does not induce expression of any neural markers. Moreover, cell dissociation of the epiblast in chick does not lead to neural differentiation, as it does in *Xenopus*, but promotes muscle development (George-Weinstein *et al.*, 1996). However, Chordin-secreting cells can stabilise expression of the pan-neural marker *sox3* in non-neural ectoderm, if these cells have previously been exposed to a node graft for five hours (too brief in itself for induction of a neural plate) (Streit *et al.*, 1998), suggesting that BMP inhibition may work in conjunction with other signals from the organiser.

The expression patterns of *BMP*s and their inhibitors in other organisms also do not quite fit with their proposed roles in *Xenopus*. *Follistatin* is not expressed in the mouse node (Albano *et al.*, 1994) and only weakly in the chick (Levin, 1998), and neither *noggin* nor *follistatin* are expressed in the zebrafish shield (Bauer *et al.*, 1998). Global exposure of stage 3 chick embryos to supernatant from Noggin-expressing CHO cells produces no observable effect, and exposure of a stage 4 node to the same supernatant has no effect on the ability of the node to induce a second axis when grafted into an unexposed host (Connolly *et al.*, 1997). *Chordin* expression, while present in the chick node at the right time to take part in neural induction, persists long after the node has virtually lost its inducing ability.

Loss of function mouse mutants also fail to show an essential function for BMP antagonists in neural induction. *Noggin* mutants develop a fairly normal neural plate and show patterning defects only at later stages (McMahon *et al.*, 1998). Even noggin/chordin double knockout embryos have the majority of the neural axis, although they do show very early anterior brain defects (Bachiller *et al.*, 2000). Null mutants for *follistatin* (Matzuk *et al.*, 1995), *BMP7* (Dudley *et al.*, 1995) or *BMP2* (Zhang and Bradley, 1996) have no early neural phenotype. A proportion of mutants lacking *BMP4* die before gastrulation, but a few survive to early limb bud stages; these do not appear to have an enlarged nervous system or absence of epidermis (Winnier *et al.*, 1995). Embryos with mutations in the BMP receptor Bmpr-1a die too early to be informative (Mishina *et al.*, 1995). All these data therefore suggest that additional pathways are involved in neural induction.

1.3.2 Other pathways for neural induction

BMP inhibitors are not the only molecules that have been reported to have direct neuralising activity. A novel secreted protein, Cerberus, is expressed in the extreme anterior endomesodermal domain of the *Xenopus* organiser during gastrulation, and can induce neural tissue in ectodermal explants, as well as second heads in whole embryos (Bouwmeester *et al.*, 1996). Biochemically, it has been shown that Cerberus protein can antagonise not only BMPs, but also the Wnt and Nodal signalling pathways; it has independent sites to directly bind BMP, Wnt and Nodal proteins in the extracellular space, and thus inhibit the actions of all three simultaneously (Glinka *et al.*, 1997; Piccolo *et al.*, 1999). Two other Wnt antagonists are expressed in the *Xenopus* organiser; *frzb-1* (Leyns *et al.*, 1997; Wang *et al.*, 1997) and *dickkopf* (Glinka *et al.*, 1998). Both are secreted molecules, and Frzb-1 has been shown to bind to Wnt proteins directly (Wang *et al.*, 1997), suggesting that *frzb-1* and *dickkopf* may prevent Wnt proteins from reaching their receptors by a functional interaction analagous to BMPs and their inhibitors. *Frzb-1* is also expressed in the mouse anterior streak (Hoang *et al.*, 1998) and chick neural ectoderm (Baranski *et al.*, 2000). These additional candidates raise the question of whether other pathways operate in parallel with the anti-BMP mechanism of neural induction.

There is some evidence that FGFs can act as direct neural inducers in both *Xenopus* (Lamb and Harland, 1995) and chick embryos (Storey *et al.*, 1998; Alvarez *et al.*, 1998). However, the role of FGFs or FGFR signalling in neural induction remains far from clear (reviewed in Mason, 1996). Cox and Hemmati-Brivanlou (1995) found that neural markers were not detected in animal cap explants treated with FGF2, unless the

caps were pre-treated with a neuralising agent. Two different groups have shown that the neuralising activity of noggin and chordin in animal caps requires an intact FGF signalling pathway (Launay *et al.*, 1996; Sasai *et al.*, 1996) suggesting that FGFs may regulate the competence of ectoderm to respond to neural inducers. Animal caps cut from embryos expressing a dominant-negative FGF receptor (XFD) were found to be insensitive to neural induction by organiser grafts or by noggin (Launay *et al.*, 1996). However, other groups have found expression of neural markers in animal caps cut from embryos co-injected with XFD and noggin (Bang *et al.*, 1997). Thus, evidence regarding the role of FGF signalling in neural induction is somewhat ambiguous.

1.3.3 Onset of neural induction

An important point that still needs to be established is when neural induction begins. As more neural inducing molecules are discovered, it is becoming apparent that full neural induction probably requires a sequence of multiple signals and cell states. It may well be that each step in the hierarchy stabilises the previous ones, altering the competence of the ectoderm to respond to future signals. Differences in the timing of these steps between species may therefore underlie the apparently different signalling requirements for neural induction in various species. In *Xenopus*, it appears that the dorsal ectoderm is already biased in favour of a neural fate by the late blastula stage (Savage and Phillips, 1989; (Kroll *et al.*, 1998), and the boundary between neural and non-neural ectoderm may be positioned before gastrulation (Zhang and Jacobson, 1993). Therefore, it could be that the dorsal ectoderm has received some neural-inducing signals before gastrulation, and that additional permissive, or stabilising signals (such as BMP inhibition) are merely required to continue its differentiation in a

neural direction. A 'pre-neurulation' state is proposed to exist in the central epiblast of the unincubated (blastula stage) chick embryo (Callebaut *et al.*, 1998). Furthermore, restricted expression of the pan-neural gene *sox3* and the carbohydrate epitope L5, both thought to reflect competence of the ectoderm to respond to neural inducing signals, are seen very early (Rex *et al.*, 1997; Streit *et al.*, 1997). However, the neuroectoderm is not specified until just before stage 4, and requires continued signalling until then (Darnell *et al.*, 1999).

1.4 Anteroposterior patterning of the neural plate

The ectopic second axis that is formed by the actions of a graft of organiser tissue frequently contains a correctly patterned nervous system. The organiser therefore, not only induces multiple tissue types, but also patterns them, suggesting an intimate relationship between the two processes.

1.4.1 Planar and vertical signalling

Basic patterning of the neural territory is likely to begin during gastrulation, once neural induction is under way. As gastrulation proceeds, the physical relationships between cell populations change, so that mesoderm and endoderm layers come to lie beneath the ectoderm. Neural patterning signals from the organiser could therefore reach the ectoderm via a planar route, while the cells still occupy the same layer, or by the vertical route, from the involuted dorsal mesoderm to the overlying ectoderm (reviewed in Doniach, 1993). In the chick, these two signalling routes would therefore be either within the plane of the epiblast or, following ingression of cells through the

streak, from the prechordal tissue and notochord to the overlying ectoderm. Neural patterning information has been shown to be transferred by both routes, mainly from work in *Xenopus*. However, the extent to which vertical and planar signals function *in vivo* remains unresolved, and may vary between species.

Otto Mangold proposed that neural pattern is achieved purely by vertical induction, where the AP pattern of the mesoderm is "imprinted" onto the overlying ectoderm (Mangold, 1933). Taking pieces of involuted dorsal mesoderm from different axial levels and inserting them individually into the blastocoel cavities of early gastrulae, he found that the AP level of neural tissue induced roughly corresponded to that of the inserted mesoderm. He suggested that the different regions of the involuted mesoderm therefore contain qualitatively different neural inducers, and that they each induce a different AP element of the neural pattern to the ectoderm directly above. However, the range of neural tissue induced by a given piece of mesoderm was broader than would have been found in the ectoderm immediately above it in the intact embryo. The evidence for generation of planar signals, mainly derived from use of Keller explants, further suggests that a strict interpretation of the Mangold model is not likely *in vivo*.

Keller explants are made by culturing dorsal mesoderm and ectoderm from early gastrulae as a single, flat sheet, with contact only along a single edge and vertical contact prevented. In this configuration, a range of neural marker genes can be induced in the ectoderm, in an AP order equivalent to that in the intact embryo (Doniach, 1993). Nieuwkoop also provided evidence for planar patterning signals

(Nieuwkoop, 1952) but, more importantly, his experiments led him to propose a model for the mechanism of AP neural patterning that is compatible with both planar and vertical signalling, and is still widely accepted today (see next section). He inserted folded flaps of competent ectoderm perpendicularly into the presumptive neural plates of early gastrulae. The ectoderm therefore had contact with the host at only one end of the fold, and any planar signals that might exist could spread into it. He consistently found that the folds displayed posterior to anterior neural pattern from the attached base of the fold to the outer tip. Furthermore, the type of neural tissue at the base matched that of the region in which the fold was implanted, and significantly, was always of a more posterior type than that at the outer tip.

1.4.2 Activation-transformation model

Based upon his ectoderm fold experiments, Nieuwkoop proposed a model for AP neural patterning involving two sets of inducer signals (Nieuwkoop, 1952). An initial "activator" signal, present in all organiser mesoderm, induces neural tissue with anterior character. A second "transformer" signal, present in a gradient with a high point in the posterior mesoderm, then progressively posteriorises the neural plate to generate the remaining regions of the CNS. He argued that the transformer is dominant over the activator, since the proximal part of the fold developed with a more posterior character, even though it must have experienced both factors. The discovery of the neural inducer molecules *noggin* and *chordin* lend support to this model, since they both induce neural tissue expressing anterior markers in *Xenopus* animal caps. Furthermore, three classes of signal- *Wnts*, *FGFs* and retinoic acid- have been shown to posteriorise neural tissue, making them candidates for the transformer signal

(reviewed in Doniach, 1995; Sasai and De Robertis, 1997). Other molecules are probably also involved; tissue grafting experiments have shown that posterior non-axial mesoderm (that comes to underlie the posterior neural plate) can exert a posteriorising influence on forebrain regions, but this activity is unlikely to be mediated by the signals just mentioned (Bang *et al.*, 1997; Woo and Fraser, 1997).

The vitamin A metabolite retinoic acid (RA) is thought to play a role in posteriorising the CNS by influencing the expression of *Hox* genes, which are involved in positional identity along the body axis (reviewed in Maden and Holder, 1992; Conlon, 1995). Treatment of *Xenopus* embryos with RA causes a reduction in forebrain volume and corresponding increase in hindbrain volume (Durston *et al.*, 1989; Sive *et al.*, 1990). More subtle effects are also seen in the hindbrain, where anterior rhombomeres are reduced or compressed (Papalopulu *et al.*, 1991). Furthermore, constitutively active RA receptors reduce anterior neural tissue while dominant negative RA receptors expand anterior neural structures (Blumberg *et al.*, 1997). Several *Hox* genes contain RA response elements, and RA can induce *Hox* gene expression in mouse ectoderm, while repressing expression of more anterior genes such as *otx2* (Conlon and Rossant, 1992; Ang *et al.*, 1994). In addition, targeted mutagenesis of RA receptors can produce homeotic alterations to the axial skeleton similar to certain *Hox* loss-of-function phenotypes (Lohnes *et al.*, 1994), although the majority of these mutant phenotypes are surprisingly mild. These results have led to the suggestion that there may be an endogenous gradient of RA within the neural plate, with a high point at the posterior end, that promotes development of posterior structures while restricting the expression of forebrain genes to the anterior of the embryo. However, recent fine-

tuned analyses of the distribution of endogenous RA during neurulation, and the localisation of the main enzymes involved in the biosynthesis and degradation of RA, challenge this concept (Maden *et al.*, 1998; reviewed in Maden, 1999; Gavalas and Krumlauf, 2000). The highpoint of the RA gradient is in fact at the hindbrain/ spinal cord boundary, and the RA concentration gradually drops towards the posterior end of the embryo (Maden *et al.*, 1998). In addition, treatment of mouse and zebrafish embryos with RA causes a reduction of anterior rhombomeres but does not affect forebrain and midbrain development (Holder and Hill, 1991; Morris-Kay, 1991; Woo and Fraser, 1997), suggesting that global effects of RA on neural structures are unique to *Xenopus*, and that a more conserved function of RA is in patterning the hindbrain.

A role for FGF signalling in posteriorising neural tissue is supported by many different experiments (reviewed in Doniach, 1995; Mason, 1996). bFGF (FGF2) can transform a frog anterior neural plate explant into posterior CNS *in vitro* (Cox and Hemmati-Brivanlou, 1995). When animal caps are co-treated with bFGF and either noggin or chordin, posterior neural markers are induced in addition to forebrain markers, at opposite poles of the explant (Lamb and Harland, 1995; Cox and Hemmati-Brivanlou, 1995; Sasai *et al.*, 1996). Injection of a dominant negative receptor (XFD) at the two cell stage results in embryos completely lacking posterior trunk and tail structures, but with relatively normal anterior neural structures (Amaya *et al.*, 1991; Launay *et al.*, 1996). Furthermore, over-expression of XFD in Keller explants inhibits posterior neural markers but not anterior or pan-neural markers (Holowacz and Sokol, 1999). The authors also found they could achieve similar effects in whole embryos, by targetting XFD to both dorsal and lateral regions (Holowacz and Sokol, 1999).

However, in transgenic embryos expressing zygotic XFD in every cell, Kroll and Amaya (1996) were unable to reveal a role for FGF in either neuralisation or posterior neural patterning.

Analysis of mouse mutants further supports a role for FGF signalling in AP neural patterning. *FGF8* -/- mice show perturbed patterning of the neural plate; anterior neuroectoderm markers are widely expressed, while posterior markers are absent (Sun *et al.*, 1999). In addition, *FGFR-1* deficient mouse embryos frequently exhibit truncations or disorganisation of posterior embryonic regions (Deng *et al.*, 1994; Yamaguchi *et al.*, 1994). Finally, a large number of *FGF*s have expression patterns confined to posterior axial and paraxial mesoderm, and not anterior mesoderm, suggesting that several members of the family could be necessary for posterior development *in vivo*. However, it is unlikely that a gradient of FGF signalling is responsible for producing AP pattern, since no clear correlation between the dose of FGF and the axial level of the neural marker induced, has been demonstrated. It is possible instead that the effects of FGF depend upon competence of the responding tissue, since the age of the responding tissue appears to affect the nature of the response produced (Lamb and Harland, 1995).

Finally, members of the Wnt family are good candidates for a posterior transformation signal. In *wnt3* -/- mice, the epiblast proliferates in an undifferentiated state that lacks AP neural patterning (Liu *et al.*, 1999). In addition, the function of *wnt3a* has been shown to be essential for posterior development in mouse (Takada *et al.*, 1994), and

wnt3a can synergise with *noggin* and *follistatin* to increase the expression of posterior neural genes in *Xenopus* animal cap explants (McGrew *et al.*, 1995).

1.4.3 Head and trunk organisers

The organiser itself is a dynamic entity; its cell population, structural organisation and profile of gene expression constantly change during gastrulation. In *Xenopus* and avian embryos, changes in the cellular composition and gene activity of the organiser can be correlated with its ability to induce the differentiation of tissues with different AP characteristics. Transplantations of early organisers result in complete axial duplications, including heads, whereas grafts of later organisers have reduced inducing ability and result in only partial axes containing just trunk (Gerhart *et al.*, 1991; Storey *et al.*, 1992). This finding, first observed by Spemann (1931), has led to the concept of distinct organisers for head and trunk regions of the embryo (reviewed in Doniach, 1993; Harland and Gerhart, 1997; Niehrs, 1999).

The developmental fate of organiser cells also changes during gastrulation, with cells contributing to progressively different parts of the axial mesoderm. Derivatives of the early node/organiser contribute to the prechordal mesoderm, while cells of the late node/organiser are destined for the more posterior notochord, and are not found in the head region (Selleck and Stern, 1991; Selleck and Stern, 1992; Beddington, 1994; Lane and Keller, 1997; Lemaire *et al.*, 1997). Since these mesoderm derivatives of the organiser retain neural-inducing activity themselves, they may underlie the head versus trunk patterning activity of the organiser in embryos of different stages.

However, evidence for compartmentalisation of separate head and trunk patterning activity within the organiser has recently been shown in *Xenopus*. The early gastrula organiser can be subdivided into an anterior (vegetal) domain that expresses *goosecoid*, and a posterior (animal) domain that expresses *Xnot* (Vodicka and Gerhart, 1995). Studies performed on isolated halves of the organiser show that each half not only has a distinct fate but also induces a unique set of region-specific neural genes. When grafted orthotopically to the organiser region of a host embryo, the anterior (*gsc*) half develops into prechordal mesoderm while the posterior (*Xnot*) half develops into notochord and somites (Zoltewicz and Gerhart, 1997). When co-cultured with animal cap ectoderm, the anterior half induces only anterior-specific genes, while the posterior half induces anterior and posterior neural genes (Zoltewicz and Gerhart, 1997). These results demonstrate that the organiser is already patterned by the early gastrula stage, with segregation of region-specific neuralising activity, and these separate functional compartments are marked by differing gene expressions.

Similar compartmentalisation of the organiser prior to gastrulation has also been demonstrated in the zebrafish organiser (Gritsman *et al.*, 2000 and references therein). Differential activation of the Nodal signalling pathway is proposed to be responsible for establishing this AP pattern within the organiser and modulating fate decisions. Higher levels or longer exposure to Nodal signals are required for expression of *gsc* and prechordal plate formation, whereas lower transient levels are sufficient to induce *floating head* expression (the zebrafish homologue of *Xnot*) and notochord development. In addition, the attenuation of Nodal signalling in zygotic *one-eyed-pinhead* mutants causes down-regulation of *gsc* and expansion of the *flh* expression

domain, followed by a fate change of cells in the prechordal plate domain to notochord (Gritsman *et al.*, 2000).

Regionalisation within the avian node has also been reported (Storey *et al.*, 1995), with the source of neural inducing ability localised to two sub-regions and associated with specific cell types contained in these regions. Gene expression patterns for *gsc* and *cNot* are similar to those observed in other species; largely restricted to prechordal mesoderm and notochord, respectively (Pera and Kessel, 1997 and references therein). However, other evidence suggests that determination of prechordal mesoderm fate in the node is not complete, and further signalling is required after these cells have extended away (Vesque *et al.*, 2000). Early extending axial mesoderm co-expresses markers of both notochord and prechordal mesoderm throughout its length, only becoming segregated at later stages. TGFβ signals from the anterior endoderm are thought to be responsible for downregulating notochord characteristics and inducing prechordal mesoderm properties in the most anterior axial mesoderm, as it extends (Vesque *et al.*, 2000).

The recent rapid increase in knowledge about molecules with head inducing activity, has led to new insights into the molecular nature of the separate head and trunk organisers. Cerberus is a secreted protein expressed in the anterior endoderm of the organiser and has the unique property of inducing ectopic heads in the absence of trunk structures (Bouwmeester *et al.*, 1996). It has been shown that Cerberus protein has independent sites to directly bind BMP, Wnt and Nodal proteins in the extracellular space, and is therefore capable of antagonising all three signalling

pathways simultaneously (Glinka *et al.*, 1997; Piccolo *et al.*, 1999). Furthermore, Piccolo *et al.* (1999) believe that all three inhibitory activities of *cerberus* are necessary for the formation of ectopic head structures. However, it is unlikely that *cerberus* interacts with the BMP pathway in the same manner as classical neural inducers, such as *noggin* and *chordin*, since *cerberus* actually inhibits trunk and tail formation and cannot dorsalise mesoderm (Bouwmeester *et al.*, 1996).

The discovery of two other secreted Wnt inhibitors, *frzb-1* (Leyns *et al.*, 1997; (Wang *et al.*, 1997) and *dickkopf-1* (Glinka *et al.*, 1998), further supports the hypothesis that head induction is achieved by simultaneous repression of TGFβ and Wnt signalling. Injection of *frzb-1* or *dkk-1* alone does not induce a second axis. However, co-injection of either of these mRNAs with that encoding the dominant negative BMP-2/4 receptor (tBR) induces complete second axes, with forebrains, eyes and cement glands (Glinka *et al.*, 1997; Glinka *et al.*, 1998). Interestingly, co-injection of *frzb-1* and tBR mRNA also leads to strong ectopic expression of *cerberus* in the ventral endoderm (Piccolo *et al.*, 1999). Other combinations of Wnt and BMP inhibitors, such as *chordin* and *frzb-1* mRNAs, or tBR and dnXwnt8 mRNAs, similarly induce complete second axes when co-injected ventrally (Glinka *et al.*, 1997; Piccolo *et al.*, 1999). It is noteworthy that *dickkopf-1* induces heads with two eyes, unlike *cerberus* and *frzb-1* which induce only one; this may reflect differences in their normal domains of expression, since *dkk-1* is normally expressed in the prechordal plate (known to play a role in splitting the eye field) whereas *cerberus* is not. The requirement for *dkk-1* in head formation is demonstrated by injection of inhibitory antibodies to Dkk-1, which produces embryos

with microcephaly and cyclopia, lacking a head altogether in some cases, while the trunk appears normal (Niehrs, 1999).

Nodal signalling has also been shown to inhibit head formation, and the combination of BMP and nodal antagonists alone is sufficient for formation of an ectopic second head (Piccolo *et al.*, 1999). Interestingly, *nodal* is actually required early on for head formation (Varlet *et al.*, 1997). Early *nodal* signalling also induces *cerberus* expression; *cerberus* is induced by injection of *Xnr1* mRNA, but not by *Xnr1* DNA, which is only expressed after the mid-blastula transition (Piccolo *et al.*, 1999). Therefore, it appears that early and late Nodal signalling have almost opposite roles in neural development (like Wnt signalling); an early Nodal signal is required to pattern the anterior endoderm and perhaps induce the organiser effectors, such as *cerberus*, which then feed back negatively on Nodal signalling in order to initiate head fate and inhibit trunk fate (Piccolo *et al.*, 1999). In zebrafish, nodal signalling is required to maintain *dkk1* expression in the gastrula; bozozok, squint and oep mutants (all defective in some aspect of nodal signalling) show normal initiation of *dkk1* in the blastula, but this is subsequently lost in the shield at early gastrula stage (Hashimoto *et al.*, 2000).

1.4.4 Summary

The organiser is not a homogeneous cell population; signals produced by different domains of the organiser and its derivatives selectively inhibit different families of growth factors, producing neural structures with different regional character. Patterning of embryonic structures at gastrulation is therefore accomplished by

balancing the inductive signals with their specific antagonists that emanate from the organiser and organiser-derived tissues. The discovery that head induction can be achieved by simultaneous repression of TGFβ and Wnt signalling provides the basis for a molecular model that incorporates the concepts of both Nieuwkoop's activator and transformer, and Spemann's head and trunk inducer (Niehrs, 1999). According to the model, BMP and Wnt inhibitors released from the anterior organiser and its derivatives neutralise both BMP and Wnt signals in the ectoderm, leading to anterior neural induction (head organiser; Nieuwkoop's activator signal). More posteriorly, Wnt inhibitors are progressively less active, so that a gradient of Wnt activity is established along the AP axis. These Wnt signals are then able to posteriorise neural fates induced by BMP inhibition alone (trunk organiser; Nieuwkoop's transformer signal). Other non-organiser-associated posteriorising factors, including FGFs and retinoic acid, could then be involved in full elaboration of the AP pattern as the axis elongates (Doniach, 1995; Sasai and De Robertis, 1997). However, even this model is unlikely to be complete, given recent evidence proposing that inter-regulation of activin and nodal-related factors with their antagonist, antivin, plays a role in generating pattern along the AP axis (Thisse *et al.*, 2000).

1.5 Tissues involved in head induction

The cell movements of gastrulation bring the neuroectoderm into contact with several different tissues, raising the possibility that any of these tissues could impart patterning information to the neural plate (Fig1.3). Organiser-derived cells form the axial mesoderm and definitive embryonic endoderm, both of which move under the

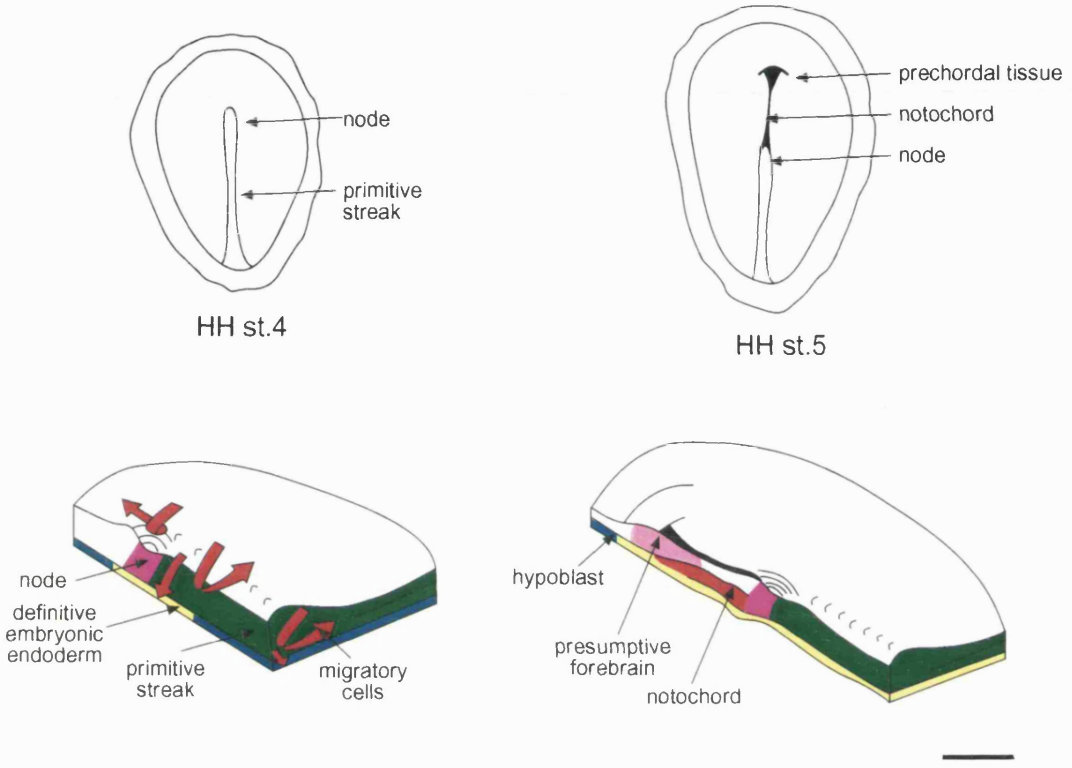


Figure 1.3 Gastrulation movements in the chick between stages 4 and 5

Dorsal surface views (top panels) showing appearance of the embryo at stages 4 and 5, and diagrams of anterior quadrants (bottom panels) to illustrate cell movements during these stages. Stage 4 = full length streak stage. Epiblast cells ingress through the streak and move laterally and anteriorly to form the mesoderm (green). Cells from the streak also insert themselves into the hypoblast (blue), pushing the hypoblast to the periphery of the *area pellucida*, and forming a new layer; the definitive embryonic endoderm (yellow). Stage 5 = head process stage. The first axial mesoderm (red) cells to leave the node form the fan of prechordal mesoderm, which ends up in the most anterior position. This is followed by cells which form the rod-like notochord. Scale bar = 750μm in top panels and 500μm in bottom panels.

neural plate and could therefore pattern it by vertical signalling. In addition, the organiser inserts cells into the midline of the neural plate, that form floorplate. Planar patterning signals within the ectoderm could therefore emanate from the neural midline, or from the peripheral neural/ non-neural boundary. Finally, early anterior patterning signals could originate in the visceral endoderm, or hypoblast. This tissue, which is established entirely separately from the streak and organiser, is extra-embryonic and will be swept to the periphery as it is replaced by definitive embryonic endoderm. However, prior to gastrulation, it too underlies the area of the embryo that will form the neuroectoderm. The evidence for signalling from each of these tissues will be discussed in turn.

1.5.1 Role of the extra-embryonic endoderm

The mouse node (and therefore its derivatives) does not appear to contain all the information necessary to induce a complete axis, since the duplicated axis formed following a node graft of any age in mouse embryos, lacks the most anterior regions of the CNS (Beddington, 1994; Tam and Steiner, 1999). Furthermore, ectopic expression of *Cwnt8* in mouse causes duplication of the primitive streak and node, just as in *Xenopus*. However, this does not result in formation of a second brain or heart in the mouse, unlike in *Xenopus* (Popperl *et al.*, 1997). These data suggest that other signals besides those of the organiser are also required for anterior patterning.

A growing variety of embryological, genetic and molecular studies propose that the anterior visceral endoderm (AVE) is important for anterior embryonic development in the mouse, and may provide the additional signals that the node lacks (reviewed in

Beddington and Robertson, 1998). AVE cells are first located in the distal tip of the egg cylinder and move, thus transferring their signalling information, to the anterior side of the embryo, prior to streak formation. The homeobox gene *hex* is expressed in these cells both at the distal tip and then on the anterior side of the embryo, and is the first sign of AP asymmetry in the visceral endoderm (Thomas *et al.*, 1998). Several other genes have been identified that are co-expressed in the AVE underlying the anterior third of the embryo; these include the VE-1 antigen (Rosenquist and Martin, 1995), *otx2* (Acampora *et al.*, 1995) HNF3β (Ang and Rossant, 1994), *lim1*, *goosecoid* and *cerberus-like* (*cerrl*) (Belo *et al.*, 1997; Shawlot *et al.*, 1998). All of these genes are localised to the anterior embryo just as the streak starts to form; this suggests that anterior identity is defined and actively established by a mechanism that is independent of the streak-derived signals in the posterior embryo. As the streak forms, the AVE is further subdivided into an anterior domain corresponding to the future site of heart development, marked by the expression of *mrg1* (Dunwoodie *et al.*, 1998) and a more posterior domain overlying the ectoderm that will form forebrain, which expresses *hesx1* (Hermesz *et al.*, 1996; Thomas and Beddington, 1996).

Several of the genes expressed in the AVE at this early stage are later expressed in the underlying anterior neuroectoderm, suggesting that the antero-posterior pattern in the visceral endoderm is imparted upon the embryonic layers by vertical signalling during gastrulation. In support of this theory, ablation of the AVE from early gastrula embryos abolishes or severely reduces the later expression of *hesx1* in the prospective forebrain region (Thomas and Beddington, 1996). Replacement of the *otx2* gene with a lacZ reporter gene produces *otx2*-/- mice in which the sites of would-be *otx2*

expression can be followed by staining for lacZ. In such mutant embryos, lacZ staining and transcription remain high in the visceral endoderm but they are abolished in the epiblast (Acampora *et al.*, 1995), suggesting that expression of *otx2* itself in the AVE is normally required for its subsequent induction in the ectoderm. Loss of *lim1* expression in the AVE also results in loss of the neuroectodermal *otx2* expression domain (Shawlot and Behringer, 1995), again highlighting the dependence of anterior brain ectoderm on the visceral endoderm to maintain appropriate expression patterns. Interestingly, a mutation in *Cripto* has been described that prevents the appropriate rotation of visceral endoderm and epiblast (Ding *et al.*, 1998). *Cripto* encodes a protein with cysteine rich and EGF-like motifs, belonging to the same family as zebrafish *one-eyed-pinhead.* Homozygous *cripto* mutants do not orientate their AP axis correctly, and fail to form a streak or node. An ectopic AVE is present however, which remains at the distal tip of the egg cylinder, and the epiblast adjacent to it abnormally assumes anterior character. Similarly, expression domains of anterior neural markers are abnormally expanded in the *FGF 8* mutant; this is thought to be at least partly because the AVE fails to be displaced, since no definitive endoderm is formed (Sun *et al.*, 1999). Finally, grafts of pre-streak rabbit AVE are able to induce forebrain markers in chick epiblast (Knoetgen *et al.*, 1999).

Studies of chimaeric embryos, containing different combinations of normal and mutant cells in the extraembryonic compartments, have provided the most convincing evidence that the visceral endoderm might be critical for normal anterior patterning of the mouse. Such chimaeras are made either by introducing mutant embryonic stem (ES) cells into wild type blastocysts or, conversely, by introducing wild type ES cells

into mutant blastocysts. ES cells are known to display a marked developmental bias and almost exclusively colonise the embryonic epiblast (Beddington and Robertson, 1989). Thus, mosaic embryos contain ES cell derivatives largely confined to the embryonic portion, while the visceral endoderm and its derivatives are of host origin.

This elegant technique has now been used to elucidate tissue-specific roles for a number of genes, including *otx2*, *lim1* and *nodal* (Acampora *et al.*, 1995; Varlet *et al.*, 1997; Rhinn *et al.*, 1998; Shawlot *et al.*, 1999). Injection of wild-type ES cells into *nodal* deficient blastocysts is sufficient to rescue their gastrulation defects; however all of these chimaeras have clear anterior defects. In contrast, all of the chimaeras produced by injecting *nodal*-deficient ES cells into wild type blastocysts showed correct patterning of the anterior CNS structures (Varlet *et al.*, 1997). Thus, nodal signalling in the visceral endoderm appears essential to confer correct anterior patterning at later stages of development. It is possible that *nodal*, expressed throughout the visceral endoderm before gastrulation, may play a role in setting up the regional identity within the endoderm.

It is interesting to note that very little cell mixing occurs in the visceral endoderm, and the cells maintain a relatively stable neighbour relationship (Lawson et al., 1986; Lawson and Pedersen, 1987; Thomas and Beddington, 1996; Thomas *et al.*, 1998). This is in marked contrast to the inner cell mass, where extensive intermingling of cells occurs until epithelialisation of the epiblast (Gardner and Cockcroft, 1998). Thus, spatially defined information could be established and relatively stably maintained in

the visceral endoderm, at least until the onset of gastrulation when this tissue is replaced by epiblast-derived definitive mesendoderm.

It appears likely, therefore, that in the mouse, anterior pattern is initiated by the visceral endoderm, prior to formation of the node. However, the AVE is not able to act as an organiser. Neither a graft of AVE from the early streak embryo alone, nor that associated with the overlying epiblast tissue displays any inductive or organising activity. Anterior neural characteristics can only be induced in the second axis by a combined graft of AVE, anterior epiblast and early gastrula organiser (EGO) (Tam and Steiner, 1999). This therefore suggests that the primary role of the AVE may be to regulate the competence of the prospective anterior tissues, thereby augmenting the activity of node-derived signals in order to form complete anterior pattern (see Koshida *et al.*, 1998).

The *Xenopus* embryo does not have any extra-embryonic tissues. However, the central yolky vegetal cells that lie directly adjacent to the organiser as gastrulation starts, are thought to be the equivalent of the mouse AVE. These deep cells are already internal and do not ingress through the organiser. They are fated to form the foregut, liver and part of the heart primordium and are distinct from those ingressing cells that give rise to the prechordal plate. These deep endodermal cells show similar patterns of gene expression to the mouse AVE; they express *cerberus* (Bouwmeester *et al.*, 1996) and *Xhex* (Newman *et al.*, 1997; Jones *et al.*, 1999). Furthermore, an early dorsoanterior movement of *hex*-expressing cells, reminiscent of that in mouse, is seen in *Xenopus* (Jones *et al.*, 1999). *Xhex* expression is first observed in cells at the centre of the

blastocoel floor; these cells then move to the dorsal side of the embryo before gastrulation begins, and populate the deep endoderm adjacent to the organiser. Dorsal endoderm appears unable to induce neural tissue, since recombinants formed from *Xenopus* dorsal endoderm and ectoderm from early gastrulae do not activate *NCAM* or *otx2* (Bouwmeester et al., 1996; Jones *et al.*, 1999). However, as mentioned earlier, mouse AVE is also unable to act as an organiser (Tam and Steiner, 1999). Therefore, a distinct anterior patterning system analogous to the mouse may also operate in *Xenopus*, but which lies adjacent to the traditional organiser.

However, removal of the *cerberus*-expressing endoderm from explants and whole embryos does not affect head formation, and expression of *otx2* and *En2* in the neural ectoderm remain normal (Schneider and Mercola, 1999). In contrast, the formation of beating hearts is reduced and the early cardiac marker *Nkx2.5* is largely absent in explants lacking endoderm. These results suggest that the deep endoderm in *Xenopus* is essential for heart development, but that it appears neither sufficient nor necessary for head induction (Schneider and Mercola, 1999). In the mouse these two activities are thought to be linked, with both head and heart induction requiring AVE signalling (Narita *et al.*, 1997; Beddington and Robertson, 1998). Although *cerberus* mRNA can induce ectopic heads when injected ventrally, it can do so without inducing ectopic expression of *hex* or *otx2*; genes suggested by mutational analysis to be essential for head induction in the mouse. Furthermore, *cer1* -/- mouse embryos develop apparently normal head structures (Simpson *et al.*, 1999). Perhaps, then, head formation is not the normal function of *cerberus*. It may simply be by virtue of its ability to inhibit Wnt

and BMP signals, thereby mimicking the normal pathway, that heads are induced in experimental situations (Schneider and Mercola, 1999).

In the chick embryo, the equivalent tissue to the mouse AVE is the hypoblast. *Crescent*, a chick member of the *Frzb* family, is first expressed in the central hypoblast of the pre-gastrulation embryo (a region analogous to the distal tip cells expressing *Hex* in mouse), and is already expressed in the anterior half of the hypoblast as the primitive streak forms (Pfeffer *et al.*, 1997). Chick *hex* (Yatskievych *et al.*, 1999) and *cerberus* are also expressed in the prestreak hypoblast (L.Zhu, M.Marvin, A.Gardiner, A.Lassar, M.Mercola, C.Stern and M.Levin, unpublished observations). Thus, the hypoblast in chick already has distinct identity when gastrulation begins and expresses genes proposed to be involved in head formation. However, it remains unclear whether the chick hypoblast can influence the AP polarity of the epiblast prior to streak formation (Khaner, 1995). Most recent experiments suggest that AP polarity is established within the epiblast (*ie.* independently of the hypoblast) by the posterior marginal zone and Koller's sickle, which induce formation of the primitive streak (Bachvarova *et al.*, 1998; reviewed in Bachvarova, 1999). In addition, grafts of the anterior hypoblast from pre-streak and mid-streak stage chick embryos are unable to induce ectopic expression of the forebrain marker *GANF*, the homologue of mouse *hesx1* (Knoetgen *et al.*, 1999). As already discussed, neither mouse AVE nor *Xenopus* deep endoderm appear capable of neural induction (Jones *et al.*, 1999; Tam and Steiner, 1999). It is therefore not surprising that chick hypoblast is unable to induce an anterior neural marker, and induction of other anterior (non-neural) markers was not tested. In addition, neither *Xenopus cerberus* nor mouse *cerberus-like* were able to

induce *GANF* in the same assay (Knoetgen *et al.*, 1999). Nevertheless, rabbit AVE is able to induce *GANF* expression in chick hosts, suggesting that differences in the location of anterior signalling centres may exist between mammalian and avian embryos.

Further evidence, however, supports the hypothesis that hypoblast signalling is not required for complete anterior development in the chick. Removal of the hypoblast does not prevent the normal early expression of *GANF* in the neuroectoderm (Knoetgen *et al.*, 1999; P.Towers, unpublished data). In addition, an early phase of *GANF* expression is not observed in chick hypoblast at any point, unlike expression of its homologue, *hesx1*, in the mouse AVE (Knoetgen *et al.*, 1999). Finally, *cOtx2* is already expressed in the epiblast at the time when the hypoblast is formed, making it highly unlikely that a signal provided by the hypoblast is required for ectodermal *otx2* expression (Bally-Cuif *et al.*, 1995). In mouse, however, *otx2* expression in the AVE is essential for its induction in the ectoderm (Acampora *et al.*, 1995).

1.5.2 Role of the axial mesoderm

Axial mesoderm has long been believed to play a role in imparting AP regional character on the neural plate, as organiser-derived information is distributed along the axis. The prechordal mesoderm, at the anterior end, is therefore implicated in patterning the anterior brain. Many of the genes expressed in the organiser, including *gsc*, *HNF3β*, *lim1* and *otx2*, are also later expressed in the prechordal mesoderm. *Dkk1*, a Wnt inhibitor involved in head formation, is expressed in organiser tissue and anterior axial mesendoderm in both *Xenopus* and zebrafish (Glinka *et al.*, 1998;

Hashimoto *et al.*, 2000). In mouse, *dkk1* is expressed in the AVE and axial mesendoderm (Pearce *et al.*, 1999), as is *cerberus-like* (Belo *et al.*, 1997).

Transplantation experiments show that the prechordal mesoderm has strong anteriorising ability. In the chick, a graft of prechordal mesendoderm adjacent to the presumptive hindbrain of a host embryo can induce forebrain and midbrain markers in the neighbouring cells (Foley *et al.*, 1997). Furthermore, grafting prechordal cells together with a HH stage 5 node, rescues the node's lost inducing ability and results in the induction of anterior neural tissue. Chick axial mesoderm has also been shown to refine the AP pattern within the neuroepithelium, particularly by providing inhibitory signals that limit the extent of anterior character (Rowan *et al.*, 1999). Explant recombination assays with mouse tissue show that positive signals from anterior mesendoderm can stabilise and maintain ectodermal expression of *otx2*, a gene essential for development of forebrain and midbrain (Ang *et al.*, 1994). In addition, posterior mesendoderm exerts a negative influence on ectodermal *otx2* expression, which presumably operates during normal development to progressively restrict the domain of *otx2* to the anterior end of the embryo. Finally, removal of prechordal mesoderm in *Xenopus* causes a significant loss of head formation, together with loss of *hex* and *otx2* expression, while spinal cord markers remain present in the neural tissue (Schneider and Mercola, 1999).

In chick, neural ventral midline cells at different AP levels of the axis acquire distinct identities in response to the different signalling activities of the underlying mesoderm (Dale *et al.*, 1997; Dale *et al.*, 1999). Floorplate develops in the ventral midline of the

neural tube throughout the majority of the axis, but a distinct group of cells, the rostral diencephalic ventral midline (RDVM) cells form at the very anterior end, overlying the prechordal mesoderm. Rat caudal neural plate cultured with prechordal mesoderm explants expresses RDVM markers rather than floorplate markers. This ability of the prechordal mesoderm to impose rostral character on ventral midline cells is thought to be due to expression of *BMP7*, in combination with *Shh* which is expressed throughout the axial mesoderm (Dale *et al.*, 1997). Onset of RDVM differentiation also requires the down-regulation of *chordin* expression, which occurs in the prechordal mesoderm at this time (Dale *et al.*, 1999).

These results strongly suggest the importance of the prechordal mesoderm in giving neural tissue its most anterior character. However, ablation of the prechordal plate in chick does not affect the rostro-caudal length of the forebrain-midbrain field (Pera and Kessel, 1997). Instead, it produces embryos lacking hypothalamus and with cyclopia, indicating a crucial role for the prechordal plate in dorso-ventral patterning of the brain at this stage. In zebrafish, removal of the presumptive prechordal plate region (gsc-expressing domain) of the late blastula does not prevent expression of the forebrain marker *opl* (Grinblat *et al.*, 1998). Furthermore, one-eyed-pinhead mutants, that lack a distinct prechordal plate, still have a distinct prosencephalon and midbrain (Schier *et al.*, 1997) and still express *opl* (Grinblat *et al.*, 1998). This mutant again highlights the importance of the prechordal mesoderm in dorso-ventral patterning, since these embryos are also cyclopic and lack hypothalamus.

Mutational analyses in mice have provided further insights into the role of the prechordal mesendoderm in anterior development (reviewed in Bally-Cuif and Boncinelli, 1997). Embryos homozygous for null mutations in either the *lim1* or *otx2* genes (Shawlot and Behringer, 1995; Ang *et al.*, 1996) both display a complete lack of head structures anterior to rhombomere 3. They do not form morphologically recognisable node, head process or prechordal mesoderm during early stages of gastrulation. Both of these genes encoding transcriptional regulators are expressed in the AVE as well as later in the node and prechordal plate. However, analysis of chimaeric embryos has shown that both genes are sequentially required in extra-embryonic and embryonic tissues to achieve normal anterior development (Rhinn *et al.*, 1998; Shawlot *et al.*, 1999).

Lim1 is required in both AVE and anterior mesendoderm for head formation. (Shawlot *et al.*, 1999). Chimaeras with wild type visceral endoderm but largely *lim1* -/- embryo proper are often microcephalic, with the forebrain region either reduced or absent. Furthermore, in explant recombination assays, anterior mesendoderm from *lim1* -/- embryos is unable to maintain the expresssion of the anterior neural marker *otx2* in wild-type ectoderm. In the converse situation, chimaeras with *lim1* -/- visceral endoderm but predominantly wild type embryo proper lack anterior head structures, identical to *lim1* -/- embryos.

Finally *noggin/chordin* double mutant embryos have been found to lack extensive forebrain regions (Bachiller *et al.*, 2000). These organiser factors are not expressed in the AVE, again highlighting the necessity for node-derived signalling in full anterior

development. Such phenotypes demonstrate that head development requires at least two essential signalling steps. The role of the mouse anterior mesendoderm appears to be to reinforce and maintain an initial anterior identity established by the AVE, thus allowing the differentiation of the anterior ectoderm as anterior neural tissue to be completed (Thomas and Beddington, 1996; Shawlot *et al.*, 1999). Expression of the same signalling molecules in AVE and anterior mesendoderm would allow the anterior ectoderm to remain in contact with the inducing signals after the AVE has been displaced.

1.5.3 Role of the definitive endoderm

Few studies have examined in detail the role of the definitive embryonic endoderm, the node-derived tissue that replaces the visceral endoderm/hypoblast, in neural patterning. However, the definitive endoderm does underlie the neural plate, making it possible for this tissue to perform a signalling role. Indeed, the anterior extremity of the neural plate lies in direct contact with the endoderm in both mouse and chick, since this is ahead of the most anterior point reached by axial mesoderm. In the mouse, roles for the definitive endoderm and prechordal mesoderm may be difficult to separate, since they are thought to emerge from the node initially as a mixed population of cells (Beddington, 1994; Tam and Behringer, 1997). Nevertheless, analysis of the *hex* mutant mouse provides compelling evidence for a role of the definitive endoderm in anterior brain formation (Martinez-Barbera *et al.*, 2000a). *Hex* is expressed in the AVE and rostral definitive endoderm of early mouse embryos (Thomas *et al.*, 1998). *Hex* -/- embryos show truncations of the rostral forebrain. Analysis of early anterior neural markers show that the prospective forebrain ectoderm is correctly induced and

patterned, but that it subsequently fails to develop. Chimaeric embryos, in which the visceral endoderm is wild-type but the embryo comprises *hex* -/- ES cells, show almost identical forebrain defects, indicating that the crucial role of *hex* is in the definitive endoderm and not the AVE. Indeed, expression of genes such as *cerrl* and *lim1* is normal in the AVE of *hex* mutant embryos. In contrast, *cerrl* expression is absent in definitive endoderm.

Knoetgen and colleagues (Knoetgen *et al.*, 1999) briefly mention that grafts of anterior definitive endoderm are unable to induce *GANF* expression in lateral ectoderm of the chick. However, as suggested above, a proposed role of the definitive endoderm is in maintenance of anterior character in already neural tissue, and not in the process of neural induction itself. Explant culture and grafting experiments in chick suggest that the anterior endoderm does play a vital role, in patterning the axial mesoderm (Vesque *et al.*, 2000). Prechordal mesoderm and notochord cells display different behaviour and form different structures as they first emerge from the node. However, characteristic molecular markers only become segregated between the two mesoderm populations after they have extended away from the node. Anterior endoderm is thought to supply additional information to specify prechordal fate and suppress notochord characteristics in the anterior mesoderm, possibly via TGFβ signalling (Vesque *et al.*, 2000). As discussed previously, the prechordal mesoderm has distinct inducing abilities and can, in turn, induce anterior RDVM cells rather than floorplate in the overlying ectoderm (Dale *et al.*, 1997; Dale *et al.*, 1999).

1.5.4 Role of the ectoderm

The anterior ectoderm, which is not derived from the organiser, is often thought of as a passive target in early neural induction, merely responding to signals emitted from the organiser. However, the neural plate has potent inducing activity (homeogenetic induction) that is regionalised, with anterior and posterior neural plate inducing head and tail structures, respectively (Servetnick and Grainger, 1991; Cho and Blitz, 1998).

In zebrafish, patterning activity of neural cells at the border between neuroectoderm and non-neural ectoderm tissues has been described (Houart *et al.*, 1998; 'row 1 cells'). Removal of row1 cells at the mid-gastrula stage leads to subsequent loss of telencephalic gene expression, with an expansion of the diencephalic domain. These forebrain defects are not restricted to tissues row1 cells normally contribute to, suggesting that these cells are involved in patterning adjacent neural tissue. Indeed, transplantation of row1 cells to more posterior regions of the neural plate at mid-gastrula stage induces forebrain markers in surrounding host cells (Houart *et al.*, 1998). An early step in neural patterning may therefore be the establishment of a small population of signalling cells within the most anterior region of the neural plate.

An important role for the junction between the most anterior neural plate and the non-neural ectoderm in forebrain development has also been described in the mouse, although at a later stage in development than the zebrafish row1 cells (Shimamura and Rubenstein, 1997). This region, the anterior neural ridge (ANR), has been shown by ablation and tissue recombination experiments to be required for induction and/or maintenance of *BF-1* expression in the anterolateral neural plate (Shimamura and

Rubenstein, 1997). *BF-1* is a gene essential for normal growth and differentiation of the telencephalon (Xuan *et al.*, 1995), suggesting that the ANR is an important signalling centre for forebrain development. *FGF8* is expressed in the ANR, and can substitute for the ANR in this signalling role (Shimamura and Rubenstein, 1997). Furthermore, both *hesx1* and *hex* mutants show forebrain defects associated with loss of *FGF8* expression in the ANR (Dattani *et al.*, 1998; Martinez-Barbera *et al.*, 2000a).

It is important to note that different parts of the brain respond differently to FGF8 signalling, and patterning centres in other parts of the brain are also thought to use FGF8 (the isthmus, for example). *FGF8* beads in the anterior forebrain induce *BF-1* (Shimamura and Rubenstein, 1997), while *FGF8* in the posterior forebrain induces *En2* and midbrain development (Crossley *et al.*, 1996). These data therefore suggest that the establishment of localised signalling centres, together with the differential competence of the ectoderm to respond, could be a mechanism to further refine the AP patterning of the neural tube.

1.5.5 Summary

In conclusion, all three germ layers might play an active role in head induction in vertebrates. The details of how these tissues acquire head-inducing activity are unknown, but the close proximity of the germ layers involved raises the possibility that an anteriorising signal can be relayed from anterior endoderm via prechordal plate to ectoderm during gastrulation. It appears that most of the information required for patterning the embryonic body is endowed in the organiser and its derivatives. However, the realisation of this patterning activity requires an interaction with tissues

of the appropriate competence or previously primed to respond to the patterning signals (Camus and Tam, 1999). This acquisition of competence can be mediated by signals that are found outside the organiser; the mouse AVE is essential for imparting forebrain character on the ectoderm (Beddington and Robertson, 1998). Organiser derivatives such as the prechordal mesoderm and definitive endoderm maintain and embellish this initial character. Subsequently, local signalling centres, such as the ANR in the forebrain region, are essential to refine the global pattern established by the organiser and complete the differentiation of the forebrain anlage.

1.6 Dorso-ventral patterning of the neural tube

Dorso-ventral (DV) patterning of the neural tube originates as medio-lateral patterning of the flat neural plate. Floorplate, that will become the ventral midline of the neural tube, is formed in the medial neural plate, directly overlying the axial mesoderm cells of the notochord. The neural tube forms by a rolling up of the neural plate, with the lateral edges folding towards the dorsal midline and fusing to form the roof plate. Neuroepithelial cells then proliferate and differentiate into neurons located at particular DV positions. Subclasses of commissural and association neurons differentiate dorsally, close to the roofplate, whereas motorneurons and ventral interneurons differentiate ventrally, near the floorplate.

At the spinal cord level, DV regional identities within the neural tube are specified in part by molecules produced by adjacent non-neural tissues. The notochord specifies ventral fates; it is capable of inducing floorplate differentiation and motorneurons

(reviewed in Jessell and Dodd, 1992; Placzek, 1995; Tanabe and Jessell, 1996). Both of these inductive activities are thought to be mediated by *Sonic Hedgehog* (*Shh*), which is synthesised by the notochord and floorplate at the time these cells groups exhibit their inductive activity, and regulates development of different cell types at particular concentration thresholds (Echelard *et al.*, 1993; Roelink *et al.*, 1995; Tanabe *et al.*, 1995; Marti *et al.*, 1995; Ericson *et al.*, 1996). *Shh* acts initially to downregulate transcription factors such as *pax3*, thereby restricting them to the dorsal neural tube where they are necessary to promote development of dorsal cell types (Goulding *et al.*, 1993; Bang *et al.*, 1997 and references therein). Regions where these genes are absent are then able to respond to the Shh signal and develop as ventral cell types (reviewed in Tanabe and Jessell, 1996).

Cell types in the dorsal neural tube, on the other hand, are specified by the adjacent epidermal ectoderm (Dickinson *et al.*, 1995; Liem *et al.*, 1995). The likely mediators of this signal are members of the *BMP* family. In chick embryos, *BMP4* and *BMP7* are expressed in the ectodermal ectoderm that borders the neural plate, and can mimic the inductive activity of the ectoderm (Liem *et al.*, 1995). However, the mechanism by which BMP signalling generates multiple dorsal cell types is unknown; particular subsets of neurons may be induced by different BMPs, or by different concentrations of signal. Furthermore, the observation that the *BMP* antagonists *chordin* and *follistatin* are expressed in the notochord and floorplate, raises the possibility that down-regulation of BMP signalling may contribute to development of ventral cell types (reviewed in Tanabe and Jessell, 1996).

It has been unclear whether patterning of the ventral (medial) forebrain is regulated by the same mechanisms as more posterior regions, since the notochord does not underlie the anterior forebrain (its anterior limit lies under the posterior diencephalon) and many molecular markers of the floorplate do not extend this far anteriorly (Dale *et al.*, 1997). However, several lines of evidence now suggest that the prechordal mesendoderm, lying directly anterior to the notochord, regulates patterning of the ventral forebrain and uses the same molecular mechanisms that are employed in more posterior CNS regions (reviewed in Rubenstein and Beachy, 1998). Grafts of prechordal mesoderm are able to induce ectopic expression of *Nkx2.1*, a gene essential for ventral forebrain development (Pera and Kessel, 1997; Shimamura and Rubenstein, 1997). Furthermore, loss of prechordal mesoderm signalling, either by tissue removal in chicks, or by genetic disruption in zebrafish mutants, leads to defects in ventral forebrain and eye development (Pera and Kessel, 1997; Schier *et al.*, 1996; Schier *et al.*, 1997). *Shh* is expressed in the prechordal mesoderm and dorsal foregut that underlie the ventral forebrain, suggesting that it could be involved in ventral patterning in all CNS regions (Echelard et al., 1993; Shimamura and Rubenstein, 1997). Indeed, Shh can induce *Nkx2.1* expression in forebrain neural plate explants (Ericson *et al.*, 1995). The phenotype of *Shh* -/- mouse embryos, which display a loss of ventral neural fates throughout the neuraxis, further confirms the patterning role of *Shh* (Chiang *et al.*, 1996). Defects in the prosencephalic derivatives include absence of ventral forebrain structures and a failure to subdivide the eye field, leading to the formation of a cyclopic eye. The remainder of the forebrain develops as a single, undivided vesicle (holoprosencephaly) that expresses dorsal telencephalic markers (Chiang *et al.*, 1996).

1.7 Development of forebrain architecture

The neural tube is formed by a rolling up of the neural plate, with the lateral edges folding towards the dorsal midline. Constrictions then form in the wall of the neural tube at specified positions along the longitudinal axis, which mark the approximate boundaries between the primordia of the major brain regions: the forebrain, midbrain and hindbrain. Each of these primary brain vesicles are modified and further subdivided as development proceeds, to eventually create the complex morphology of the CNS and diversity of cell types within it. The forebrain (prosencephalon) becomes subdivided into the secondary prosencephalon and more caudal diencephalon. The ventral part of the secondary prosencephalon consists of the hypothalamus, and the dorsal part is predominantly made up of the telencephalon, as well as the optic vesicles. The telencephalon will ultimately be the largest part of the mammalian brain, forming the cerebral cortex.

The forebrain is a topographically complex structure; as the forebrain matures, a series of evaginations, inward growth and flexure greatly alter its morphological appearance. However, it is thought that many aspects of this complex organisation can be broken down into a relatively simple framework (Puelles and Rubenstein, 1993; Rubenstein *et al.*, 1994; Rubenstein *et al.*, 1998). Fate map studies of 3-4 somite stage chick embryos (and comparable stages in several other organisms) show that the arrangement of the forebrain primordia in the neural plate is roughly a flattened representation of their topological relationships in the mature brain (Fig1.4; reviewed in Rubenstein *et al.*, 1998). Furthermore, the restricted expression patterns of many

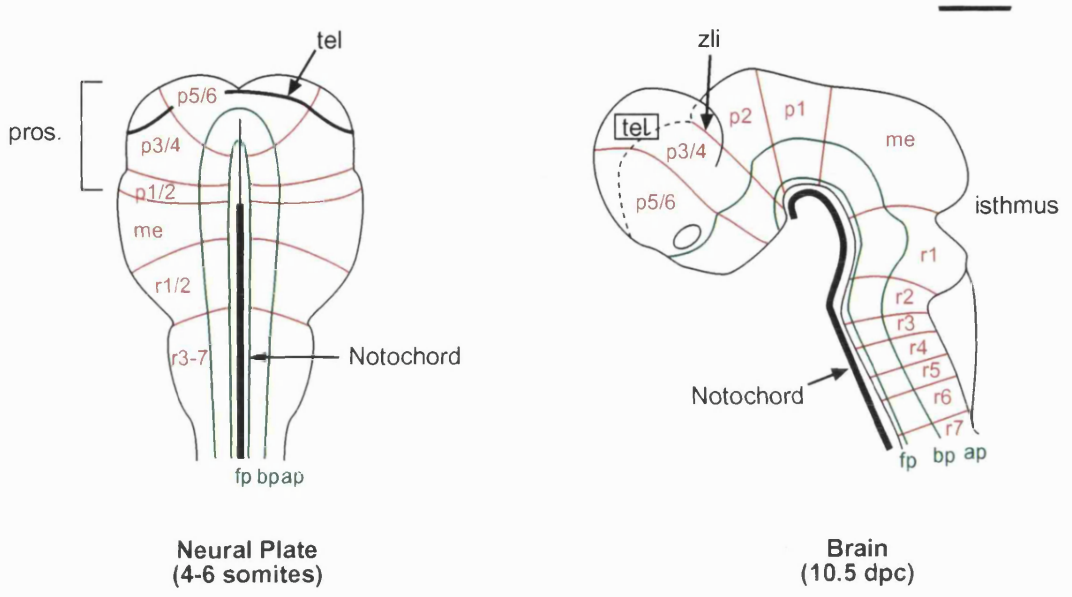


Figure 1.4 Morphology and regional organisation of the anterior brain

Schematic diagrams showing the approximate positions of the prosencephalon (pros.), mesencephalon (me) and rhombencephalon (r1-7) primordia at the neural plate stage, and the later topographical relationship of these regions in a sagittal section of the brain. Longitudinal subdivisions (green) and transverse subdivisions (red) generate a grid-like arrangement. fp = floorplate, bp = basal plate, ap = alar plate. zli = zona limitans intrathalamica, r = rhombomere, p = prosomere. Note that in this framework, the telencephalon (tel) forms from the dorsal parts of prosomeres 4-6. Scale bar = 150μm in neural plate stage, 325μm in brain stage (both diagrams of mouse embryos).

Diagram is modified from Rubenstein and Beachy, 1998.

genes in the mid-gestation forebrain continue to reflect the simpler organisation of the neural plate (Puelles and Rubenstein, 1993; Rubenstein and Puelles, 1994). The forebrain therefore appears to have a grid-like arrangement of molecularly distinct domains that are initially set up at the neural plate stage (Fig1.4), and is patterned by similar mechanisms to more posterior regions of the CNS.

The AP and medio-lateral (DV) patterning mechanisms discussed in the previous sections are responsible for subdividing the neural plate. Patterning along the mediolateral dimension of the neural plate produces the primordia of the principal longitudinal columns of the CNS: the floor, basal, alar and roof plates. At the anterior end of the neural plate these longitudinal domains course in a semicircle around the rostral end of the floorplate and are concentrically parallel to the anterior neural ridge (Shimamura *et al.*, 1995). AP patterning generates transverse blocks of neuroectoderm with distinct competence to respond to inductive signals (Shimamura and Rubenstein, 1997). As the distinct histogenic domains are generated, boundaries begin to form between them. In the hindbrain, transverse boundaries separate segmental units called rhombomeres (Lumsden and Krumlauf, 1996). Rubenstein and Puelles propose that analagous segments, or 'prosomeres', are present in the forebrain (Rubenstein *et al.*, 1994). The prosomeres are numbered from posterior to anterior, so that prosomeres p1-p3 make up the diencephalon, while p4-p6 comprise the secondary prosencephalon. Prosomere boundaries may act as barriers for transmission of patterning signals or cell movement (Figdor and Stern, 1993). Segment-restricted programmes of proliferation, differentiation and migration could then generate the diverse tissues of the forebrain.

It is important to note that not all aspects of forebrain patterning are similar to those in more posterior regions; local patterning centres arising in specialised ectoderm tissues specify additional levels of complexity and generate forebrain structures that are unique within the CNS. Rathke's pouch is a small outpocketing that brings the stomodaeal (oral) ectoderm into direct contact with the ventral diencephalon, necessary for formation of the anterior pituitary. In addition, interaction between the optic vesicles and the overlying thickened ectoderm, the lens placodes, is required for complete development of the eyes.

1.9 Aims of this thesis

The chick embryo can be readily cultured *in vitro* and, due to its large size and planar organisation, is suitable for performing spatially localised tissue manipulations. This project takes advantage of these properties in order to study the processes of neural induction and AP patterning of the chick neural plate. The role of the node in neural induction and patterning is examined by testing its ability to induce a complete neural axis in naïve ectoderm. A comparison between the properties of mouse and chick nodes is made in this way, and their differences discussed in light of recent data regarding the tissues involved in AP patterning in the two species. The role of the definitive endoderm, the node-derived tissue that replaces the extra-embryonic hypoblast, in patterning anterior brain regions is investigated in detail. Removal of this tissue results in defects in brain patterning; analysis of these embryos therefore required probes for numerous pan-neural and region-specific neural marker genes, together with probes for potential signalling molecules. Normal expression patterns of these genes during gastrulation and anterior brain development were characterised, and are discussed in the early part of this thesis. In addition, analysis of cell movements by DiI labelling gives further insight into the signalling environment neuroectodermal cells are exposed to during early development of the chick embryo.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chicken tissues

Fertilised White Leghorn chicken eggs were obtained from Needle Farm, Hertfordshire and Winter Farm, Cambridge on the day of laying, and stored in cooled conditions (about 13°C). In order to achieve the correct stage in development, eggs were incubated at approximately 38°C for the appropriate length of time. Embryos were staged according to Hamburger and Hamilton (1951, reprinted 1992).

2.1.2 Bacterial strains

The strain DH5α was purchased from New England Biolabs (Beverley, USA).

2.1.3 Enzymes

All restriction endonucleases, DNA modifying enzymes, DNA and RNA polymerases used in this work were purchased from Gibco/BRL (Paisley, Scotland) unless otherwise stated.

2.1.4 Miscellaneous

General use chemicals of the highest standard of purity available were obtained from standard suppliers, unless otherwise stated. All solutions were prepared with distilled water and sterilised by autoclaving, unless recommended otherwise by the manufacturer. Separate stocks of solutions and plasticware were used for RNA work. RNase-free solutions were either made up in sterilised water or autoclaved following DEPC treatment.

2.1.5 Recipes for general use buffers and culture media

Agarose gel loading buffer: 0.25% Orange-G, 25mM EDTA, 50% v/v glycerol.

Hanks balanced salt solution (low divalent cation version): 0.137M NaCl, 5mM KCl, 0.01mM $CaCl_2.2H_20$, 0.04mM $MgS0_4.7H_20$, 0.05mM $MgCl_2.6H_20$, 0.36mM $Na_2HP0_4.12H_20$, 0.44mM KH_2P0_4, 5.5mM Glucose, 0.001%w/v Phenol Red, phosphate buffered to pH7.5.

Pannett and Compton (PC) Saline: 40ml Solution A, 60ml Solution B, 0.3M Glucose, made up to 1 litre with H_20, pH 7.2- 7.6.

Solution A: 2M NaCl, 0.2M KCL, 71.25mM $CaCl_2.2H_20$, 62.5mM $MgCl_2.6H_20$ made up to 40ml in H_20.

Solution B: 15mM Na_2HP0_4, 1.7mM $NaH_2P0_4.2H_20$, made up to 60ml with H_20.

Phosphate buffered saline (PBS): 8g NaCl, 0.2g KCl, 1.44g Na_2HP0_4, 0.24g KH_2P0_4, made up to 1 litre with H_20, pH adjusted to 7.4.

20x SSC: 175.3g NaCl, 88.2g sodium citrate, made up to 1 litre with H_20, pH adjusted to 7.0.

50x TAE: 2M Tris, 50mM EDTA, adjusted to pH 8.0 with glacial acetic acid.

1x TE: 10mM Tris-HCl, 1mM EDTA, pH 8.0.

Bacterial growth media:

LB- broth: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, adjusted to pH 7.2 with NaOH.

LB- agar: 1.5% (w/v) technical agar in LB- broth. Selective LB-agar contained 100μg/ml ampicillin, final concentration (ampicillin stocks 10mg/ml).

2.2 DNA Methods

2.2.1 Restriction enzyme digestion

Restriction digests were carried out in buffers recommended by the manufacturer. Typically, 5μg of DNA was digested with 2μl enzyme for at least one hour, in a total volume of 100μl (10μl buffer, the rest dH_20). Following digestion, the DNA was precipitated for at least 30 minutes at -20°C, in 200μl 100% ethanol and 10μl NH_4Ac. After centrifuging, the pellet was resuspended in 10μl dH_20 and a 1μl sample was run on a 1% agarose gel.

2.2.2 Agarose gel electrophoresis of DNA

Normal agarose (SIGMA) was used for the preparation of gels. Restriction digests were analysed on horizontal gels containing 1% (w/v) agarose in 1x Tris-Acetate-EDTA (TAE) buffer, with 0.5μg/ml ethidium bromide (final concentration). Samples were mixed 2:1 (v/v) with agarose gel loading buffer, loaded onto the gel, and electorophoresed at 5 volts/cm in TAE buffer. The gel was stopped when a clear band of nucleic acid could be visualised on a short wave transilluminator (254nm), and the band was photographed with Polaroid type 667 film.

2.2.3 Preparation of competent cells

A single colony from an LB agar plate was used to innoculate 2.5ml LB broth and incubated overnight at 37°C. The following day, the entire overnight culture was used to innoculate 250ml LB broth containing 20mM $MgS0_4$. The cells were grown in a 1 litre flask until the A_{600} reached 0.4- 0.6 (typically 1- 2 hours). The cells were harvested by centrifugation at 4500x g in a Beckman J25 for 5 minutes at 4°C. They were then gently resuspended in 100ml ice-cold TFB1 and incubated on ice for 5 minutes. The cells were again centrifuged at 4500x g for 5 minutes at 4°C, and gently resuspended in 10ml ice-cold TFB2. Finally, the cells were incubated on ice for 15-60 minutes. They were then split into 200µl aliquots, quick-frozen in a dry ice/ isopropanol bath and stored at -70°C.

TFB1: 30mM KAc, 10mM $CaCl_2$, 50mM $MnCl_2$, 100mM RbCl, 15% glycerol, made up in dH_20.

TFB2: 10mM MOPS, 75mM $CaCl_2$, 10mM RbCl, 15% glycerol, made up in dH_20.

2.2.4 Transformation of competent cells with plasmid DNA

50µl competent cells and 10ng plasmid DNA were added to chilled transformation tubes. After swirling to mix, the tubes were stood on ice for 45 minutes. The cells were heat-shocked at 37°C for 45 seconds, and then chilled on ice for 2 minutes. 0.5ml LB broth was added to each tube and they were incubated at 37°C for at least 1 hour. 50µl was plated onto ampicillin selective LB agar plates and incubated overnight at 37°C.

2.2.5 Large scale plasmid preparation

The procedure for plasmid maxi-preps outlined here is based upon the method described in (Sambrook *et al.*, 1989).

A single colony from an ampicillin selective LB agar plate was used to innoculate 500ml LB broth (with 500µl ampicillin stock), and incubated overnight in a 3 litre conical flask at 37°C. The following day, the culture was transferred to 500ml nalgene bottles and centrifuged at 3000x g (4000 rpm) in a Beckman JLA 10,500 rotor for 15 minutes. The media was removed and the pellet resuspended in 20ml chilled Solution I, with vortexing. 40ml fresh Solution II was then added to lyse the cells, and the tubes gently inverted to mix. After leaving at room temperature for 5-10 minutes, 20ml ice-cold Solution II was added and the tube softly shaken 3-4 times. A snowy precipitate of protein and chromosomal DNA was immediately formed. After leaving on ice for 10 minutes, the tubes were again centrifuged at 3000x g for 15 minutes at 4°C. The supernatant was filtered through a tissue into a fresh nalgene bottle, leaving a clear liquid. 0.6 volumes of isopropanol was added to precipitate the DNA and, after vortexing, this was left at room temperature for 10 minutes. The DNA pellet was recovered by centrifuging at 3000x g for 15 minutes and discarding the supernatant. The pellet was washed with 70% ethanol and left at room temperature to dry.

Solution I: 50mM glucose, 25mM Tris-HCl pH8.0, 10mM EDTA.

Solution II: 0.2N NaOH, 1% SDS.

Solution III: 60ml 5M potassium acetate, 11.5ml acetic acid, made up to 100ml with dH_20.

The DNA pellet was dissolved in 5.6ml TE (pH8.0), and transferred to a 50ml Falcon tube wrapped in foil. To this was added 6g CsCl and 430µl EthBr of a 10mg/ml stock. The solution was loaded into a Beckman centrifuge tube, leaving no air, and the top sealed. The tubes were very carefully balanced and centrifuged for 16 hours at 50,000 rpm in a Beckman Ti 70.1 rotor. The following day, the band of DNA was carefully removed using a syringe and transferred to a foil-wrapped Sterilin tube. An equal volume of water-saturated butanol was added, and the mixture shaken and left to separate. The bottom layer was removed with a pipette and put into a new Sterilin tube, with another volume of butanol. This was repeated until neither layer was pink. Finally, the lower layer was taken and to it was added 3 volumes of dH_20 plus 8 volumes cold 100% ethanol. The mixture was vortexed and left on ice for 20 minutes. The pellet of DNA was recovered by centrifuging at 4800 rpm (3800x g) in a SIGMA 3K10 benchtop centrifuge for 15 minutes at 4°C. The pellet was washed with 70% ethanol, dried and resuspended in 400µl TE. The DNA was quantitated using a spectrophotometer, by measuring the absorbance of an aliquot of the solution at 260 and 280nm. Its quality was also checked by gel electrophoresis, after which it was stored at a concentration of 1µg/µl, at -20°C. This method typically gave approximately 1.5mg extremely clean DNA.

2.2.6 Synthesis of RNA probes

The reagents were mixed together at room temperature in the following order:

Sterile distilled water	10µl
5x transcription buffer	4µl
0.2M DTT	2µl

Nucleotide mix (10mM each of GTP, ATP, CTP and 6.5mM UTP, 3.5mM digoxygenin-UTP pH8.0)	2µl
Linearised plasmid (1µg/µl)	1 or 2µl
Placental ribonuclease inhibitor (100U/ml)	0.5µl
SP6, T3 or T7 RNA polymerase (10U/ml)	1µl

The mixture was incubated at 37°C for 2 hours. After 1 hour, a further 1µl of polymerase was added. RNA was precipitated by adding 100µl TE, 10µl 4M LiCl, 300µl 100% ethanol and 1µl tRNA. This solution was incubated at -20°C for at least 30 minutes, and then centrifuged for 15 minutes. The pellet was washed in 70% ethanol, air dried and resuspended in 10µl TE. A 2µl aliquot was taken and run on a 1% agarose gel, to check the size of the band and estimate the concentration of RNA. Prehyb mix (see section 2.4.1) was added to the remaining 8µl of probe, in order to obtain a concentration of 1µg/100µl. Probes were then stored in this manner at -70°C.

2.3 Culture and Manipulation of Early Chick Embryos

2.3.1 Culture of early chick embryos using the ring culture method

The method described is based on that of New (1955).

Eggs were carefully cracked open and the yolks submerged in a shallow dish of warmed Pannett and Compton bird embryo saline (PC saline). The thin albumen was collected for use as culture medium, with a standard plastic pastette; the thick 'ropey' albumen was discarded. Each yolk was cut around its equator to give a disc of vitelline membrane with the embryo attached near the centre, on the inner surface.

Using blunted no.5 forceps, the disc of membrane was peeled off the yolk and transferred to a watch glass, also submerged in the dish of PC saline. The membrane was placed on the watch glass with blastoderm uppermost, and a tissue culture stainless steel ring was put on top of the membrane, to hold it down. The membrane was stretched under the ring, and the blastoderm localised in the centre. The entire assembly was then lifted out of the saline dish and placed into a 50mm petri dish, containing a ring of moist filter paper to maintain constant humidity. The centre of the filter paper disc had previously been cut out, to aid viewing of the embryo. All embryos were set up in this way, as shown in Fig2.1, before proceeding any further.

The assembly was placed under the lowest power of a dissecting microscope, preferably one with incident light. Blunt forceps were used to complete the process of centralising the blastoderm within the metal ring, evenly stretching the membrane and pulling it taut by wrapping its edges over the upper surface of the ring. This also created a seal around the bottom of the ring. The saline below the membrane was removed and replaced with albumen solution (90% albumen, 10% PC saline, 5μg/ml gentamycin). Saline within the ring was removed and replaced with 50% Liebowitz L-15 tissue culture medium (air-buffered): 50% Hanks balanced salt solution while the operations were performed. (Hanks BSS for this work contained only 1/10 the normal Ca^{2+} and Mg^{2+} ions [see 2.1.5], so that cell adhesion in the embryo tissues remained more like those during development *in ovo*.) Following operations, this medium was removed, leaving the upper surface of the membrane, and the embryo, completely drained.

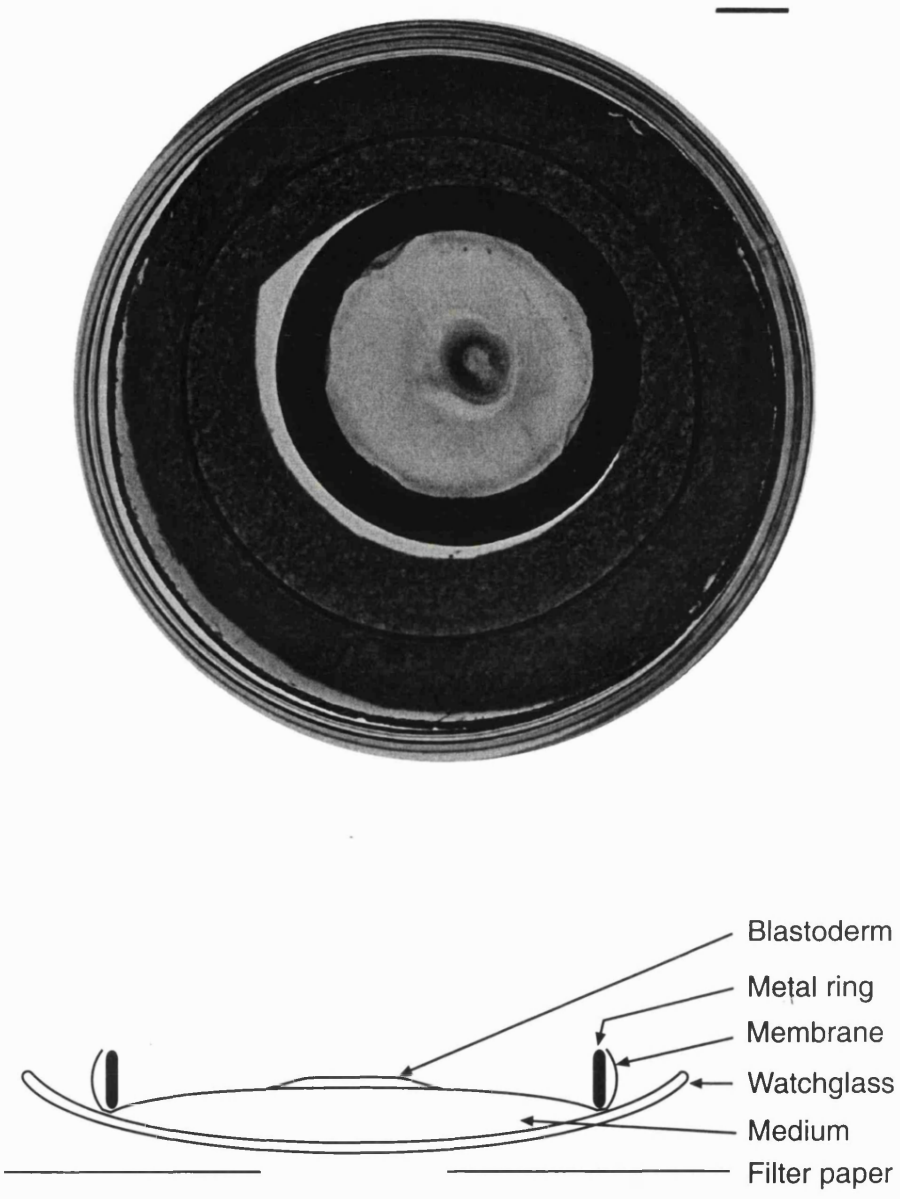


Figure 2.1 A chick embryo set up in ring culture

Aerial view and schematic cross section showing an embryo set up in ring culture. Note that the blastoderm (embryo) sits on the upper surface of the membrane, so that tissue layers initially lying innermost in the yolk are directly accessible for manipulation. Scale bar = 0.5cm.

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More albumen medium was added under the membrane so that it became domed. Set-ups were then returned to the 38°C incubator and cultured further.

2.3.2 Grafting of chick nodes

Host embryos of HH st.3-4 were set up in ring culture. Donor embryos, HH st.4 (the full streak stage), were removed from the vitelline membrane while in the dish of PC saline and transferred to a petri dish containing 50% L-15: 50% Hanks. One at a time, the nodes were removed from donor embryos using needles, and each transferred to a separate host in ring culture, using a p20 Gilson (set at approx. 3µl). Needles were used to make a pocket in the hypoblast on one side of the host, near the edge of the anterior *area opaca*. The graft was inserted into the pocket between the host hypoblast and epiblast layers, with the epiblast layer of the node facing uppermost; this meant that the layer orientation in the graft was reversed with respect to the host. A control graft of posterior streak was inserted into a similar pocket on the opposite side of the host. Host embryos were cultured further for a range of periods, between about 6 and 18 hours.

Several other tissues were also used as grafts, the basic procedure for host and donor embryos being the same as above. In addition, clumps of transiently transfected cells were grafted into various positions in HH st.3-4 hosts, as described in more detail later under cell transfections (2.6.3).

2.3.3 Grafting of mouse nodes

Mouse embryos were collected from pregnant females on the appropriate day of gestation. Mid day of the morning following appearance of the vaginal plug was defined as 0.5 days post coitum (dpc). After sacrificing the mother, decidua were removed from the uterus and placed in mouse dissecting medium (M2) (Hogan *et al.*, 1994). Embryos were then carefully dissected out of the decidua under a microscope. Embryos were kept rocking in M2 medium at 37°C until the appropriate time for further dissection (when chick host embryos were set up). 7.25 dpc mouse embryos were transferred to a petri dish containing M2 medium and the distal tip (including all tissue layers) was dissected off each one, using needles. This tissue graft therefore included the entire node, together with any prechordal mesoderm which may have just left the node. However, AVE derivatives were not included, as they would be located much further anterior by then. Control grafts of posterior streak were cut from the proximal part of the egg cylinder, close to the boundary with the extra-embryonic tissue. Anterior visceral endoderm (AVE), together with underlying tissue, was cut from the proximal egg cylinder on the opposite side to the streak. For the younger mouse embryos (6.5 dpc), the site of the primitive streak was estimated, and the proximal part of the egg cylinder (AVE and underlying tissue) removed from the opposite side of the embryo.

Meanwhile, host chick embryos (HH st. 3-4) were set up in ring culture, as described in 2.3.1. A shallow layer of L-15:Hanks medium was left on top of the embryos, and a pocket was made in the hypoblast, at the anterior edge of the *area pellucida*. One by one, mouse node or AVE grafts were transferred to a chick host using a p20

Gilson, and inserted into the hypoblast pocket. Control grafts were also transferred and inserted in a similar way. The medium was drained down from over the embryo and the vitelline membrane domed from beneath, to ensure tight apposition of the grafted tissue and host. Host embryos were cultured at 38°C for a further 24 hours.

2.3.4 Detection of mouse tissue following node grafts

Homozygous *Rosa26* mouse embryos, expressing the *lac Z* reporter gene in every cell (Zambrowicz *et al.*, 1997), were obtained and dissected in the standard way, as described in 2.3.3. The distal tip (containing the node) was removed from the egg cylinders of these embryos and grafted into stage 3+ to 4 chick embryos set up in ring culture (as in 2.3.3). Host embryos were cultured at 38°C for a further 24 hours. They were then fixed in special fixing solution appropriate for *lacZ* staining, for 15 minutes on ice. Embryos were washed three times in PBS + NP40 for 10 minutes at room temperature, or the final wash overnight at 4°C. Xgal stain buffer was added to the embryos and they were incubated at 37°C. After the colour had developed to the appropriate extent (about 30 minutes), embryos were washed in PBS, photographed and sectioned using the vibrotome.

Fixing solution: 2.5ml 4% PFA, 80µl 25% glutaraldehyde, 20µl 1mM $MgCl_2$, 0.5ml 100mM EGTA, made up to 10ml in PBS + NP40.

PBS + NP40: 500ml PBS, 1ml 10% NP40.

Xgal stain buffer: 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.3H_20$, 0.01% Na deoxycholate, 2mM $MgCl_2$, 0.02% NP40, 1mg/ml Xgal from stock solution (added just prior to use).

2.3.5 Removal of definitive embryonic endoderm

Stage 4+ chick embryos were set up in ring culture as described in 2.3.1. The top surface of the embryos was drained and they were returned to the 37°C incubator for 3-4 hours. During this time the embryos developed further (stage 4+ to 5+), and their warmed condition made it easier to remove the thin lower layer. Embryos were flooded with a shallow layer of warmed L-15:Hanks medium. A region of lower layer/ endoderm spanning from the anterior periphery of the *area pellucida* back over the node was then gradually loosened and removed using needles. Extreme care and delicacy were needed to ensure the embryonic layer(s) below were not damaged, in particular the prechordal mesoderm. (In a small sample of embryos, the entire emerged axial and prechordal mesoderm were also intentionally removed.) Following operations, the medium above the membrane was once again drained, and the medium beneath replenished to dome the membrane slightly. Doming the membrane too much often resulted in incomplete dorsal closure, especially in experimental embryos with reduced neural pattern. However, in experiments where the removed tissue was replaced with heterotopic lower layer, thorough draining and high doming of the membrane was essential for flattening the graft, to ensure good contact and healing. Hosts embryos were incubated further for between 6 and 24 hours, until they had reached a stage suitable for analysis by *in situ* hybridisation for particular neural marker genes.

2.4 Wholemount *In Situ* Hybridisation, Digoxygenin Version

2.4.1 Embryo pretreatment

Embryos were dissected into PC saline and washed free of excess protein. They were fixed by rocking in 4% PFA (dissolved at 60°C in RNase-free PBS) overnight at 4°C or at room temperature for 2 hours. After fixation, the embryos were washed twice, each for 10 minutes, in PBT (PBS + 0.1% Tween 20). They were then dehydrated in 50% methanol for 10 minutes and twice in 100% methanol, each for 10 minutes. Embryos were left in 100% methanol at -20°C for at least overnight, although they could also be stored for several days at this stage.

Embryos were rehydrated with 10 minute washes of 75%, 50% and 25% methanol, and then two 10 minute washes in PBT. The embryos were then treated with 10µg/ml proteinase K in PBT, to aid penetration of the probe. This step was carefully timed; stage 4 embryos were treated for 6 minutes, while 12 somite embryos were treated for 10 minutes. Embryos were then washed twice in PBT for 5 minutes. Care was taken with these washes, since the embryos were very fragile following the proteinase K treatment. The embryos were re-fixed in 4% PFA in PBT with 0.25% glutaraldehyde (*ie.* 100µl of 25% glut. per 10mls) for 20 minutes at room temperature. After washing twice in PBT for 5 minutes, embryos were transferred to a fresh tube containing 1ml prehybridisation mix. Embryos could be stored at this stage at -20°C for several weeks, without any detrimental effect.

Prehybridisation mix: 50% formamide, 5% 20x SSC, 20mg/ml Blocking reagent (Boehringer), 5mg/ml CHAPS, 0.5mg/ml Heparin, 1mg/ml cleaned total RNA, 5mM EDTA, 0.1% Tween 20, made up in DEPC water and stored at -70°C.

2.4.2 Hybridisation

1µg of thoroughly warmed digoxygenin-labelled RNA probe (*ie.* 100µl stored probe, see 2.2.6) was added to 1ml fresh prehyb mix. Following removal of the original prehyb mix, this solution was added to the embryos, and incubated with rocking overnight at 70°C.

2.4.3 Post-hybridisation washes

Embryos received a series of washes, each for 30 minutes, at 70°C; twice in fresh Solution 1 and then twice in Solution 3. The embryos were then washed at room temperature three times in fresh TBST, for 5 minutes each. Embryos were pre-blocked by rocking in 10% heat inactivated goat serum in TBST, for 60-90 minutes at room temperature. They were then incubated overnight at 4°C with 1µl/ml anti-DIG antibody in fresh blocking solution. Throughout the following day, embryos were washed in TBST at room temperature; three rapid washes (5 minutes each) were followed by as many 1 hour washes as possible (at least 5). Embryos were left rocking in TBST overnight at 4°C.

Solution 1: 50% formamide, 5x SSC (pH4.5), 1% SDS, made up in dH_20.

Solution 3: 50% formamide, 2x SSC (pH4.5), 0.5ml Tween 20, made up in dH_20.

TBST (10x): 8g NaCl, 0.2g KCl, 25ml 1M Tris pH7.5, made up to 100ml in dH_20 and autoclaved.

TBST working solution: 20ml TBST, 0.5ml Tween 20, made up to 200ml in dH_20.

2.4.4 Development of the colour reaction

Embryos were washed three times in fresh NTMT for 5 minutes, at room temperature. The embryos were then transferred to 7ml glass vials (FBG-ANCHOR). 1ml fresh NTMT including 3.5µl NBT (100mg/ml in 100% DMF) and 3.5µl BCIP (50mg/ml in 100% DMF) was added to each tube, and they were rocked in the dark at room temperature. Once the colour had developed to the desired extent (time varied greatly according to probe), embryos were washed twice with PBT and kept at 4°C. For storage of embryos, particularly prior to sectioning, they were re-fixed by rocking for 30 minutes in 2% phosphate buffered formal saline in PBS, with 40µl/ml 25% glutaraldehyde. They were then transferred to 2% formal saline in PBS, and stored at 4°C.

NTMT: 1.5ml 4M NaCl, 5ml 1M Tris pH9.5, 2.5ml 1M $MgCl_2$, 0.5ml Tween 20, made up to 50ml with dH_20.

Phosphate buffered formal saline: 30% dH_2O; 30% PC Saline; 30% 100mM phosphate, pH7.6; 10% formaline (40:40 volume).

2.5 DiI Labelling

DiI (Molecular Probes) from a 0.5% ethanolic stock solution was diluted 1:10 with 0.3M filter-sterilised sucrose, and stored at 4°C in the dark. Embryos were set up in ring culture and incubated for about an hour, so that the top surface of the embryo was as dry as possible. Glass needles were pulled (to a diameter of approx.10 μm) from 1.2mm borosilicate glass capillaries using a Kopf needle puller, and DiI was inserted into the capillary using Eppendorf GELoader tips. Small groups of surface cells were labelled by injecting pulses of dye into immediate contact with the cells. A Picospritzer II (General Valve Corporation, USA) delivering a square pulse of air pressure was used, with adjustment of the pressurehead (~10 psi) and pulse length (~20 millisec.) to give suitable-sized labelled cell groups. If deeper (mesoderm) cells were to be labelled as well, the capillary was bent in a Bunsen flame and the pulse increased. Following further development for various time periods, embryos were individually fixed in multiwell plates with 4% PFA, and examined with a Zeiss Axiophot microscope under phase and epifluorescence optics. They were stored in 4% PFA at 4°C and sectioned using a Cryostat.

2.6 Transient Cell Transfection using Electroporation

2.6.1 Expression constructs used in transfections

Vg 1 and nodal expression constructs were both chimaeras consisting of the region encoding the functional (C-terminal) ligand peptide coupled in frame to that encoding the N-terminal proprotein of a different TGFβ superfamily member. This

was because native Vg protein certainly, and nodal probably, have special processing restrictions and are not secreted efficiently by heterologous cells. The Vg 1 construct, made by E. Seleiro when in the lab, encoded the Vg 1 functional protein coupled to the BMP4 proregion. The nodal construct, made by J.Collignon in E. Robertson's lab, encoded the mouse nodal functional protein coupled to the dorsalin proregion. Both constructs were myc-tagged.

The Xnr 3 construct, made in W. Smith's lab, contained 1.42kb of the gene from the 5' end, which included the entire coding region (Hansen *et al.*, 1997). This construct was not myc-tagged.

2.6.2 Transfection method

Cos 7 cells were grown to 75% confluence and harvested by trypsinisation. They were washed twice in HEPES-buffered saline (HeBs) and counted using a haemocytometer grid. $6x10^6$ cells were resuspended in 800µl HeBs and transferred into a 0.4cm Bio-Rad electroporation cuvette containing 10µg plasmid DNA and 100µg sonicated herring sperm carrier DNA. The suspension was pulsed at 250V and 960µF using a Bio-Rad Gene Pulser with capacitance extender, giving a time-constant for current decay of 10-12msec. The cells were allowed to rest for 10 mins at room temperature before being plated onto two 100mm circular tissue culture dishes (400µl on each). They were cultured overnight in complete medium so that cells killed by the electroporation procedure could be removed.

HEPES-buffered saline (2x): 1.6g NaCl, 0.074g KCl, 0.027g $Na_2HP0_4.2H_20$, 0.2g dextrose, 1g HEPES, made up to 100ml with dH_20, pH adjusted to 7.05. Sterilised by passing through a 0.22μm filter and stored in 5ml aliquots at -20°C.

2.6.3 Implanting transfected cells grafts into host embryos

Live cells were harvested with enzyme-free cell dissociation buffer, counted and resuspended in Dulbecco's modified Eagle's medium (DMEM) to give a suspension of approx. $1x10^6$ cells/ml. Drops of 20μl (containing about 20,000 cells) were then deposited all over the inside of the lid of a 100mm circular tissue culture dish. The lid was turned the right way up and placed over the bottom of the dish, which contained 6ml DMEM. These hanging drops were cultured overnight, allowing the cells to fall and aggregate at the bottom of each drop and form a graftable tissue.

The following day, host embryos were set up in ring culture. The cells were removed from the hanging drops and transferred to a petri dish of L-15 medium. They were then cut using needles into pieces about 300μm square and transferred onto the embryos using a p20 Gilson. The cells were inserted into a pocket made in the hypoblast of the host embryo, and the medium within the ring was drained thoroughly so that the cells were held tightly next to the embryonic layers. In some cases, the transfected cells were combined with pieces of streak or node tissue in a double graft. In addition, cells were sometimes placed much more centrally, adjacent to the node. However, the procedure for grafting and insertion of the tissue remained the same. Host embryos were then cultured for a further 18 hours.

2.6.4 Detection of transfected cells by X-gal staining

Following electroporation and overnight culture, complete medium was removed and the cells washed twice with PBS at room temperature, for 5-10 minutes each. Cells were fixed with 0.5% glutaraldehyde in PBS (approximately 5mls per plate). They were then washed three times in 10ml PBS with 2mM $MgCl_2$ for 5 minutes. Xgal stain buffer was added and the plate incubated at 37°C. Once the colour had developed, plates were washed twice with PBS. The percentage of blue cells in a representative area of each plate were then counted under the microscope, using a haemocytometer. For storage, cells were kept in 50% ethanol in PBS.

Embryo wash buffer: 2mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P40, made up in PBS.

Xgal stain buffer: 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.3H_20$, 1mg/ml Xgal, made up in embryo wash buffer. Stain buffer is made up without adding Xgal and stored at 4°C. An Xgal solution of 40mg/ml in dimethylformamide is made up and stored in a dark glass bottle at -20°C. Xgal is added to the stain buffer just prior to use.

2.6.5 Detection of processed proteins on western blots

Following electroporation and overnight culture, cells were lysed on ice for 15minutes in lysis buffer. Plates were scraped and the sample spun at 13000 rpm, 4°C for 15 minutes to remove heavy debris. 20µl of the supernatant was mixed with 10µl loading buffer and boiled at 100°C for 10 minutes. The sample was again spun in a bench centrifuge for 1 minute. Samples were loaded onto an SDS-polyacrylamide gel, stacked at 50V and run at 150V through a 10% resolving gel

(Sambrook *et al.*, 1989). The gel was washed in transfer buffer and the protein transferred onto hybond membrane, for 1 hour at 100V. The membrane was blocked for 1 hour at room temperature (or at 4°C overnight) using 5% Marvel in PBS with 0.1% Tween 20. Primary antibody (9E10) was added to fresh blocking solution at 1:1000, and the membrane incubated for 1 hour at room temperature. After washing, secondary antibody (anti-mouse HRP) was added at 1:3000, and the membrane blocked for a further hour at room temperature. After washing in PBT for 1 hour, proteins were revealed using ECL mix (Amersham).

Lysis buffer: 50mM Tris pH7.5, 150mM NaCl, 1% NP40, 2mM EDTA, made up in dH_20. Just before use, 10µl/ml PMSF plus 1µl/ml Aprotinin and Leupeptin were added.

Loading buffer: 1ml glycerol, 0.5ml β mercaptoethanol, 3ml 10% SDS, 1.25ml 1M Tris pH6.8, 1.2mg bromophenol blue. Loading buffer was stored in aliquots at -20°C.

Transfer buffer: 2.9g glycine, 5.8g Tris base, 0.37g SDS, 200ml methanol, made up to 1 litre with dH_20.

2.7 Sectioning of Embryos using Vibrotome and Cryostat

2.7.1 Vibrotome embedding mixture

Embedding mixture was prepared in batches of 100ml each time. 0.45g Gelatine (300 bloom) was heated in 10ml PBS with stirring until dissolved. This was then made up to 80ml with PBS, and left to cool. 27g Albumin Grade II was added and

left to dissolve for several hours, with stirring. Finally, 18g Sucrose was added and stirring continued until dissolved. The thick mixture was then divided into 5ml aliquots and stored at -20°C.

2.7.2 Processing embryos for vibrotome sectioning, after *in situ* hybridisation

Following *in situ* hybridsation, embryos were stored at 4°C in 2% formal saline in PBS (see 2.4.4). Prior to sectioning, embryos were equilibrated in embedding mixture (see 2.7.1) for approximately 10 minutes. During this time, 200µl embedding mixture was mixed with 20µl of 25% glutaraldehyde and left to set in a 7mm x 7mm Dispomould (Raymond Lamb). Embryos were placed on top of the set mixture and correctly orientated. A further 200µl embedding mixture was mixed with 20µl of 25% glutaraldehyde and added on top of the embryos. Blocks were left to set fully for about 30 minutes. They were removed individually from their moulds, trimmed and mounted onto the vibrotome stage. They were immersed in PBS and sections 50µm thick were cut using a Leica Vibrotome (VT 1000S). Sections were placed on slides and mounted permanently in Glycergel mounting medium (DAKO). They were viewed using a Zeiss Axiophot with Nomarski optics, and photographed with Ektachrome 64T film.

2.7.3 Preparation of embryos for cryostat sectioning, following DiI labelling

Embryos were stored individually in multi-well plates at 4°C in 4% PFA. For sectioning, they were washed twice in PBS for 5 minutes and equilibrated overnight in 25% sucrose at 4°C. Embryos were placed into Dispomoulds (as for vibrotome sectioning) and the remaining sucrose removed with filter paper. OCT compound

(BDH) was then added to fill up the well, and the embryos correctly orientated. Blocks were placed on dry ice for about 30 minutes to set. They were then removed from their moulds and fitted onto a circular base using additional OCT compound. Sections 16μm thick were cut using a Leica Cryostat (Jung CM3000) and collected on Superfrost Plus slides (BDH). They were examined using a Zeiss Axiophot microscope under phase and epifluorescence optics, and photographed using 1600 film.

CHAPTER III

IN-DEPTH STUDY OF PAN-NEURAL AND REGIONALLY SPECIFIC NEURAL MARKER GENES DURING GASTRULATION AND ANTERIOR BRAIN DEVELOPMENT

3.1 Introduction

The work presented in this thesis explores the possible mechanisms of neural induction and neural patterning in the chick, based upon embryo manipulations that disrupt these processes or cause them to occur in ectopic locations. In order to gain a read-out of the effects of such manipulations, it was necessary to build up an array of neural markers, so that tissue could be identified both structurally and by the combination of genes it expressed, as visualised by *in situ* hybridisation. Genes were therefore selected that were expressed throughout the nervous system, as well as those whose expression was restricted to well-defined regions. In addition, genes that were expressed early in neural development were chosen as well as those expressed much later, so that early and prolonged effects of a particular manipulation could both be examined. Many of the genes chosen not only marked a particular brain region, but their expression was also essential for the formation of that region, thus providing a link between my gene expression analyses and the development of specific brain structures.

Before using my array of molecular markers to study the effects of particular manipulations, it was necessary to study their normal expression patterns using my *in situ* hybridisation protocol (different laboratories have previously reported varying spatio-temporal patterns for some dynamically expressed genes). In addition, expression patterns for a particular gene are not always published over a wide range of stages, so it was also important to confirm and then consolidate all the different

fragments of information for each gene, into a more complete pattern. Expression patterns for the gene markers used most often are described in detail in this chapter.

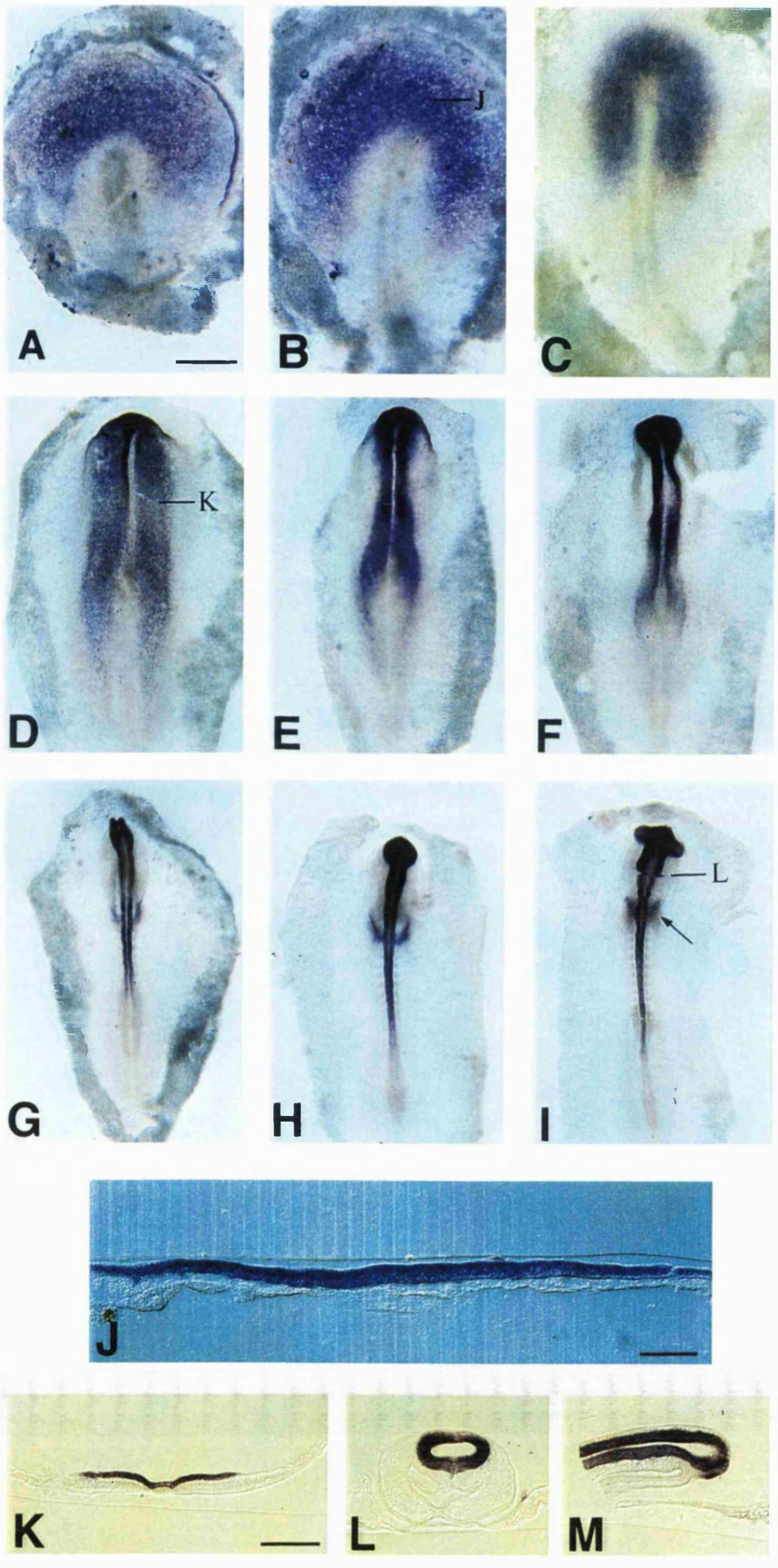
3.2 *Sox 3* expression

Sox 3 belongs to a large family of transcription factors that contain a DNA binding motif (HMG-box) similar to that found in the testis-determining gene Sry. A cDNA clone of *cSox 3* was obtained from Mike Parsons (NIMR) and digoxygenin- labelled antisense RNA probes were synthesised from the linearised DNA. *In situ* hybridisation was carried out on wholemount chick embryos for a range of developmental stages (Fig3.1).

RNA transcripts for *sox 3* are detected as early as HH stage 2. At this stage, expression is seen over most of the *area pellucida*, although entirely absent from the streak rudiment. This expression is confined to the epiblast, with none seen in the hypoblast (Rex *et al.*, 1997). As the streak extends to full length (HH stage 4), *sox 3* expression becomes more restricted to the anterior half of the embryo, forming a wide arc in the ectoderm ahead of and lateral to the streak (Fig3.1A,B,J). The streak itself, including the node, remains *sox 3*- negative. Gastrulation movements during stage 4 and 5 bring the posterior ends of the *sox 3* arc in towards the shoulders of the streak (Fig3.1C). Also during this time, *sox 3* expression becomes more medially restricted to the thickened neural ectoderm, rather than the more lateral, thinner non-neural ectoderm. The emerging head process and notochord do not express *sox 3*, although the ectoderm overlying this axial mesoderm still shows expression, as revealed in section (Fig3.1K).

Figure 3.1 Profile of *sox 3* expression during gastrulation and neurulation

Wholemount embryos viewed from the dorsal aspect, anterior to the top of the page. **A** Stage 3; **B** Stage 4; expression in a wide arc in the ectoderm ahead of and lateral to the streak. **C** Stage 4+; expression becomes medially restricted to the thickened neural ectoderm. **D** Stage 7; **E** Stage 8-; **F** 3 somites; **G** 6 somites; **H** 9 somites; **I** 12 somites; *sox 3* expression remains throughout the neural tube and forming brain structures. Arrow in **I**, expression in emerging neural crest. **J** Transverse section through stage 4 embryo, at level shown in **B**; *sox 3* expression is restricted to the ectoderm layer. **K** Transverse section through stage 7 embryo, at level shown in **D**; expression in thickened neural ectoderm, including over the emerging head process/ notochord. **L** Transverse section through 12 somite embryo, at level shown in **I**; expression is uniform along the DV axis of the neural tube and remains strong in the floorplate. **M** Sagittal section through the midline of a 12 somite embryo, anterior to the right. Scale bar in **A** = 500μm for **A**- **F**; 600μm for **G**- **I**. Scale bar in **J** = 200μm. Scale bar in **K** = 300μm for **K**- **M**.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During stage 6, as the node regresses further, *sox 3* expression extends backwards. Intensity of expression along the rostrocaudal axis varies, with strongest expression seen in the head fold and just anterior to the node, and weaker expression in between (Fig3.1D). Expression is also seen in the anterior ectoderm, now on the ventral side of the headfold. From stage 8 onwards (the latest stage collected being 12 somites), *sox 3* expression remains throughout the neural tube and forming brain structures (Fig3.1E-I,L,M). Expression is uniform along the DV axis of the neural tube and remains strong in the floorplate (Fig3.1L). Expression is also largely uniform along the AP axis, although always weaker at the very posterior end (Fig3.1I,M; (Rex *et al.*, 1997). Neural crest emerging in the branchial arches also expresses *sox 3* (arrow in Fig3.1I).

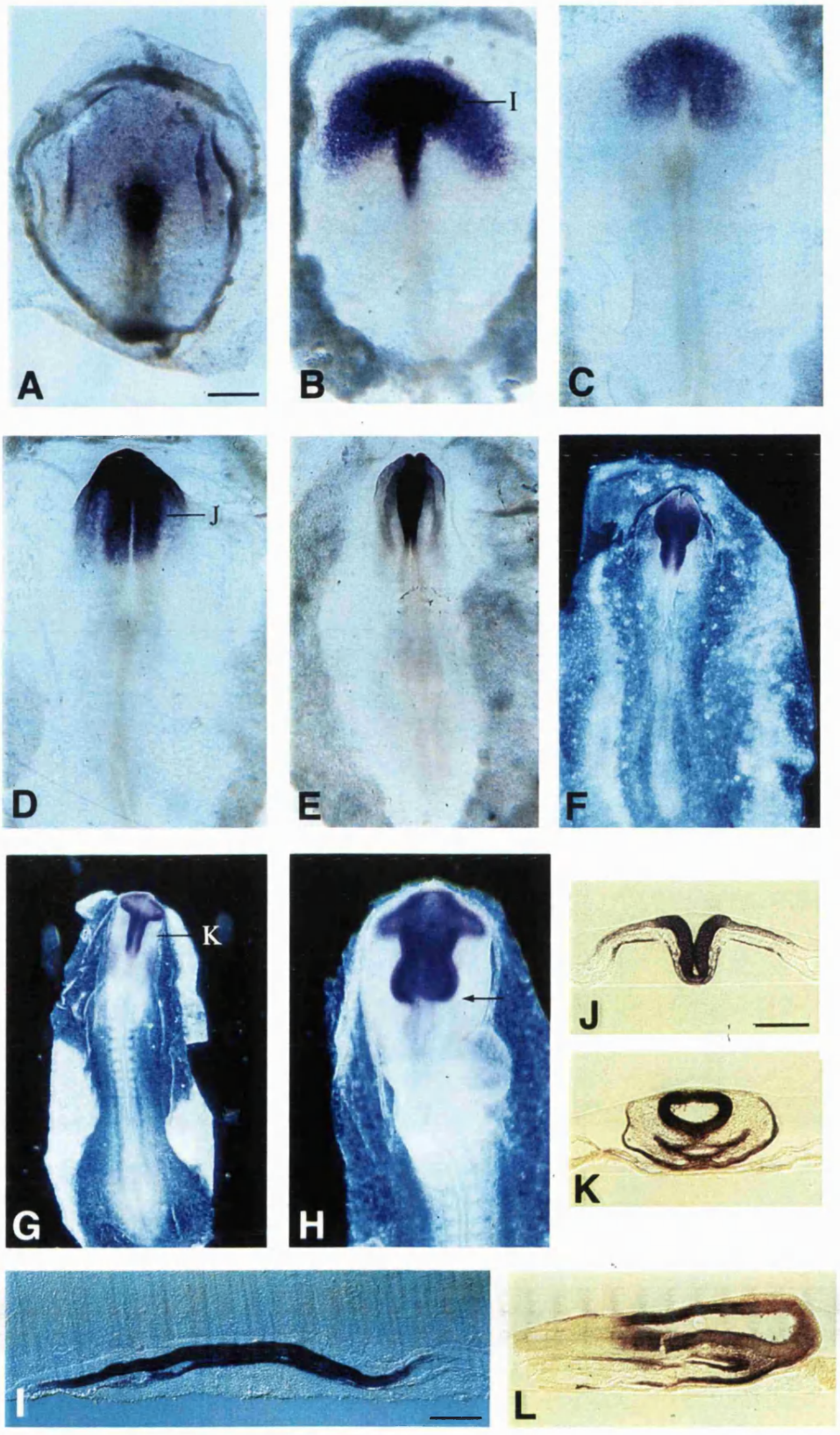
3.3 *Otx 2* expression

Otx 2 is a homeobox-containing gene, isolated in vertebrates as one of the two homologues of the *Drosophila orthodenticle* gene (reviewed in Finkelstein and Boncinelli, 1994). I obtained a cDNA clone of c-*otx 2* from Massimo Gulisano and used it to synthesise riboprobes for wholemount *in situ* hybridisation.

Otx 2 expression is already visible in the earliest stage I tested (HH stage 2), with strong expression associated with the streak rudiment and weaker expression over most of the *area pellucida*. This expression is in both epiblast and hypoblast layers (Bally-Cuif *et al.*, 1995). As the streak elongates during stage 3, the weaker expression becomes more restricted to the anterior half of the embryo (Fig3.2A), and then

Figure 3.2 Expression pattern of *otx 2* during gastrulation and neurulation

Wholemount embryos viewed from the dorsal aspect, anterior to the top of the page. **A** Stage 3; weak expression in the anterior *area pellucida* (in both epiblast and hypoblast layers) and strong expression in the node. **B** Stage 4; new wave of strong *otx 2* expression in the node and the *area pellucida* ahead of it. The first axial mesoderm cells to leave the node, the prechordal mesoderm, also express *otx 2*. **C** Stage 4+; as the node starts to regress, expression is lost in the node, and all remaining head process mesoderm is *otx 2*- negative. **D** Stage 7+; expression remains restricted to the anterior of the axis, always well ahead of the regressing node. **E** 4 somites; **F** 7 somites; **G** 10 somites; **H** 13 somites; expression remains strong throughout all structures of the anterior head. Arrow in **H**, constriction at the midbrain/hindbrain boundary, coinciding with the caudal limit of *otx 2* expression. **I** Transverse section through stage 4 embryo, at level shown in **B**; *otx 2* is expressed in both ectoderm and endoderm layers. **J** Transverse section through stage 7+ embryo, at level shown in **D**; expression is in thickened neural ectoderm, as well as underlying mesoderm, endoderm and more lateral non-neural ectoderm. **K** Transverse section through 10 somite embryo, at level shown in **G**; expression is seen throughout forebrain and midbrain, as well as in the foregut, head mesenchyme, and ventral and lateral non-neural head ectoderm. **L** Sagittal section through the midline of a 10 somite embryo, anterior to the right. Scale bar in **A** = 500μm for **A**-**E**; 600μm for **F**, **G**; 250μm in **H**. Scale bar in **I** = 350μm. Scale bar in **J** = 250μm for **J**- **L**.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disappears altogether. In addition, streak expression becomes localised to the node region (Fig3.2A,B).

From stage 4+ onwards, a new wave of *otx 2* expression is seen. Strong expression is found in an arc ahead of the node, in both anterior ectoderm and underlying endoderm cells (Fig3.2B,I). All layers of the node express *otx 2*, as do the first axial mesodermal cells to leave the node, the prechordal mesoderm. However, as the node starts to regress, *otx 2* expression in the node is lost, and notochordal cells leaving the node from that point on are also *otx 2*- negative (Fig3.2C). The domain of *otx 2* expression remains restricted to the anterior neural plate, the caudal limit of expression always well ahead of the regressing node (Fig3.2C,D). Sections reveal *otx 2* expression in the thickened neural ectoderm, as well as more lateral non-neural ectoderm, and underlying mesoderm and endoderm of the anterior embryo (Fig3.2J). From stage 8 onwards, until at least 14 somites, *otx 2* expression remains strong throughout all structures of the anterior head (Fig3.2E-H,K,L). The developing forebrain and midbrain express *otx 2* throughout, with the caudal boundary of expression gradually becoming sharper to coincide with the morphological midbrain/ hindbrain boundary, as it is formed (arrow on Fig3.2H). Lateral and ventral non-neural ectoderm surrounding the anterior brain also expresses *otx 2*, as does head mesenchyme in between these two layers (Fig3.2K). Expression is also seen at the anterior tip of the foregut (Fig3.2L). Thus, anterior structures derived from all three embryonic layers continue to express *otx 2*.

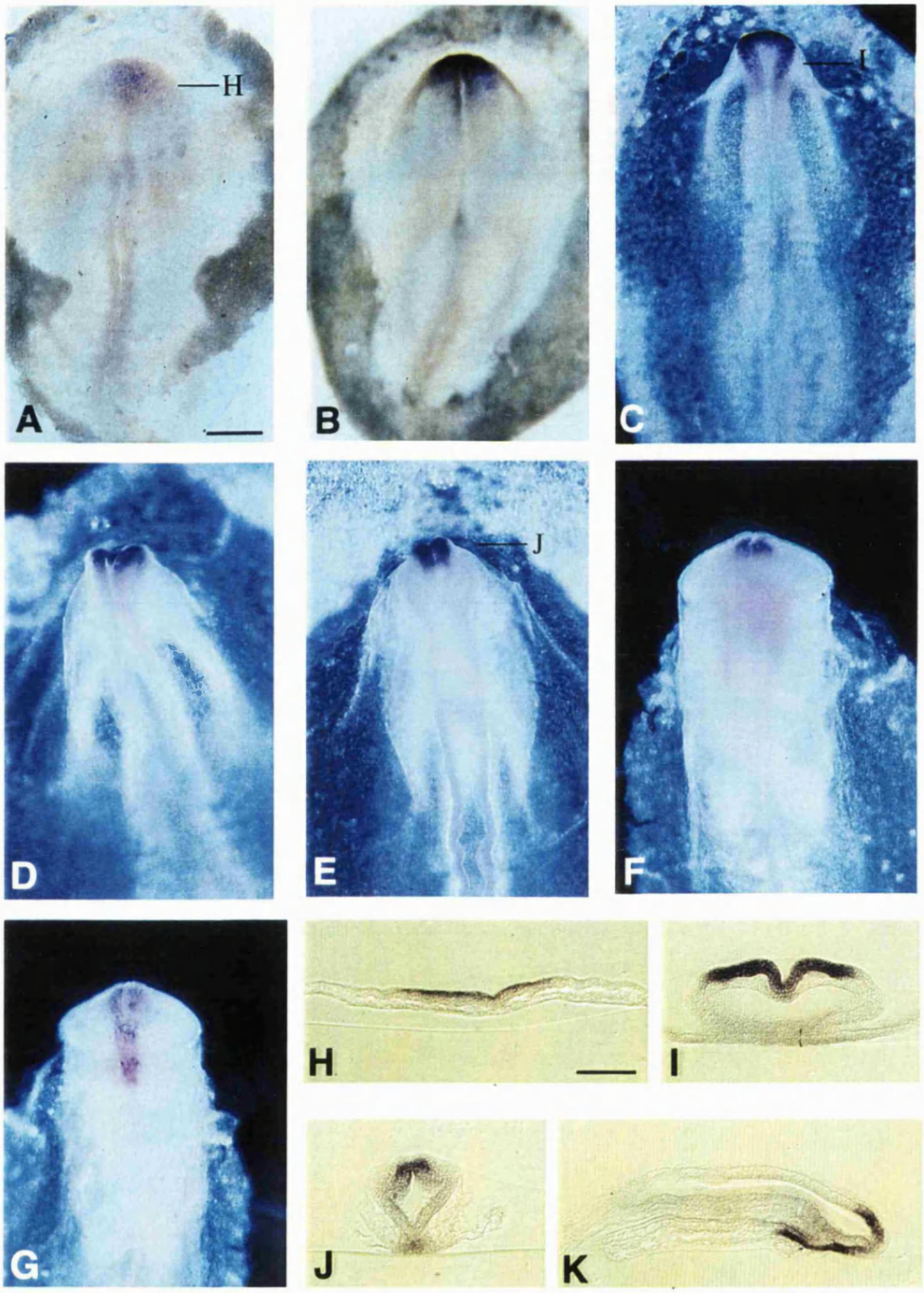
3.4 *GANF* expression

GANF belongs to the *Anf* (anterior neural fold) class of paired- like homeobox genes (Kazanskaya *et al.*, 1997). My *GANF* probe was obtained from Michael Kessel, and wholemount *in situ* hybridisations were performed on chick embryos covering a range of stages (Fig3.3).

GANF expression is first observed at stage 5-, localised to the very anterior neural ectoderm (Fig3.3A,H; Knoetgen *et al.*, 1999). This expression is therefore over and anterior to the prechordal mesoderm, following extension of the head process. Weak expression is also seen in the prechordal mesoderm, underlying the ectodermal expression. As the headfold forms (stage 6), strong *GANF* expression is maintained in the anterior neural plate (Fig3.3B). This expression becomes even more restricted by stage 8 (4 somites); only the anterior neural folds and the medial ectoderm that connects these folds express *GANF* (Fig3.C,D,I). At the 7 somite stage, *GANF* expression is seen as two symmetrical strips in the dorsal forebrain (Fig3.3E). Expression in the facial ectoderm, ventral to the CNS, is also first observed at this stage (Fig3.3J). Both aspects of this expression are maintained until the 12 somite stage, with *GANF* expression again seen as two strips in the dorsal telencephalon and in the oral ectoderm, on the ventral side of the head (Fig3.3F,G,K). This region of oral ectoderm, directly adjacent to the foregut and ventral forebrain, will form Rathke's pouch. After the 12 somite stage, the only domain of *GANF* expression that remains is in Rathke's pouch and the developing anterior pituitary (Knoetgen *et al.*, 1999).

Figure 3.3 Profile of early *GANF* expression

Wholemount embryos viewed from the dorsal aspect (except in **G**), anterior to the top of the page. **A** Stage 5; **B** Stage 6; expression in a very restricted patch of ectoderm at the anterior end of the neural plate, and also weak expression in underlying prechordal mesoderm. **C** 3 somites; **D** 5 somites; expression is restricted to the anterior neural folds and medial ectoderm that connects these folds. **E** 7 somites; **F** 12 somites; *GANF* expression seen as two strips in the dorsal forebrain. **G** 12 somites, ventral view of embryo in **F**; shows expression in strip of oral ectoderm that will form Rathke's pouch. **H** Transverse section through stage 5 embryo, at level shown in **A**; expression is restricted to the ectoderm layer. **I** Transverse section through 3 somite embryo, at level shown in **C**; expression in anterior neural folds and the connecting medial ectoderm. **J** Transverse section through 7 somite embryo, at level shown in **E**; expression is seen in dorsal forebrain and ventral head ectoderm. **K** Sagittal section through the midline of a 12 somite embryo, anterior to the right. Shows expression in dorsal telencephalon and oral ectoderm, on the ventral side of the head. Scale bar in **A** = 500μm for **A**- **C**; 250μm for **D**- **G**. Scale bar in **H** = 250μm for **H**- **K**.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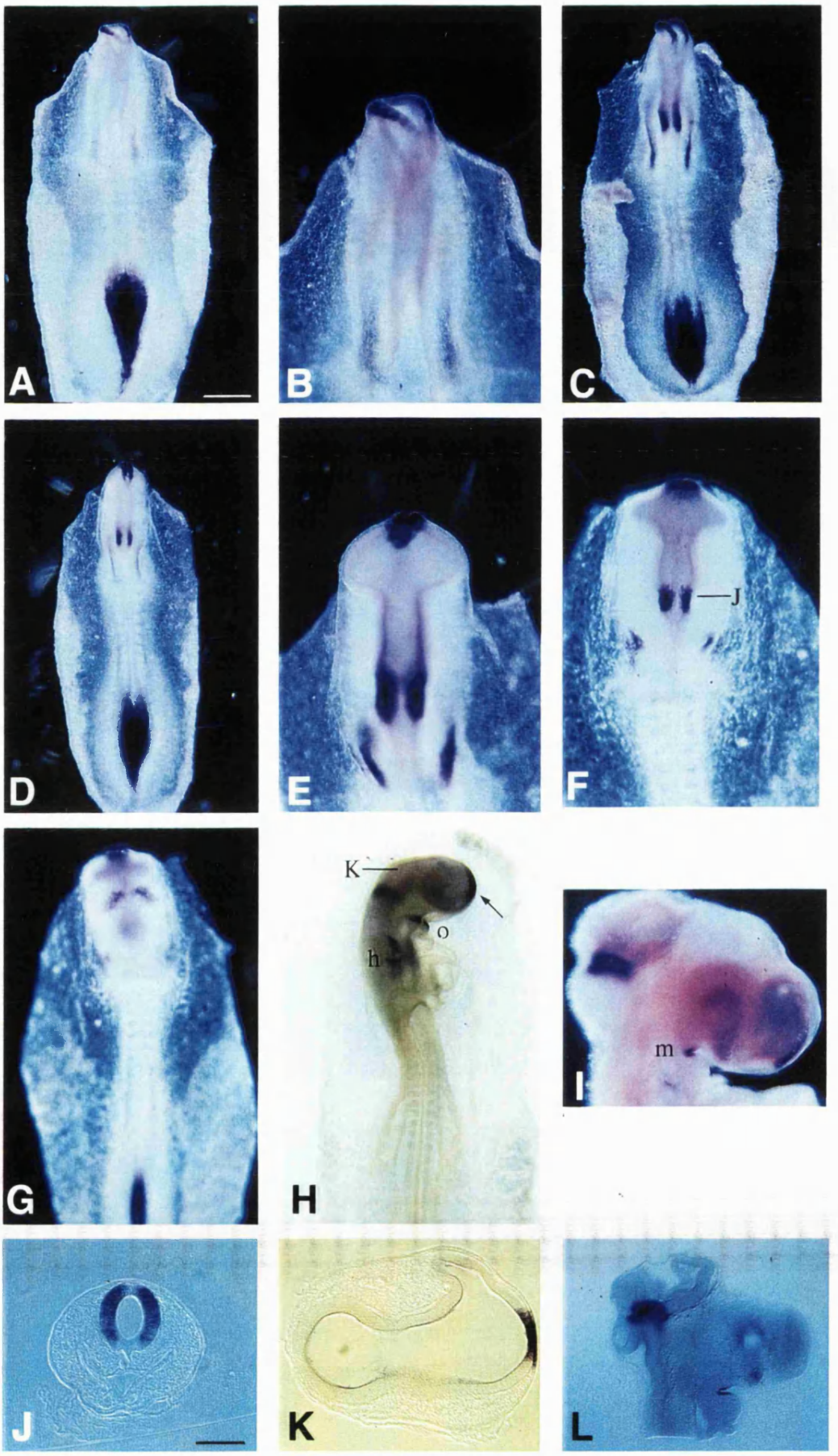
3.5 *FGF 8* expression

FGF 8 is a member of a family of signalling molecules (reviewed in Basilico and Moscatelli, 1992) that act locally to control the growth, developmental decisions and differentiation of cells in many regions of the developing embryo. *FGF 8*, like most members of the family, is a secreted protein that binds to high affinity receptor tyrosine kinases. Digoxygenin-labelled probes to chick *FGF 8* were synthesised from the DNA obtained from Ivor Mason, and wholemount *in situ* hybridisations performed on chick embryos of various stages (Fig3.4).

FGF 8 expression is first observed at HH stage 4, throughout the majority of the streak, but absent from the node. Between stages 6 and 8 (4 somites), asymmetric expression is seen in the right posterior portion of the node, as well as still throughout the streak (Boettger *et al.*, 1999). During later stages of development, the remnants of streak expression are seen in the tailbud. From the 5 somite stage onwards, several new domains of *FGF 8* expression are observed in the anterior embryo. Firstly, expression is observed in the very anterior neural folds (Fig3.4A,B), which is maintained in the anterior brain at all stages examined (Fig3.4A-F), and continues in the telencephalic commissural plate, when the anterior folds have fused (Fig3.4H,I,K). In addition, strong expression is seen in a more caudal position, in the region between the prospective midbrain and hindbrain (Fig3.4C-F; Crossley *et al.*, 1996). Sections indicate that this expression does not include cells in the ventral midline (Fig3.4J). This domain becomes restricted to a narrow band as the morphological constriction (isthmus) forms at the midbrain-hindbrain junction (Fig3.4H,I,L). Finally, *FGF 8*

Figure 3.4 Expression profile for *FGF 8* during neurulation

Wholemount embryos viewed from the dorsal aspect (except in **G** and **H**), anterior to the top of the page. **A** 5 somites; **B** Higher magnification of embryo in **A**; first expression observed in the anterior neural folds. Also, remnants of streak expression seen in the tailbud. **C** 7 somites; expression is first seen at the midbain/ hindbrain junction, and in two symmetric lateral bands corresponding to cranial and cardiac neural crest. **D** 8 somites; **E** 10 somites. **F** 12 somites; anterior expression maintained in the telencephalon, all other aspects of *FGF 8* expression also maintained. **G** 12 somites, ventral view of embryo in **F**; shows expression in two chevrons of oral ectoderm. **H** Stage 14, ventral view with forebrain turned to the right. Expression seen in the telencephalic commissural plate (arrow), midbrain/ hindbrain boundary, oral ectoderm (o) and cardiac crest close to the developing heart (h). **I** Stage 20; expression in maxilla and mandible (m), midbrain/hindbrain boundary and telencephalon. **J** Transverse section through midbrain of 12 somite embryo (level shown in **F**), showing expression absent from the ventral midline. **K** Horizontal section through forebrain and midbrain of stage 14 embryo (level shown in **H**), showing telencephalic expression. **L** Sagittal section through stage 20 embryo (taken through plane of page), confirming expression in ectoderm of maxilla and mandible. Scale bar in **A** = 500μm for **A**, **C**, **D**, **G**; 250μm for **B**, **E**; 350μm in **F**; 750μm for **H**, **I**. Scale bar in **J** = 200μm for **J**- **K** and 750μm for **L**.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expression is observed at the 7 somite stage in two symmetric lateral bands, which correspond to cranial and cardiac neural crest (Fig3.4C). The cardiac crest expression can be seen close to the developing heart at later stages (Fig3.4F,H). *FGF 8* expression in the cranial crest can later be found in two chevrons of oral ectoderm, either side of the *GANF* domain, in the ventral embryo (Fig3.4G) and then in the ectoderm of the maxilla and mandible (Fig3.4I,L).

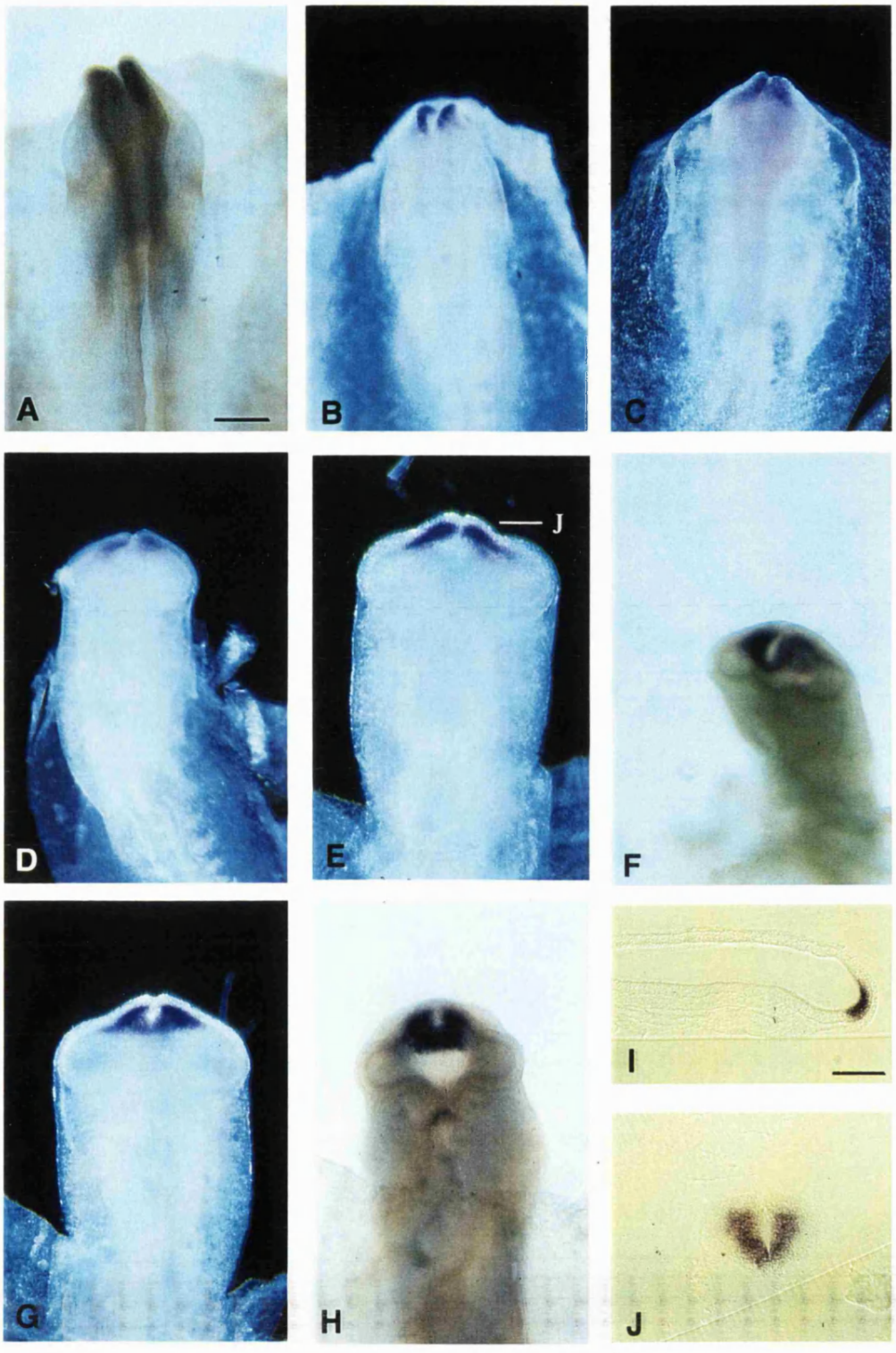
3.6 *BF-1* expression

BF-1 (Brain Factor-1) is a winged-helix transcription factor, containing a DNA binding motif first identified in vertebrate HNF-3 and *Drosophila forkhead* proteins (Tao and Lai, 1992). I synthesised digoxygenin-labelled antisense riboprobes to chick *BF-1*, from DNA given by Angela Nieto, and used them to perform *in situ* hybridisations (Fig3.5).

Strong expression of *BF-1* is first seen at the 7 somite stage, in two symmetrical stripes restricted to the anterior neural folds of the forebrain (Fig3.5A,B,C). *BF-1* expression remains confined to the rostral forebrain at all stages examined, and is not observed elsewhere in the embryo. Strong *BF-1* expression is maintained in most dorsal and ventral telencephalic neuroepithelium (Fig3.5E-J; Alvarez *et al.*, 1998). Expression also extends laterally, on the rostral border of the optic vesicles (Fig3.5E,F). As the anterior neural folds fuse to form the telencephalic commissural plate, the two stripes of *BF-1* expression join at the ventral midline, forming a horse-shoe shape (Fig 3.5F,H).

Figure 3.5 Early expression profile for *BF-1*

Wholemount embryos viewed from the dorsal aspect (except in **F** and **H**), anterior to the top of the page. **A** 6 somites; expression first observed in a very restricted domain in the anterior neural folds. **B** 7 somites; **C** 8 somites; **D** 10 somites; expression is maintained in the rostral forebrain and becomes stronger. **E** 12 somites, showing expression in telencephalon and extending laterally on the rostral border of the optic vesicles. **F** 12 somites; anterior end view of embryo in **E**. **G** 13 somites; **H** 13 somites, ventral view of embryo in **G**, showing horse-shoe shape of *BF-1* domain. **I** Sagittal section through 12 somite embryo, showing expression in dorsal and ventral telencephalon. **J** Transverse section through 12 somite embryo, at level shown in **E**; skims through expression in telencephalon. Scale bar in **A** = 250μm for **A**- **D**, **F**, **H**; 150μm for **E**, **G**. Scale bar in **I** = 250μm for **I**- **J**.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3.7 *Pax 6* expression

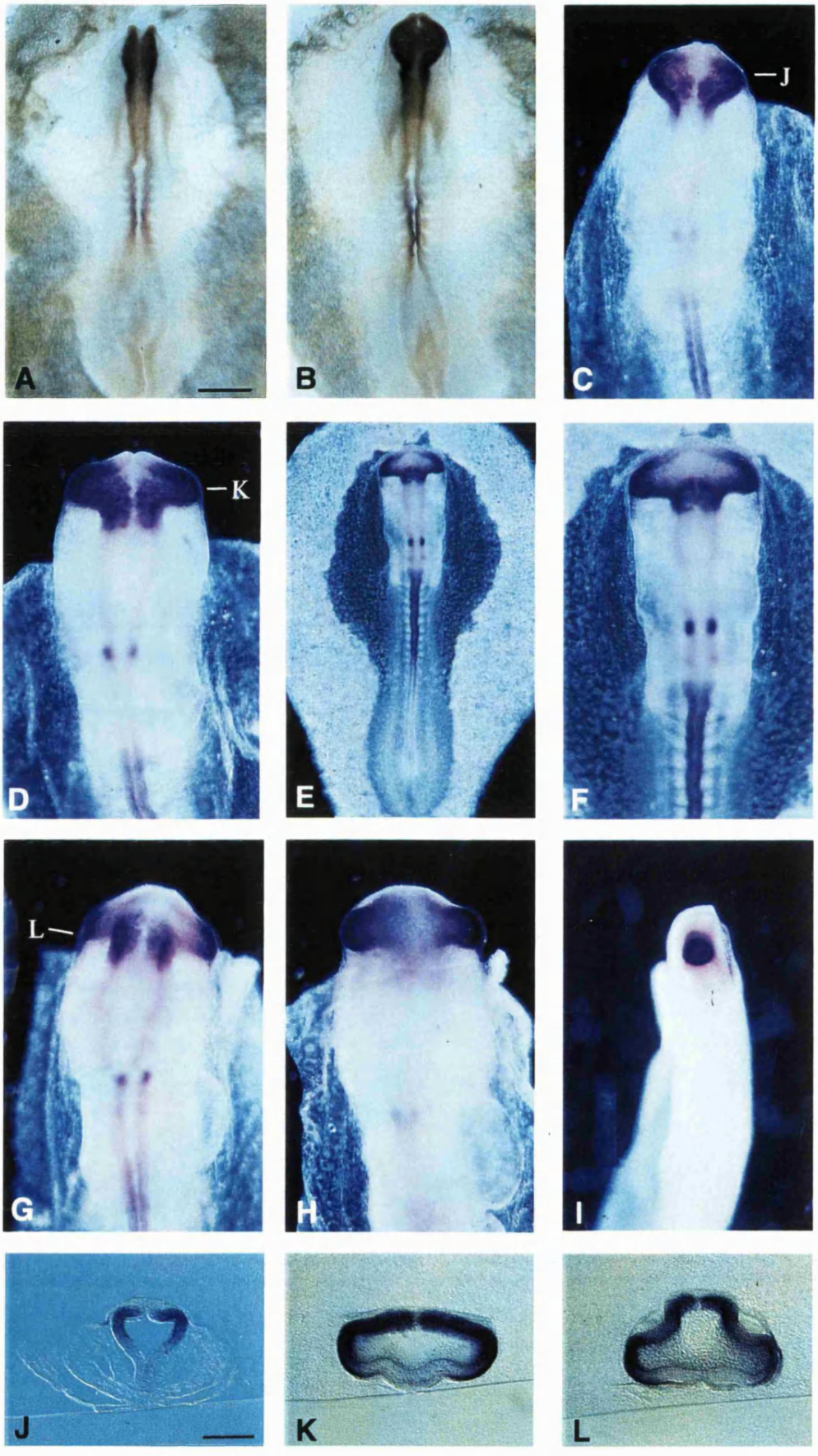
Pax 6 belongs to a family of genes that encode transcription factors, and contains both a paired domain and a homeodomain DNA binding motif (Walther and Gruss, 1991). *Pax 6* is the earliest expressed gene of the *Pax* family and is almost exclusively confined to the developing CNS. I obtained a chick *pax 6* probe from Fabienne Pituello, and used it to perform *in situ* hybridisations on chick embryos of various stages (Fig3.6).

Pax 6 expression is first detected in the chick at stage 6 (Li *et al.*, 1994). This expression is seen in a band of non-neural ectoderm close to the anterior margin of the neural plate, in tissue fated to become facial and lens ectoderm. Neural expression of *pax 6* is first seen at the 6 somite stage, in the presumptive forebrain and the developing spinal cord (Fig3.6A). Expression in the spinal cord is directly adjacent to all previously segmented somites, but is not seen in the somites themselves, nor in the neural tube adjacent to presomitic mesoderm (Fig3.6A,B,E). In addition, *pax 6* expression is seen in the hindbrain region from 9 somites onwards, particularly in rhombomere 2 (Fig3.6C-G).

Strong *pax 6* expression is maintained broadly throughout the forebrain as it expands (Fig3.6A-D). Transverse sections reveal that transcripts are present in a wide area of the neural tube, but are absent from the dorsal midline and from ventral cells (presumptive rostral diencephalic ventral midline- RDVM- and adjacent cells; Fig3.6J). Expression is also absent from the very anterior neural folds of the forebrain

Figure 3.6 Expression profile for *pax 6* during neurulation

Wholemount embryos viewed from the dorsal aspect (except in **H** and **I**), anterior to the top of the page. **A** 6 somites; first expression observed in presumptive forebrain, and also in spinal cord adjacent to somites. **B** 7 somites. **C** 9 somites; expression is first seen in rhombomere 2 of the hindbrain. **D** 12 somites; expression maintained broadly throughout the forebrain, but absent from the anterior neural folds. Caudal boundary of *pax 6* expression sharpens at the forebrain/ midbrain junction. **E** 13 somites; **F** higher magnification of embryo in **E**, showing expression receding from the dorso-anterior brain. **G** 15 somites; **H** ventral and **I** lateral views of same embryo as in **G**, showing localisation of expression to the lateral optic vesicles. **J** Transverse section through 9 somite embryo, at level shown in **C**, showing broad expression throughout the forebrain but absent from ventral neural cells. **K** Transverse section through 12 somite embryo, at level shown in **D**; shows widespread expression in forebrain and surrounding head ectoderm, but still absent from ventral midline. **L** Transverse section through 15 somite embryo, at level shown in **G**. Scale bar in **A** = 500μm for **A**, **B**; 750μm in **E**; 250μm for **C**, **D**, **F**, **G**- **I**. Scale bar in **J** = 200μm for **J**- **L**.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(Fig3.6C,D). The caudal boundary of the *pax 6* domain sharpens at the forebrain-midbrain boundary (Fig3.6D).

The non-neural ectoderm surrounding the forebrain also expresses *pax 6*. By the 12 somite stage, the telencephalon has started to expand dorsally and the vesicles from which the eye cups will form have extended laterally. *Pax 6* expression recedes from the dorso-anterior brain and becomes particularly concentrated in the lateral optic vesicles (Fig3.6F-I). In concert, *pax 6* expression in the surrounding non-neural ectoderm also becomes laterally restricted, remaining strong in the region that will form the lens placode, directly adjacent to the neural tissue of the optic vesicles (Fig3.6I,L). Expression is still absent from ventral cells, due to the presence of inhibitory signals from the midline mesendoderm (Fig3.6K,L; Macdonald *et al.*, 1995). *Pax 6* expression remains in several tissues of the eye at much later stages, and is thought to be involved in several steps of eye development.

3.8 Analysis and discussion of results

Patterns of neural expression for an array of genes are described in detail in this chapter, concentrating on those expressed in the anterior brain. Evidence, particularly from work in mouse, shows that several of these genes play a necessary functional role in forming specific brain regions. These results are discussed below, and they explain why these particular genes were selected for use as molecular markers in my experiments, as described in later chapters.

As shown here and in other published reports (Rex *et al.*, 1997), *sox 3* is expressed throughout the CNS at later stages (HH st.6 onwards); it is seen in all neural cells along both the AP and DV axes. In addition, expression of *sox 3* is specific to neural tissue, and is not detected in any other tissues of the embryo, at these stages. These features make *sox 3* a very good marker of neural tissue, and indeed it is frequently used to detect the presence of neural tissue.

However, very early expression of *sox 3* (HH st.2-6) is rather widespread and, although confined to the ectoderm, clearly covers a wider area than that which will form neural tissue. This makes *sox 3* less useful as a neural marker in very early embryos, since early expression of *sox 3* does not necessarily mean that cells will follow a neural fate. Rex and colleagues suggest that *sox 3* initially marks the area of ectoderm competent to respond to neural induction, and only later (after st.5) becomes refined to just the cells fated to become the CNS (Rex *et al.*, 1997). It is interesting to note that refinement and full establishment of the boundary between the neural and epidermal ectoderm is also thought to occur at this time. Pronounced thickening of the neuroectoderm in comparison to the surrounding non-neural ectoderm makes the neural plate morphologically more obvious. In addition, expression of the epidermal marker *BMP 4* recedes from the neural plate during stage 4 and 5 (Towers *et al.*, 1999). *BMP 4* expression in the periphery of the *area pellucida* initially significantly overlaps with the domain of *sox 3* expression and obviously encroaches upon the neural area. *BMP 4* is thought to establish an epidermal identity in the ectoderm, thereby restricting the extension of the neural plate (Pera *et al.*, 1999). Restriction of *sox 3* expression to the neural plate therefore coincides with restriction of *BMP 4* to

epidermal ectoderm, suggesting that the boundary between these two tissues is refined at this time by a mutually inhibitory mechanism.

Studies of *otx 2* expression in mouse show that it has a specific pattern, restricted to head embryonic structures following neurulation (Simeone *et al.*, 1992). Furthermore, embryos homozygous for null mutations in the *otx 2* gene display a complete lack of head structures anterior to rhombomere 3 (Ang *et al.*, 1996), showing that *otx 2* function is essential for formation of forebrain and midbrain structures. The first site of *otx 2* expression in the mouse, prior to gastrulation, is in the anterior visceral endoderm (AVE); the tissue known to be necessary for complete head development (Simeone et al., 1993; Acampora *et al.*, 1995). Analysis of chimaeric embryos has shown that *otx 2* is sequentially required in this extra-embryonic endoderm and then in the anterior neuroectoderm itself, for normal anterior brain development (Rhinn *et al.*, 1998). In addition, positive signals from the anterior mesendoderm are thought to stabilise and maintain *otx 2* expression in the anterior ectoderm (Ang *et al.*, 1994). A very similar expression pattern for *otx 2* is seen in the chick, as described here and in other published reports (Bally-Cuif *et al.*, 1995). *Otx 2* is expressed in head structures derived from all three embryonic layers, and is therefore a very good marker for broad anterior fate.

The first member of the *Anf* class of genes, *Xanf-1*, was cloned in *Xenopus* (Zaraisky *et al.*, 1992). Since then homologues have been identified in mouse, *Hesx-1* (Thomas *et al.*, 1995) also known as *Rpx* (Hermesz *et al.*, 1996), in zebrafish, chick (*GANF*) and humans (Kazanskaya *et al.*, 1997). In all species investigated, these genes show

very similar patterns of expression during early embryogenesis, with most intense expression always seen at the anterior extremity of the body axis.

Loss of *Hesx1* expression in the mouse leads to significant reduction in prospective forebrain tissue, especially the telencephalon, and absence of developing optic vesicles (Thomas and Beddington, 1996; (Dattani *et al.*, 1998). This suggests that *hesx1* plays an important role in the initial allocation of forebrain neuroectoderm. *Hesx1* is first expressed in the AVE very early in development, and then later in the adjacent anterior neuroectoderm (Hermesz *et al.*, 1996; Thomas and Beddington, 1996). However, chimaeric analysis suggests that *hesx1* function in the AVE is redundant or not necessary for anterior neural development, and that its essential site of function is in the anterior neuroectoderm itself (Martinez-Barbera *et al.*, 2000b). In the chick, the early expression domain in extra-embryonic endoderm (hypoblast) is not observed, and *GANF* is only detected in the ectoderm as the prechordal mesoderm appears, when neural plate formation has already begun (Knoetgen *et al.*, 1999).

GANF is restricted to the most anterior neural plate from relatively early stages and remains restricted to the dorsal forebrain throughout the stages of development studied here. It is therefore a good marker of very specific anterior neural fates at a relatively wide range of stages, allowing early and late brain patterning to be directly compared. *GANF* is also expressed in a central stripe of ventral head ectoderm, including the region that forms Rathke's pouch and the anterior pituitary (Hermesz *et al.*, 1996). It is therefore also a very early marker of stomodaeal ectoderm.

In mouse, *FGF 8* is expressed in several organising centres in the brain, including the anterior neural ridge (ANR) and the midbrain/ hindbrain boundary, or isthmus (Crossley and Martin, 1995). The ANR is necessary and sufficient for *BF-1* expression in anterior neural plate explants. Furthermore, *FGF 8* is expressed in the ANR at early somite stages, and recombinant FGF 8 protein is capable of inducing *BF-1* (Shimamura and Rubenstein, 1997). These results therefore strongly suggest that the ANR is an important signalling centre for forebrain development in the mouse, and *FGF 8* expression is required for this function. In addition, both *hesx1* and *hex* mutants show forebrain defects associated with loss of *FGF 8* expression in the ANR (Dattani *et al.*, 1998; Martinez-Barbera *et al.*, 2000a).

Many aspects of the chick *FGF 8* expression pattern (described here) are similar to those reported in the mouse (Crossley and Martin, 1995). *FGF 8* is seen strongly in the very anterior neural folds from the 5-6 somite stage, just prior to the onset of *BF-1* expression. Therefore, the anterior neural folds may play an important forebrain patterning role in the chick, equivalent to the ANR in the mouse (Shimamura and Rubenstein, 1997). *FGF 8* has proved to be a very useful marker of the anterior neural folds and telencephalon. In addition, the domains of *FGF 8* expression in several other regions of the embryo serve as a very good internal control, in experimental embryos where the telencephalic expression domain has been lost.

Homozygous null *BF-1* mutants have a dramatic reduction in the size of the cerebral hemispheres, establishing a central role for the gene in forebrain development (Xuan *et al.*, 1995; Dou *et al.*, 1999). In these mutants, telencephalic cells are specified

normally, but they either differentiate prematurely or their proliferation is reduced, suggesting that *BF-1* may act by controlling the rate of cell proliferation and the timing of neuronal differentiation. Normal expression of *BF-1* in the developing mouse brain is restricted to the telencephalon and the nasal half of the retina and optic stalk. This expression is prior to the morphological appearance of the telencephalon/ diencephalon boundary, suggesting that *BF-1* may also play a role in sub-dividing the forebrain territories.

In chick, strong *BF-1* expression is first seen at the 7 somite stage (described here), entirely restricted to the neural folds in the anterior forebrain. This very specific expression is reliably maintained in the telencephalon throughout the stages of development studied here, making *BF-1* a very useful, relatively late marker for telencephalic identity (Alvarez *et al.*, 1998).

Correctly regulated expression of wild type *pax 6* is necessary for normal development and patterning of the forebrain, particularly the diencephalon (Grindley *et al.*, 1997; Mastick et al., 1997; Warren and Price, 1997). The developing eye, where expression of *pax 6* eventually becomes focussed, is also extremely sensitive to *pax 6* gene function; homozygous *pax 6* mutants (*small eye*) lack eyes altogether (Hogan *et al.*, 1986; Hill *et al.*, 1991).

In the stages of development studied here, *pax 6* is broadly expressed throughout the forebrain vesicle. This makes it a good general marker of prosencephalic identity, covering a much wider area of tissue than *GANF*, *FGF 8* or *BF-1*. Interestingly, by the

12 somite stage, *pax 6* has an expression pattern complementary to that of *BF-1* and *FGF8*, since it is excluded from the anterior neural folds of the telencephalon but is expressed throughout the rest of the prosencephalon. The expression pattern of *pax 6* can also provide additional information on DV patterning of the forebrain. *Pax 6* is not expressed in the ventral region of the CNS, and it is thought that *Shh,* or a closely related signalling molecule, emanating from midline tissue in the ventral forebrain acts to inhibit *pax 6* expression (Macdonald *et al.*, 1995). The presence of midline signalling can therefore be confirmed by absence of *pax 6* expression in ventral neural cells.

CHAPTER IV

INDUCED EXPRESSION OF NEURAL MARKERS FOLLOWING A NODE GRAFT

4.1 Introduction

Hensen's node was first shown to be able to induce a second neural axis in the chick by Waddington (1933). Since then, detailed studies using molecular markers (Storey *et al.*, 1992) have concluded that the second axes induced by young nodes (HH st.3-4) are complete. However, the most anterior marker used was the 3A10 antibody, which stains neurons in the diencephalon. I therefore wanted to confirm the completeness of anteroposterior pattern in second axes, using telencephalic markers such as *BF-1* and *GANF* (see Chapter III).

Several observations suggest, however, that the mouse node (and therefore its derivatives) does not contain all the information necessary to induce a complete axis. Second axes formed in mouse hosts, following mouse node grafts of any age, are missing forebrain and midbrain structures and do not express *otx 2* (Beddington, 1994; Tam and Steiner, 1999). Furthermore, an additional signalling centre has been identified in mouse that is necessary for generating the most anterior parts of neural pattern (reviewed in Beddington and Robertson, 1998). This tissue, the anterior visceral endoderm (AVE), comprises a group of extra-embryonic cells that are established entirely separately from the node, and move to the anterior side of the embryo before overt streak formation commences on the posterior side. I have examined the ability of mouse nodes to induce anterior neural markers when grafted into chick hosts. I am therefore able to draw direct comparisons between the inducing ability of chick and mouse nodes, as discussed in this chapter.

Finally, I have examined whether members of the *nodal* and *Vg* families play a role in neural induction. In the mouse, *nodal* is required in the AVE for head formation (Varlet *et al.*, 1997), and is also essential in embryonic tissues for formation of the primitive streak; homozygous mutant embryos lacking *nodal* expression cannot initiate streak formation

and arrest during gastrulation (Conlon *et al.*, 1994). A similar early role for *Vg1* in streak formation has also been proposed; ectopic expression of *cVg1* in pre-streak blastoderms simultaneously induces the formation of a second primitive streak and the activation of organiser-specific genes (Seleiro *et al.*, 1996; Shah *et al.*, 1997). I therefore tested the ability of grafts of *nodal* and *cVg1*-expressing cells to induce ectopic neural tissue. I also tested *Xnr3*, a nodal-related family member shown to be a direct neural inducer in *Xenopus* (Hansen *et al.*, 1997).

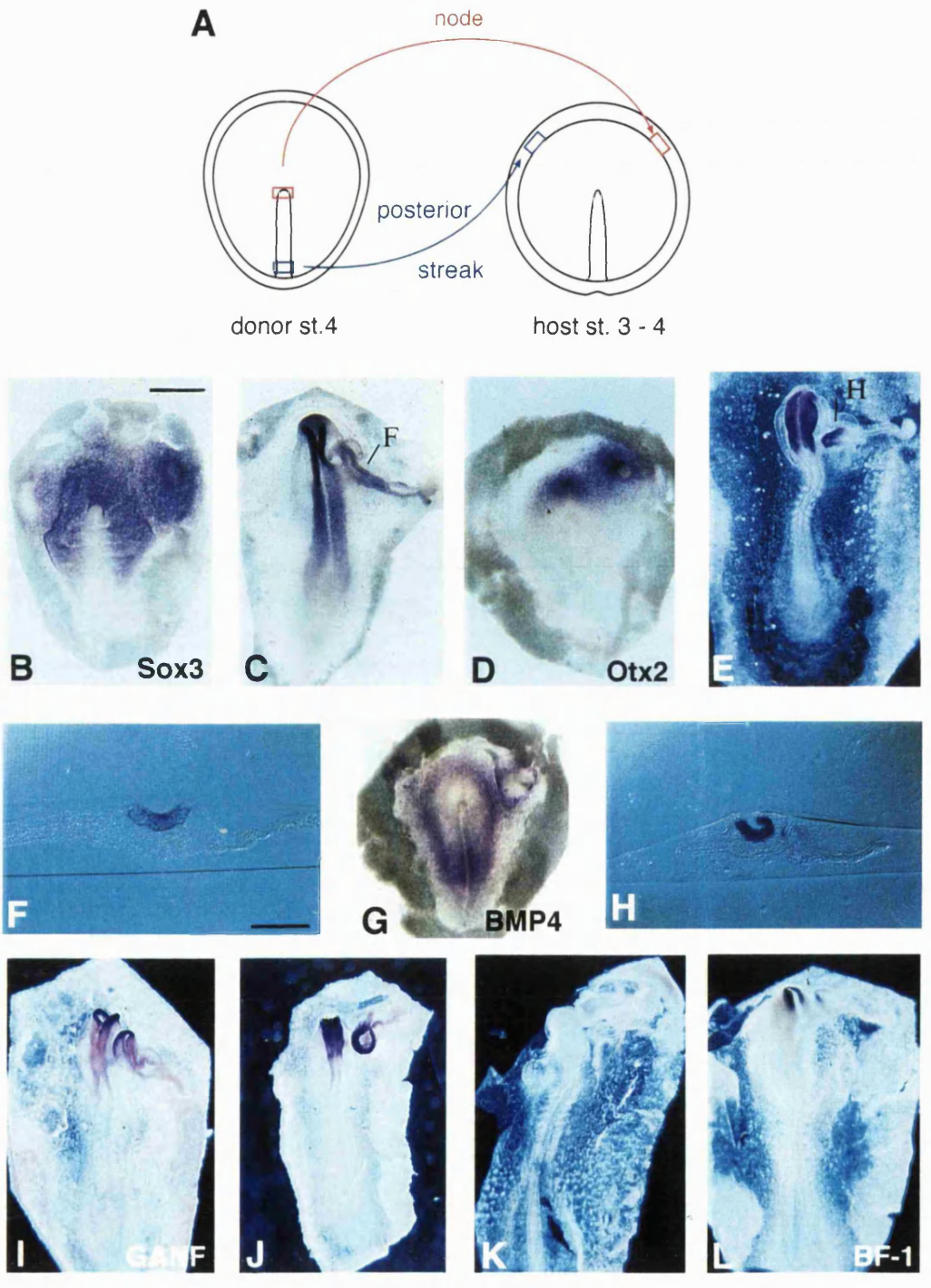
4.2 Gene expressions following chick node grafts

Using the results of Storey *et al.* (1992), together with my own explorations, it was found that optimal neural induction was obtained by grafting a HH st.4 node into a st.3+ to 4 host. Furthermore, the optimal position for placing the graft was on the outer side of the *area opaca/ area pellucida* boundary, in the region anterior to the node (Fig4.1A). The epiblast there was competent to respond to inducing signals and produced a fair-sized second axis, which was far enough away from the host axis that morphological interference was minimal. The control graft of posterior streak tissue appeared to integrate into the host tissue, producing no obvious structure.

Strong *sox 3* expression was seen associated with node grafts at all timepoints tested (from 6 hours onwards). This expression was confined to the host ectoderm, as a patch before any overt structure was visible, and later throughout the extent of the second axis as it began to take shape (Fig4.1B,C,F). Previous studies in the lab, using quail grafts into chick hosts and staining for the QCPN antibody (Patel *et al.*, 1996), showed that the entire neural plate, including floorplate at all axial levels, was host-derived. Thus, *sox 3* expression here was induced in host cells. Weaker *sox 3* expression was also seen associated with the control grafts (Fig4.1B). This latter expression was transient, being

Figure 4.1 Gene expressions in the second axis induced by a chick node graft

A Schematic showing location of node and posterior streak grafts, on the outer side of the *area opaca/ area pellucida* boundary, in the region anterior to the host node. All host embryos shown are viewed from the dorsal aspect, anterior to the top of the page, with the node graft on the right hand side. **B** *Sox 3* expression induced in the host by both grafts, 6hours after operations. **C** *Sox 3* expression after 18 hours; strong expression is seen throughout the second axis induced by the node graft, but is not maintained by posterior streak graft. **D** *Otx 2* expression after 7 hours, induced in the host by the node graft. **E** *Otx 2* expression after 18 hours, at the anterior end of the second axis. **F** Transverse section through second axis in **C**, showing *sox 3* expression in the neural ectoderm. **G** *BMP 4* expression after 6 hours, induced as a ring in the host ectoderm, at a distance from the node graft. Expression is also associated with the posterior streak graft. **H** Transverse section through second axis in **E**, showing *otx 2* expression in the neural tube and some adjacent non-neural ectoderm. **I** *GANF* expression 20 hours after operations, at the anterior end of the second axis. **J** *GANF* expression in the reversed second axis, produced by reversing the AP polarity of the node at the time of grafting. **K** Lack of *GANF* expression in both host and second axis, following removal of host definitive endoderm. **L** *BF-1* expression 20 hours after operations, at the anterior end of the second axis. Scale bar in **B** = 1mm for **B- E, G, I- L**. Scale bar in **F** = 250μm for **F** and **H**.


A
node
posterior
streak
donor st.4
host st. 3 - 4
B
Sox3
C
F
D
Otx2
E
H
F
G
BMP4
H
I
J
K
L
BF-1

observed only in embryos fixed a short time after grafting (up to 10 hours) and not maintained in embryos fixed later (Fig4.1C).

Otx 2 expression was also seen in host cells, from 7 hours onwards after a node graft (Fig4.1D,E). Sections revealed this expression to be in both ectoderm and endoderm layers (as it is in the anterior region of a normal embryo at this stage), prior to the formation of any overt structure. No expression was seen associated with control grafts. At later timepoints after node grafting, *otx2* expression was seen confined to one end of the second axis (usually the end closest to the host head region). This expression was predominantly in the neural tube, but also in some adjacent non-neural ectoderm, as shown in sections (Fig4.1H).

GANF expression was observed in the ectoderm of all second axes 20 hours after a node graft. This was a very restricted patch of expression, seen in the end of the axis closest to the host axis/ host anterior end (Fig4.1I). Interestingly, if the AP polarity of the node was reversed with respect to the host, at the time of grafting, the location of the *GANF* domain suggested that the AP polarity of the second axis was also the opposite of that in the host (Fig4.1J). This indicates that patterning of the induced structures is dependent on the grafted node, and not greatly influenced by the AP polarity of the host environment. However, in a preliminary experiment where the anterior endoderm of the host was removed between stage 4+ and 5, a procedure likely to result in anterior truncation of the host axis (see Chapter 6), *GANF* expression was not observed in either the host or the second axis (Fig4.1K). Therefore, patterning of the induced structures may still be sensitive to host signals, and if anterior patterning information is missing in the host (from the definitive endoderm), the second axis can also be truncated.

BF-1 showed a similar pattern of expression to *GANF*. Twenty hours after a node graft, *BF-1* expression was seen in a very restricted patch in the ectoderm at one end of each second axis (Fig4.1L).

Interestingly, node grafts actually caused an up-regulation of the ventralising, neural-inhibiting *BMP4* (Fig4.1G). Unlike the *BMP4* expression associated with the control graft of posterior streak (which would normally be expressing anyway), expression was in the host ectoderm surrounding the node graft, at a specific distance from it. Thus, the node graft itself, and the area immediately around it, were *BMP4*- negative, even though they had induced ectopic *BMP4* expression at a distance. This ring of *BMP4* expression likely overlaps with the outer edge of the early *sox3* and *otx2* domains (compare Fig4.1B,D,G), just as endogenous expression domains of these genes do in the normal early embryo. *BMP*s are involved in refining the boundary between the neural and epidermal ectoderm, and restrict the extension of the neural plate by establishing an epidermal identity. Therefore, it appears that a grafted node not only induces an ectopic neural plate, but also induces a halo of epidermal ectoderm surrounding it. Such a phenomenon has recently been reported by Pera and colleagues (1999), who find a similar ring of the early epidermal marker *DLX 5* is induced at a distance following a node graft.

4.3 Gene expressions following mouse node grafts into chick

Mouse embryos were collected and dissected out of the decidua by S. Dunwoodie and J P. Martinez-Barbera. Mouse node grafts were cut by J. Cooke. All preparation of chick hosts, insertion of grafts and further processing was performed by myself.

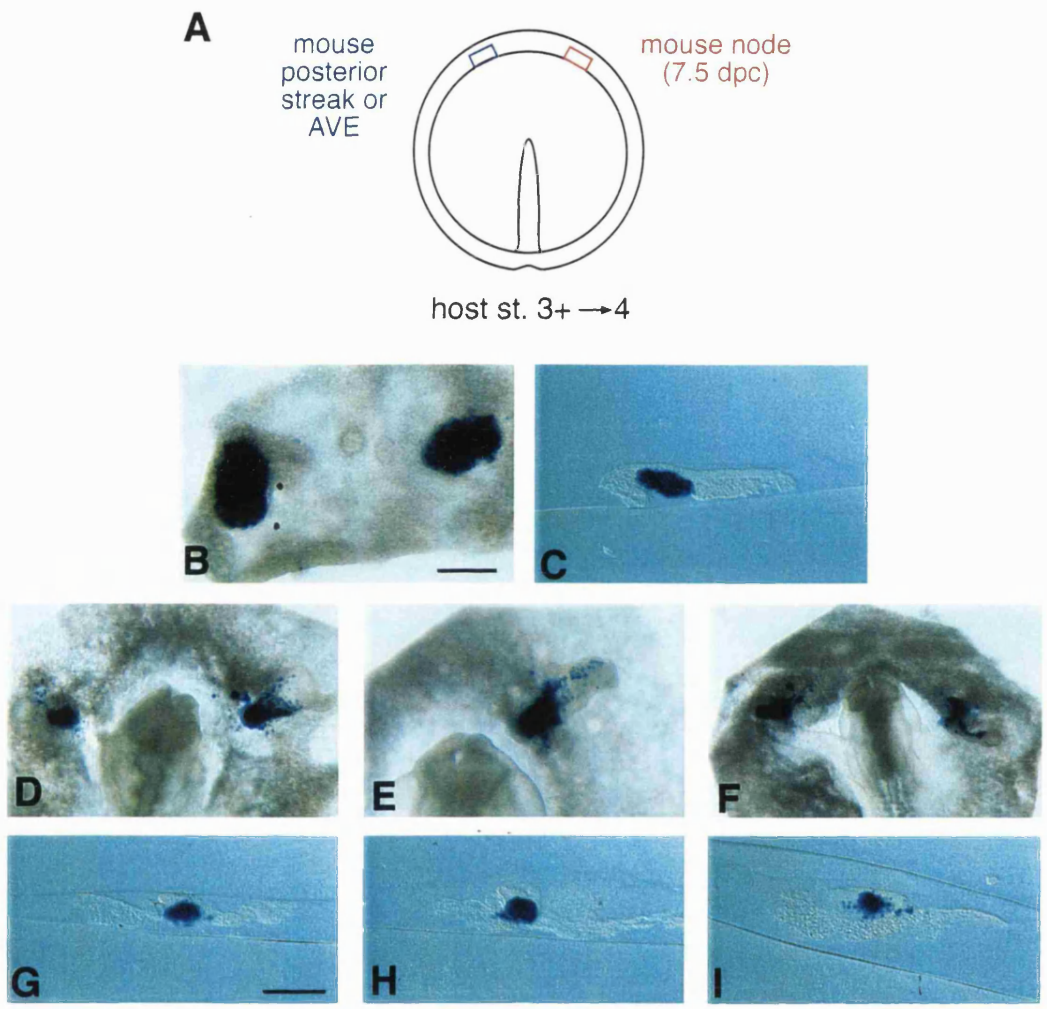
4.3.1 Age and position of node grafts

We found that we were able to induce a second axis in 69% of cases (133/193) by grafting a node from a 7.25 dpc mouse embryo (late streak) into a mid stage 4 chick host. The second axis produced by a mouse node was never as large as that produced by a chick node graft, but this was not surprising due to the effects of cross- species grafting. In addition, the size of mouse tissue grafted was considerably smaller than a chick node (square of side 200μm instead of 300μm). To address this point, I grafted two mouse nodes together; Storey *et al.* (1995) used double grafts when looking at subpopulations of cells in chick nodes. I found that two nodes grafted together did not produce noticeably larger second axes than one node, nor did they induce *sox 3* expression more reliably.

Nevertheless, the mouse tissue exhibited strong convergent extension movements and often burrowed right into the host ectodermal layer, causing it to bulge out on the opposite surface. However, the mouse node did not appear to be as strong a neural inducer as chick nodes. Neural tubes were rarely as well established in the second axes from mouse node grafts, and more of the cells in the second axis displayed mesoderm character. Control grafts of posterior streak also produced some local disturbance in the host. However, sections showed that these areas lacked structure and no thickened neural ectoderm was ever present. Interestingly, I found that position of the graft within the host affected the size of axis formed. Grafts placed anteriorly in the host consistently produced larger axes than those placed more laterally (compare graft positions in Fig4.2A with Fig4.1A), but still anterior to the node (all grafts were placed on the outside of the *area opaca / area pellucida* margin.) However, analysis of *otx2* expression suggested that although axes from more lateral grafts were smaller, they were patterned as extensively as those from more anterior grafts. Storey and colleagues (A. Streit; pers. comm.) also found that the position of a chick node graft within the host, provided it was in the anterior half, did not affect the patterning in the induced axis.

Figure 4.2 Contributions of mouse tissue to a second axis induced in chick

A Schematic showing location of mouse node and posterior streak/ AVE grafts in the chick hosts, on the anterior *area opaca/ area pellucida* boundary. **B** Staining for Lac Z throughout graft in embryo fixed immediately after operations. **C** Section through graft shown in **B**. **D**- **F** Embryos fixed 24 hours after operations, viewed from the dorsal aspect. Node grafts are on the right hand side in each case. Graft on the left in **D** is posterior streak, and graft on the left in **F** is anterior visceral endoderm (AVE). **G** and **H**, transverse sections through second axis induced by node graft, showing mouse tissue located below thickened neural ectoderm. Neural tube is therefore of host (chick) origin. **I** Section through posterior streak graft, showing lack of neural thickening. Scale bars = approximately 300μm throughout.


A
mouse
posterior
streak or
AVE
mouse node
(7.5 dpc)
host st. 3+ →4
B
C
D
E
F
G
H
I

Finally, I observed that slightly older hosts were at least as responsive as younger ones, if not more so. Thus, chick embryos as late in stage 4 as possible, responded well to mouse node grafts; this is slightly older than the age of host usually used for chick node grafting experiments. The stage of the mouse node graft itself did not seem to make any noticeable difference to its inducing ability. In mouse to mouse node grafts, the early gastrula organiser (EGO) and the node also appear to have similar inducing and patterning abilities (Beddington, 1994; Tam and Steiner, 1999). In the experiments described here, the first prechordal mesoderm may have left the node at the time of grafting, in some embryos; however, the graft was cut in such a way that these cells would still be included. In chick, the emerged prechordal mesoderm is able to recover some of the lost patterning properties of the stage 5 node (Foley *et al.*, 1997).

4.3.2 Survival of mouse tissue and contributions to the second axis

An immediate concern was to establish how long the mouse tissue was able to survive within a chick host. In order to address this, we made use of the *Rosa 26* line of mice, which expresses the *lac Z* reporter in every cell (Zambrowicz *et al.*, 1997). I was therefore able to identify all mouse cells by assaying for β galactosidase activity (18 embryos examined). I found that mouse cells were still visible 24 hours after grafting into chick hosts (Fig4.2D-I). Since all my assays of neural marker expression were performed 24 hours after grafting, it was likely therefore that mouse tissue would still have been alive and capable of signalling in these specimens.

Assaying for β galactosidase activity also allowed me to examine the contributions of mouse cells to tissues of the second axes. I found that, in the cases where a thickened neural ectoderm was present, these cells were chick origin (not staining), and the mouse tissue remained in a clump below this ectoderm (Fig4.2G-I). This is similar to the contribution of an avian node to the second axis; graft cells form notochord, endoderm

and contribute to any somites formed, whereas the whole neural plate is host derived (Patel *et al.*, 1996). Observation of wholemounts also suggested the mouse cells remained clumped together; the majority of the peg-like extrusion through the ectoderm was chick cells (Fig4.2D,E). Unfortunately, node grafts from the *Rosa 26* strain of mice did not induce as large second axes as were usually formed, thereby limiting the conclusions that could be drawn from this experiment.

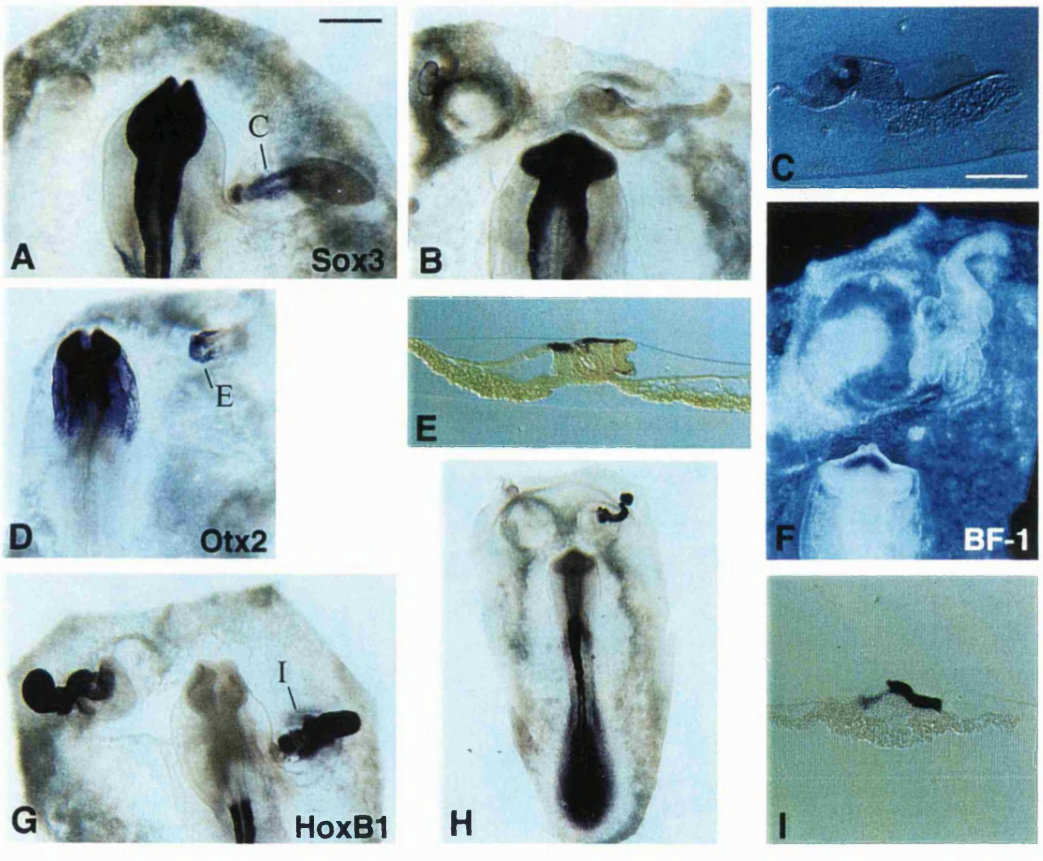
4.3.3 Expression of chick neural markers in the second axis

cSox3 expression was only observed in 42% of second axes (13/31) induced by a node graft (Fig4.3A,C). In embryos showing the strongest expression, a band of *sox3* was seen running along the length of the second axis, either side of the midline. Sections revealed this expression to be in the neural tube (Fig 4.3C). However, in the majority of cases, *sox3* expression was poor and only seen in small patches of the axis. There appeared to be no correlation between the size of second axis and whether it expressed *sox3* or not, with some large axes (with obvious neural induction) showing no expression at all (Fig4.3B). This was initially very puzzling, given that *sox3* is normally expressed throughout the nervous system from very early stages. However, Streit and colleagues (1997) found that *sox2*, also a pan-neural marker, was only induced by nodes capable of inducing a full range of both anterior and posterior structures. I therefore tested whether the same could be true for *sox3*, by comparing expression following a stage 4 and a stage 4+ to 5 chick node. I too found that induction of *sox3* in neural tissue induced by an older chick node, incapable of inducing the most anterior neural pattern, was poor (data not shown).

cOtx 2 was found to be expressed in 68% of second axes produced by node grafts (43/63 cases). This expression was strong but very restricted to a small patch at one end of the axis (Fig4.3D). However, sections revealed that the majority of this expression was

Figure 4.3 Gene expressions in the second axis induced by a mouse node graft

All host embryos shown were fixed 24 hours after operations and are viewed from the dorsal aspect, anterior to the top of the page, with the node graft on the right hand side. **A** Good induction of *sox 3* expression in the second axis. **B** Almost no *sox 3* has been induced by the node graft, although the structure and size of the second axis is very good. **C** Transverse section through second axis in **A**, showing *sox 3* expression in the neural tube. **D** *Otx 2* expression at the anterior end of the second axis. **E** Transverse section through second axis in **D**, showing *otx 2* expression in non-neural ectoderm and not in the neural tube. **F** *BF-1* expression is seen in the host but not in the second axis, even though the second axis is well-formed. **G** *HoxB1* expression throughout the majority of the second axis. In this embryo, both grafts were nodes. **H** *HoxB1* expression. In this case, the graft on the left is posterior streak. **I** Transverse section through second axis in **G**, showing *HoxB1* expression in neural tissue and adjacent non-neural ectoderm. Scale bar in **A** = 300μm for **A**, **B**, **D**, **F**, **G**; 750μm in **H**. Scale bar in **C** = 150μm for **C**, **E** and **I**.


A
Sox3
C
B
C
D
Otx2
E
E
F
BF-1
G
HoxB1
I
H
I

confined to non-neural ectoderm directly adjacent to the neural tube, and no neural expression of *otx2* (except maybe in the most anterior 50μm; 1 vibrotome section) was observed (Fig4.3E). Control grafts of posterior streak induced *otx 2* expression in only one case (1/41).

Chick *BF-1* expression was never seen in any second axis produced by a node graft (0/25). Nor was any expression seen associated with control grafts (0/25). However, all 25 host embryos showed good, strong *BF-1* expression (Fig4.3F).

In order to confirm whether more caudal levels of neural patterning were present, I examined chick *hox b1* expression in second axes. I found strong expression in 83% of second axes (34/41). This expression was throughout the majority of the axis (including the peg-like extrusion), with a small amount of tissue at the presumed anterior of the axis left blank (Fig4.3G,H). Sectioning revealed the expression to be in neural tissue, together with adjacent mesoderm and ectoderm (as it is in a normal embryo) (Fig4.3I). There was in fact very little neural tissue in the axis that did not express *hoxb1*. This is quite different from the expression of *hoxb1* in second axes following a chick node graft, where a much larger expanse of non-expressing tissue is seen at the anterior end of each axis (data not shown; see also Connolly *et al.*, 2000). 50% of the control, posterior streak grafts (8/15) also showed *hoxb1* expression.

4.3.4 Inducing ability of anterior visceral endoderm (AVE)

In the mouse, the anterior visceral endoderm (AVE) is known to be necessary for generating the most anterior parts of neural pattern (see Beddington and Robertson, 1998). I therefore examined the ability of the AVE to induce neural tissue and/ or a second axis. I found that an AVE graft (together with the underlying anterior neural plate) from a 7.25 dpc mouse embryo did not induce any structure in the majority of cases, and was visible as a cleared circular region within the host *area opaca* 24 hours after grafting. *Sox3*

expression was only observed in the vicinity of 3/16 AVE grafts, suggesting that the AVE is even poorer at inducing *sox3* expression in chick than the mouse node. In addition, the AVE did not appear to have any effect on the second axis induced by a mouse node. Combined grafts of node and AVE from a 7.25 dpc mouse were still unable to induce *BF-1* expression in the host (0/8).

However, it is likely that by 7.25 dpc the AVE has been displaced by anterior definitive endoderm, and now overlies extra-embryonic tissue. Therefore, I tried to examine the inducing and/or patterning ability of AVEs taken from younger mouse embryos (6.5 dpc). In a preliminary experiment, an AVE graft alone induced no structure, in the majority of cases (5/7), and appeared to have no patterning effect when co-grafted with a 6.5 dpc node (9 embryos).

4.4 Ability of transfected cells to induce neural tissue

In the mouse, *nodal* is initially expressed at low levels throughout the epiblast and overlying visceral endoderm, but becomes rapidly localised to the posterior epiblast, at the site where the streak will form (Varlet *et al.*, 1997). Homozygous mutant embryos lacking *nodal* expression cannot initiate streak formation and are arrested at the gastrulation stage of development (Conlon *et al.*, 1994). A similar early role for *Vg1* in streak formation has also been proposed, and *Vg1* has been shown to be a powerful inducer of both streak and organiser tissue in several organisms, including work conducted in our lab (Thomsen and Melton, 1993; Kessler and Melton, 1995; Watabe *et al.*, 1995; Cui *et al.*, 1996; Seleiro *et al.*, 1996; Shah *et al.*, 1997). I therefore tested the ability of mouse *nodal* and *cVg1* to induce neural tissue; I also tested *Xnr3*, a nodal-related family member shown to be a direct neural inducer in *Xenopus* (Hansen *et al.*, 1997).

A good rate of transient transfection of Cos 7 cells was achieved using the electroporation method (Fig4.4A). Western blots showed that the C-terminal processed proteins were effectively expressed from both *cVg1* and *nodal* constructs (Fig4.4B; see Methods for details of constructs). However, the *Xnr3* expression construct was not myc- tagged, so I was unable to prove that functional protein was being expressed.

I found that grafting either *cVg1*, *nodal* or *Xnr3* cells alone, to a lateral position just inside the *area pellucida* boundary of a HH st.3-4 host, was unable to cause ectopic induction of either *sox 3* or *otx 2* (Fig4.4C-E). Interestingly, grafts of *nodal* cells placed more centrally did appear to have an effect, and caused local expansion of the endogenous *sox 3* expression pattern (data not shown). This may have only been a transient effect, since grafts of mid and posterior streak (where *nodal* is expressed) are also able to induce *sox 3* expression transiently (see Fig4.1B).

The chick homologue of *nodal*, *cNR1*, was shown in the lab to have a very striking expression pattern (P. Towers, unpublished data). *cNR1* is expressed throughout the majority of the streak as it develops but is positively excluded from the node, with its expression always stopping sharply at the posterior limit of the node. The region of the streak just posterior to the node is proposed to play a role in inducing and/or maintaining node identity (Joubin and Stern, 1999). I therefore examined whether nodal signalling could positively maintain node character in neighbouring non-expressing cells. I used mouse *nodal* cells, since I did not have a *cNR1* expression construct. I found that *nodal* cells co-grafted with either a node or a piece of mid streak were unable to alter the response or gene expressions normally observed for such grafts. I also found that grafting *nodal* cells just anterior to the node neither enlarged nor reduced the endogenous area of *chordin* expression in the host (data not shown). Removing the area of streak just posterior to the node (*ie.* the anterior limit of *cNR1* expression), at the same time as grafting *nodal* cells anterior to the node, also had no effect on host *chordin* expression.

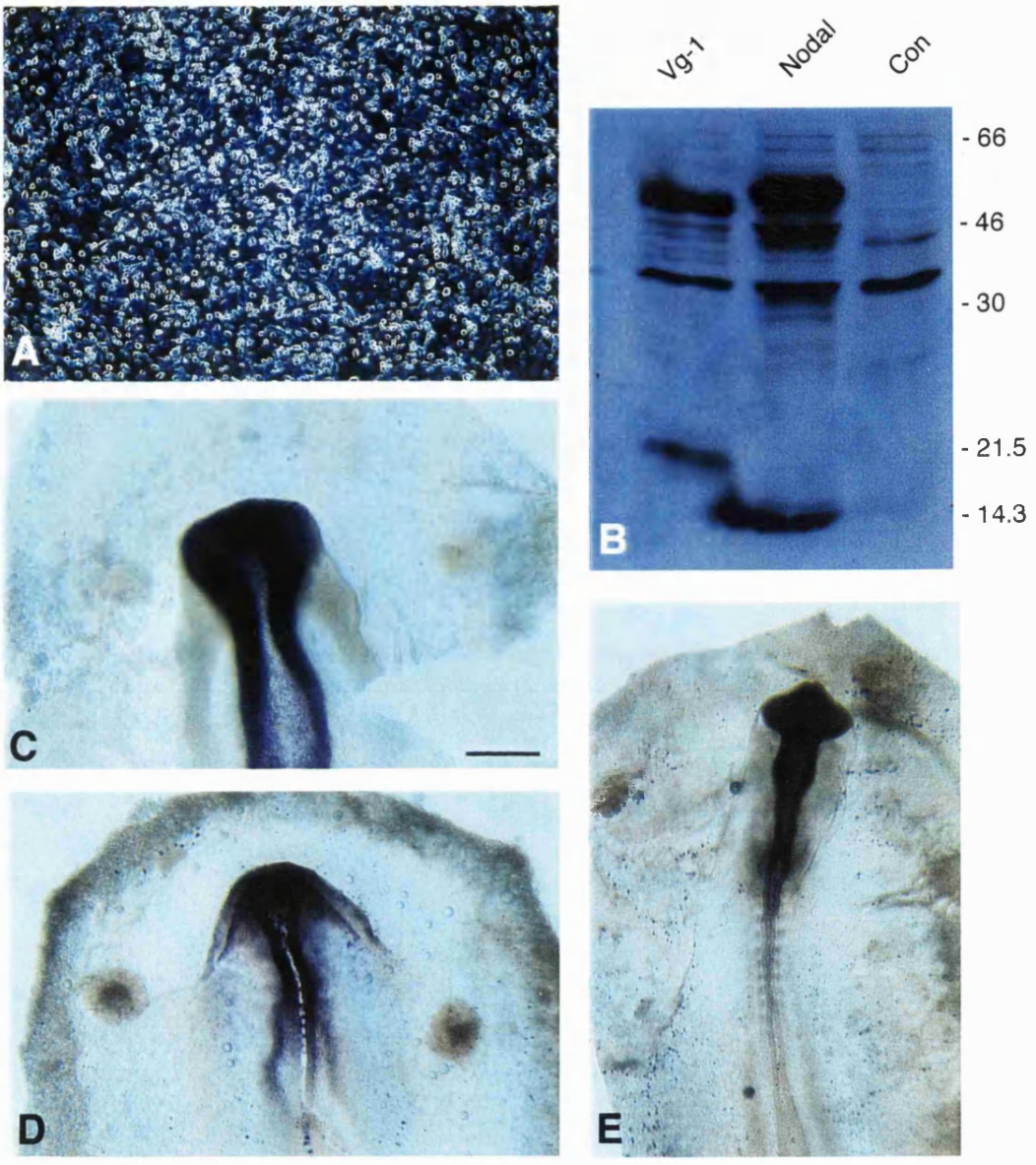


Figure 4.4 Lack of neural induction by grafts of transfected cells

A Detection of β galactosidase activity following transient transfection with *lac Z* construct. Approximately 40% of cells are blue, therefore expressing *lac Z*. **B** Western blot showing effective expression of processed protein from *Vg1* and *nodal* constructs. **C** No *sox 3* expression is induced by nodal cell grafts. **D** No *otx 2* expression is induced by nodal (left) and Xnr3 (right) cell grafts. **E** No *sox 3* expression is induced by Xnr3 cell grafts. Scale bar in **C** = 200μm in **C**, **D** and 400μm in **E**.

The removed area was quickly filled in, but the extent of *chordin* expression was not increased posteriorly down the streak and did not include the replacing cells (data not shown).

4.5 Analysis and discussion of results

The results presented in this chapter confirm previous findings (Storey *et al.*, 1992) that the chick node is able to induce a second axis with complete neural pattern. All markers tested, including the very restricted telencephalic markers *GANF* and *BF-1*, were expressed in every second axis produced. In addition, the experiments described enable me to directly compare the inducing ability of chick and mouse nodes, since the same responding tissue (chick epiblast) is used for both operations. Nodes from late streak mouse embryos were consistently able to induce second axes in the chick hosts; however molecular analysis revealed important differences in the patterning of these axes, as compared with those induced by chick nodes. Neural *otx2* expression was rarely seen in second axes induced by mouse node grafts, and *BF-1* expression was never seen, suggesting the absence of anterior neural domains. Examination of the posterior marker *hoxb1* further suggested the specific loss of anterior neural pattern. There was very little neural tissue in the axis induced by a mouse node graft that did not express *hoxb1*. This is markedly different from the expression pattern following a chick node graft, where large areas of non *hoxb1*-expressing neural tissue are present at the anterior end of each second axis.

Analysis of *sox3* expression following node grafts produced a very puzzling result. Strong *sox3* expression was observed throughout the second axes induced by a chick node graft. However, good *sox3* expression was only observed in less than 50% of second axes induced by a mouse node graft. Furthermore, there appeared to be no correlation between the size of the second axis and whether it expressed *sox3* or not.

Sox3 is a pan-neural gene (Rex *et al.*, 1997), so would presumably be expected to be expressed wherever any neural tissue is present. However, Streit and colleagues found that an older chick node (stage 4+ to 5) was unable to elicit expression of *sox2*, a closely related family member, in host tissue (Streit *et al.*, 1997). After stage 4, the chick node loses its ability to induce a complete neural axis, although it is still capable of inducing more posterior neural tissue until stage 7 (Storey *et al.*, 1992). These results therefore suggested that *sox2* could only be induced by nodes capable of inducing a full range of both anterior and posterior structures (Streit *et al.*, 1997). I found that the same was true for *sox3*; induction of *sox3* in neural tissue induced by an older chick node was poor. It is important to note that an older chick node together with its emerged prechordal mesoderm, could still elicit strong *sox3* expression. Emerged prechordal mesoderm is known to be able to recover some of the patterning properties of an older node (Foley *et al.*, 1997). Therefore, the poor induction of *sox3* following a mouse node graft can be explained by the inability of the mouse node to induce complete AP neural pattern. The reason for this strange phenomenon is very unclear. It suggests that full neural induction involves a series of steps and a hierarchy of molecular events, with localisation of *sox* genes to the nervous system being an early step in the cascade (Streit *et al.*, 1997).

Neural induction was achieved in approximately 70% of chick hosts following a graft of a mouse node (as judged by local ectoderm thickening, since *sox3* was a poor marker of neural induction in this case). However, neural induction is reported to occur in only 25% of cases following mouse node grafts into mouse hosts (Beddington, 1994; Tam and Steiner, 1999). This reduced incidence of induction may reflect the technical difficulties of the operation, given the small size of the mouse embryo. However, it may also be due to a suppressive activity emanating from the host node. In the chick, the node is proposed to have a suppressive influence on surrounding blastoderm tissues, since if the node is removed, surrounding tissue can reconstitute the organiser and its derivatives (Psychoyos and Stern, 1996). This regenerative ability is suppressed by the presence of a node in

blastoderm isolates (Yuan and Schoenwolf, 1998). Node grafts placed on the *area pellucida* margin are well away from the host node, and are presumably not influenced by it. However, in the mouse, the graft is placed much closer to the host node, due to the smaller size and different shape of the embryo. Any inhibitory effects of the host node would therefore be more pronounced in the mouse than in the chick. If this theory is correct, it suggests that the induction of a second axis by a node graft may be more effective in mouse host embryos that lack a functional node (Camus and Tam, 1999).

In mouse, the anterior visceral endoderm (AVE) is required for the formation of anterior structures (Beddington and Robertson, 1998), and the combination of AVE and node-derived signalling is proposed to be necessary for producing complete neural pattern (Shawlot *et al.*, 1999; Tam and Steiner, 1999). However, I found that the AVE, when grafted in combination with a mouse node, had no noticeable effect on the second axis produced, and these combined grafts were still unable to induce *BF-1* expression in the chick host. Klingensmith and colleagues also reported that the presence of the AVE produced no observable effects in recombinant cultures of mouse node and anterior ectoderm (Klingensmith *et al.*, 1999). In my experiments, the lack of effect may well be due to the age of the AVE. By late streak stages, the AVE has been displaced to overlie extra-embryonic tissues, and no longer expresses genes such as *hesx1* (Hermesz *et al.*, 1996; Thomas and Beddington, 1996). Therefore, the anterior signalling capacities of the AVE may well be over by the time that grafts were taken. More detailed analysis with the AVE from younger embryos (6.25 dpc) could address this question.

On its own, the AVE from prestreak or early streak mouse embryos appears unable to act as an organiser and does not display any inductive or organising activity (Tam and Steiner, 1999). Indeed, I also found very little response to an AVE graft; no structural effects in the host epiblast were observed, and *sox3* was present in less than 20% of cases (apparently lower than after a node graft). The primary role of the AVE may therefore be

to regulate the competence of the prospective anterior tissues, thereby augmenting the activity of node-derived signals in order to form complete anterior pattern. However, pre-streak rabbit AVE has been shown to induce *GANF* expression when grafted into chick hosts (Knoetgen *et al.*, 1999). This is the only case where the mammalian AVE has been reported to have a neural inducing ability, and may reflect the more robust nature and larger size of the rabbit tissue compared to the mouse. Alternatively, it is possible that responsiveness of the host tissue may differ between species. In the chick, signals for anterior neural pattern are thought to reside in the node and its derivatives, with any earlier signalling from the extra-embryonic hypoblast not essential. This suggests that the timing of head induction and development may occur much later in chick (and other vertebrates) than in mammalian embryos (Knoetgen *et al.*, 1999). Chick ectoderm may therefore remain responsive to anterior information from the AVE until a later stage than mouse host embryos. My results showing a lack of response of chick ectoderm to mouse AVE support the first alternative. However, it is likely that the mouse embryos used in my experiments were too old at the time the grafts were taken, to fully test the anterior signalling function of the AVE.

Despite all its remarkable inductive properties in ectopic grafts, the actual requirement for the organiser in the intact embryo is unclear, since surgical removal of the organiser has surprisingly little effect on the body plan. In the chick, the node is quickly reconstituted following its ablation, and can regenerate even after very large pieces of tissue are removed (Psychoyos and Stern, 1996), so it is very difficult to study development in the absence of a node. However, unique insights can be gained in the mouse, since null mutations in the *HNF3β* gene prevent node development (Ang and Rossant, 1994; Weinstein *et al.*, 1994). Embryos homozygous for targeted null alleles of *HNF3β* completely lack the node and its derivatives; nevertheless, 50% of such embryos develop a neural tube which displays AP pattern, with all neural markers expressed at

approximately their normal axial position (Ang and Rossant, 1994; Weinstein *et al.*, 1994; Klingensmith *et al.*, 1999). This phenotype suggests that the mouse node is not necessary for early AP neural patterning, and may instead be involved in localising and concentrating inducing signals at particular regions of the embryo (Klingensmith *et al.*, 1999). However, early regionalisation of the neural tube is not necessarily translated into later brain morphology; indeed, later brain structure is not complete in *HNF3β* mutants. The loss of forebrain regions seen in *noggin/chordin* double mutants (Bachiller *et al.*, 2000), as well as the phenotype observed in *lim1* or *hex* chimaeric embryos (Shawlot *et al.*, 1999; Martinez-Barbera *et al.*, 2000a) further suggest that the node and its derivatives are essential for maintaining and embellishing an initial labile state in anterior ectoderm. In this way, full maturation of neural patterning and brain morphology can be achieved.

It is interesting to note that chimeras comprising wild type extra-embryonic tissues and homozygous *HNF3β* -/- epiblast cells, form a normal primitive streak but still do not form a node (Dufort *et al.*, 1998). This suggests that formation of the primitive streak and a morphologically definable node may be separate events in mouse. In the chick embryo, it is also unclear whether separate mechanisms are involved in forming the streak and the node. Ectopic expression of *cVg1* in pre-streak blastoderms simultaneously induces the formation of a second primitive streak and the activation of organiser-specific genes (Seleiro *et al.*, 1996; Shah *et al.*, 1997). However, this may reflect two separate roles for *Vg1*-like signals, performed by different tissues. *Vg1* is thought to act, in concert with Wnt signalling pathways, as part of a conserved mechanism for the positioning and preliminary patterning of the vertebrate embryo axis, since it has been shown to be a powerful inducer of organiser tissue in several organisms (Thomsen and Melton, 1993; Kessler and Melton, 1995; Watabe *et al.*, 1995; Cui *et al.*, 1996; Seleiro *et al.*, 1996; Shah *et al.*, 1997). In chick, the posterior marginal zone, where functional Vg1 protein is first thought to be localised, is considered analogous to the *Xenopus* Nieuwkoop centre; it

acts before gastrulation to induce the organiser, but does not contribute any cells to it. However, a second, later node-inducing centre has also been identified in the streak, just posterior to the node (Joubin and Stern, 1999). This is made up of a different cell population from the posterior marginal zone, but also appears to use the Vg1 and Wnt signalling pathways (Joubin and Stern, 1999).

Early roles related to that proposed for *Vg1* are also suggested for genes of the *Nodal* group. In the mouse, *nodal* is initially expressed at low levels throughout the epiblast and overlying visceral endoderm, but becomes rapidly restricted to the posterior epiblast, at the site of primitive streak formation. As the streak elongates, *nodal* expression is rapidly down-regulated and is confined to the endoderm around the node (Varlet *et al.*, 1997). Homozygous mutant embryos lacking *nodal* expression cannot initiate streak formation and are arrested at the gastrulation stage of development (Conlon *et al.*, 1994).

As described in this chapter, I examined possible effects of nodal and Vg1 signalling by implanting grafts of transiently transfected Cos cells. I observed no noticeable effects when cells expressing mouse nodal or cVg1 proteins were implanted into the periphery of the *area pellucida* of stage 3+ to 4 hosts. The lack of streak induction is likely to be due to the age of the host and its loss of competence to respond, since ectopic formation of a streak or organiser following *Vg1* grafts is not observed in hosts after the very early primitive streak stage (Shah *et al.*, 1997). The lack of neural induction following grafts of *nodal* or *cVg1* cells is probably because of the position of the grafts. Joubin and Stern found that combined grafts of *Wnt1* and *Vg1* cells could induce *chordin* expression in 50% of cases if placed half way between the node and the lateral edge of a stage 4- host (Joubin and Stern, 1999); however, *chordin* was never induced if the same cells were grafted in the lateral periphery. This failure of more peripheral regions to respond to node-inducing signals is thought to be due to the presence of *BMP4* in peripheral ectoderm. Accordingly, the *BMP* inhibitor *noggin* can relieve the ability of host tissue to respond to

grafts of *Vg1* and *Wnt1* cells at the periphery (Joubin and Stern, 1999). Interestingly, *nodal* cells did appear to produce an effect in my experiments when placed more centrally; they caused local expansion of the endogenous *sox3* expression pattern. Continuation of these preliminary experiments may therefore yield some interesting results.

The chick homologue of *nodal*, *cNR1*, is expressed throughout the majority of the streak, but expression always stops sharply at the posterior limit of the node. This expression pattern is striking, given that the region of the streak just posterior to the node is proposed to play a role in inducing and/or maintaining node identity (Joubin and Stern, 1999). However, I found that grafting sources of ectopic nodal signalling, or removal of endogenous regions of *nodal*-expressing tissue, had no effect on *chordin* expression in the host node. It may have been that any potential effects were masked by the inhibitory influences that the node has on surrounding cells. The *BMP3*-related gene *ADMP* (anti-dorsalising morphogenetic protein) is expressed in the organiser yet, paradoxically, it inhibits the formation of dorsal structures, including those derived from the organiser (Moos *et al.*, 1995; Joubin and Stern, 1999). Indeed, *ADMP* reduces the induction of *chordin* by grafts of the streak 'node inducing centre' or *Vg1* and *Wnt1* cells (Joubin and Stern, 1999).

ADMP is also a candidate molecule for acting together with *BMPs* to induce the early epidermal marker *Dlx5*. Expression of *BMPs* in the periphery is thought to establish an epidermal identity in the ectoderm, thereby restricting the extension of the neural plate and refining the boundary between the neural and epidermal ectoderm (Pera *et al.*, 1999). It is therefore very interesting to note that a node graft not only induces an ectopic neural plate, but also induces a halo of epidermal ectoderm surrounding it. In my experiments, I observe a ring of *BMP4* induced at a distance from the grafted chick node. Similarly, other reports show a ring of *Dlx5* induced around the ectopic neural plate (Pera *et al.*, 1999).

Finally, *Xnr3* is unlike the rest of the nodal family members, since it induces neural tissue and cannot induce mesoderm (Hansen *et al.*, 1997). However, I found that *Xnr3*-expressing Cos cells were unable to induce neural tissue in chick embryos, when grafted to the periphery of the *area pellucida.* Although *Xnr3* has been shown to be a direct neural inducer in *Xenopus*, it is somewhat less potent than the 'classical' inducers such as *noggin* and *chordin* (Hansen *et al.*, 1997). Since *chordin* and *noggin* have each been shown to be insufficient for neural induction in the chick (Streit *et al.*, 1998; A.Streit and C.D.Stern, unpublished), it may not be surprising therefore, that *Xnr3* produced no response in chick. Interestingly, *Xnr3* has been shown to work with *Xwnt11* to cause neural induction in *Xenopus* (Glinka *et al.*, 1996). Co-grafting *Xnr3* cells with *Wnt*-expressing cells may therefore produce a more robust response in chick hosts.

CHAPTER V

CELL MOVEMENTS DURING THE FORMATION OF DEFINITIVE ENDODERM, AS FOLLOWED BY DiI LABELLING

5.1 Introduction

Gastrulation is a time when huge changes are taking place within the embryo. Cells of the single-layered epiblast are displaced and rearranged on a massive scale, in order to form the three definitive embryonic layers. During this time, therefore, positional relationships between cell populations are constantly changing, and intercellular signalling becomes extremely complex as communication is rapidly established and then lost, many times over. Studying patterns of gene expression during gastrulation, as documented in Chapter III, is very informative. However, it must be appreciated and remembered that these snapshots of gene expression are taken within the context of a developing embryo. Thus, patterns of expression that appear static, in the same region of an embryo at different stages, may in some cases be produced by a constantly changing, dynamic population of cells. As cells move into the appropriate domain of the embryo, transcription of a particular gene is initiated, only to be terminated if and when the cells leave that domain (for example, Joubin and Stern, 1999).

This chapter discusses the results of a study of cell movements within the chick embryo during gastrulation, in order to build up a more complete picture of events occuring in the embryo at this time. Cell movement was followed by injecting the fluorescent carbocyanine dye DiI onto very small regions of the embryo. This molecule is amphiphilic, allowing it to interact tightly with phospholipid bilayers. It can therefore be used as a cell tracer, since it remains restricted to the membranes of the cells initially labelled and their progeny.

DiI labelling was used to study cell movement within the endoderm layer, in particular. The definitive embryonic endoderm is known to be formed by cells moving down through the node and streak, and inserting themselves into the extra-embryonic hypoblast, thus pushing the hypoblast to the periphery of the *area pellucida* (Sanders *et al.*, 1978). It is believed that this process starts before the mesodermal layer is formed; however it is not known at what point cells stop becoming incorporated into the endoderm layer. The formation of axial mesoderm can easily be seen, and the relative movements of ectoderm and axial mesoderm have been well-documented (Dale *et al.*, 1999). However, it is not clear whether axial mesoderm is able to slide over the endoderm, or if the two layers move together. In the mouse, prechordal mesoderm and endoderm are thought to leave the node as a mixed population of cells, only later segregating into separate layers (Beddington, 1994; Tam and Behringer, 1997). Although this does not appear to be the case in the chick, it is still possible that transfer of information between endoderm and mesoderm may not just be by secreted molecules, but also by movement of actual cells. This chapter presents results that address all of the topics outlined above.

5.2 Cell movement in the lower layer between stages 4 and 5

In order to trace cell movement in the lower layer, embryos were set up in ring culture so that the endoderm was now uppermost and directly accessible for labelling. A small pulse of DiI was applied to the surface of the embryo at a particular point. Control embryos were fixed immediately, in order to assess the reproducibility of DiI position

at the outset. Remaining embryos were cultured further, for up to 6 hours, and the position of labelled cells recorded under fluorescent light.

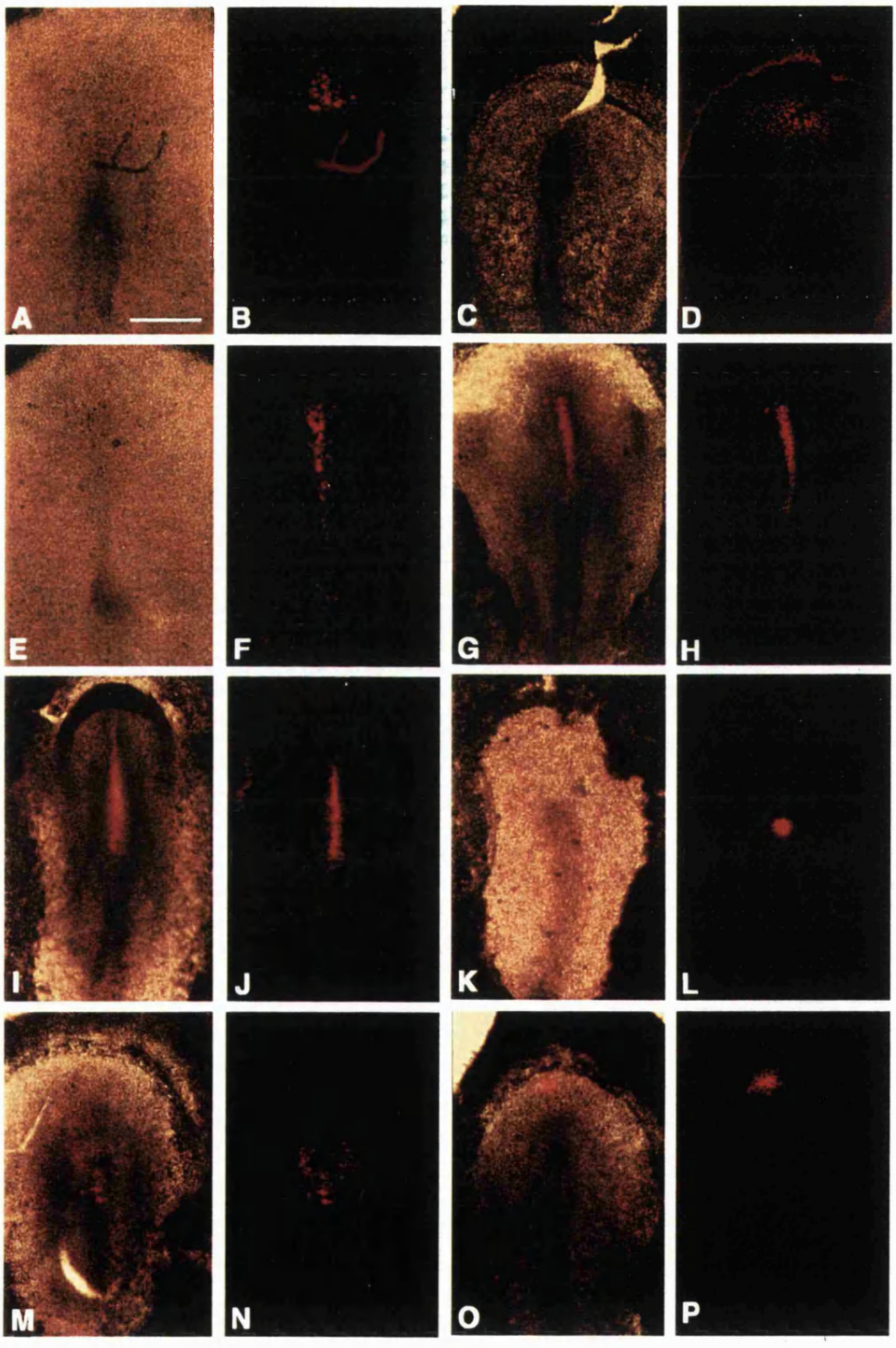
5.2.1 Labelling the node at stage 4

In the majority of cases, the pulse of DiI was applied to endoderm cells directly overlying the node, at stage 4. Control embryos, fixed immediately after labelling, showed that in about 60% of cases (18/29) the spot of DiI was extremely localised and well within the shoulders of the node (Fig5.1K,L). In the other embryos (11/29) the labelled patch was slightly larger and included some surrounding cells, although still obviously centred on the node. Control embryos were sectioned to ensure that labelling was confined to the endoderm layer. In addition, some control embryos were cultured for up to an hour, to confirm that the patch of DiI did not expand by diffusion or any means other than cell movement.

All other labelled embryos were cultured further, for approximately 6 hours, by which point they had reached a range of different stages. In those embryos which were now late stage 4/4+, labelled cells were found scattered in the region anterior to the node (12/14 embryos; Fig5.1A-D). The labelled cells were no longer in a clump but had spread and appeared to have mixed with unlabelled cells. However, all labelled cells had moved anterior to the node, leaving the cells over the node itself blank. Sectioning confirmed that the labelled cells were in the endoderm layer. In addition, removal of the anterior endoderm, six hours after labelling over the node, was sufficient to remove all labelled cells from this region (in 5/5 cases), while some fluorescence could still be seen in the endoderm surrounding the area removed.

Figure 5.1 Cell movement in the lower layer between stages 4 and 5

Embryos viewed from the ventral aspect, anterior to the top. Bright field and corresponding fluorescent images are shown for each embryo. **A- D** Two stage 4+ embryos, 6 hours after labelling the node. Labelled cells are scattered in the endoderm anterior to the node. **E- H** Two stage 5 embryos, 6 hours after labelling the node. Labelled cells are restricted to the midline, but have extended in an AP direction, in a region closely correlating with the emerged axial mesoderm. **I-J** Stage 7 embryo, 6 hours after labelling the node. Labelled cells are found extending from just behind the headfold, back along the midline. **K- L** Stage 4 embryo, immediately after labelling, showing that label is initially extremely localised and well within the shoulders of the node. **M- N** Stage 5 embryo, 6 hours after labelling posterior to the node. Labelled cells are found in bull's horns shapes extending from the node, leaving the midline area immediately ahead of the node unlabelled. **O- P** Stage 4+ embryo, 6 hours after labelling half way between the node and the anterior of the embryo. Labelled cells are found right on the anterior boundary of the *area pellucida*. Scale bar in **A** = 300μm for **A**, **B**, **E**, **F**; 650μm for **C**, **D**, **K- P** and 800μm for **G- J**.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Some embryos had reached stage 5 after the short incubation. In these embryos, labelled cells were much less scattered and were instead restricted to the midline (Fig5.1E-H). They had, however, extended in an anteroposterior direction, and were seen in a region closely correlating with the emerged prechordal mesoderm and head process (12/15 embryos). Labelled cells were found overlying the prechordal mesoderm in all cases, although extending back along the head process to variable degrees. The cells directly over the node were unlabelled in most cases, and labelling was never seen posterior to the node.

Finally, the oldest set of embryos had reached the 1-2 somite stage by the time of fixation. Once again labelled cells were found close to the midline, and had extended in an AP direction. In 11/15 embryos, labelled cells were found extending from just behind the headfold back along the midline (Fig5.1I,J), while in 3/15 cases the labelled cells were found more posteriorly, at the level of the somites, although still anterior to the regressing node. In these latter embryos, the fact that labelled cells were not found as far anteriorly is likely to be because these embryos were the oldest at the time of operating, and could have been just beyond stage 4. Therefore, the anterior cells may have already moved slightly ahead of the node, and so were not labelled.

5.2.2 Labelling anterior to the node at stage 4

The movement of lower layer cells anterior to the node was also analysed using DiI. Patches of cells in the midline, half way between the stage 4 node and the anterior of the embryo, were labelled (n=4). Six hours later, these cells were found to be lying right on the anterior boundary of the *area opaca/ area pellucida*, well ahead of any

axial mesoderm that had emerged (Fig5.1O,P). The group of labelled cells tended to become elongated along the boundary. Interestingly, cells labelled in this anterior margin at stage 4 were later found to have moved even further anterior and were lying over the *area opaca.*

5.2.3 Labelling just posterior to the node at stage 4

In order to determine whether the above results indicated a global anterior movement of lower layer cells, pulses of DiI were applied just posterior to the node at stage 4. Following 6 hours further incubation, all embryos (n=5) had reached stage 5 and labelled cells were found in and around the node and anterior streak. Interestingly, the area of labelled cells had extended anteriorly to some extent, but only in more lateral regions. Thus, labelled cells were found in diagonal lines or bull's horns shapes extending from the node, while the midline area immediately ahead of the node was largely unlabelled (Fig5.1M,N).

5.3 Fate mapping of stage 5 lower layer

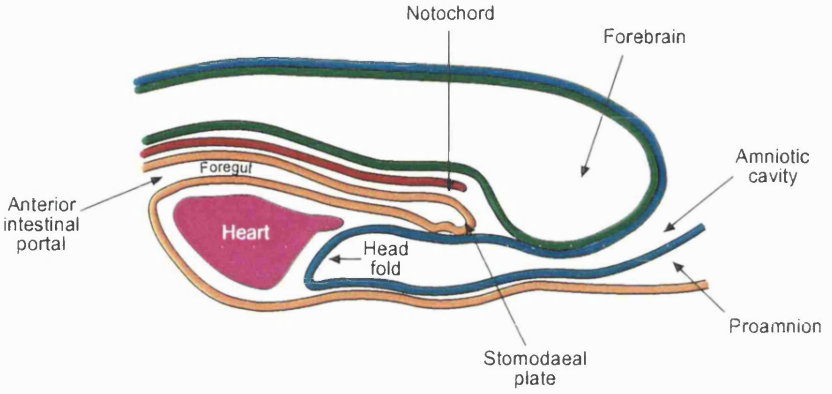
The definitive endoderm is formed during gastrulation by node-derived cells inserting themselves into the hypoblast layer, displacing the hypoblast to the edge of the *area pellucida*, where it contributes to extra-embryonic structures. This means that the lower layer in the anterior part of the *area pellucida* consists of two types of cell, which not only have different origins but also have different fates. I labelled groups of lower layer cells at particular positions in stage 4+ to 6 embryos, and documented their location at the 12 somite stage. I was therefore able to construct a rough fate map for

Figure 5.2 Fate mapping of stage 5 lower layer

A Schematic sagittal section of a 9 somite embryo, anterior to the right, showing arrangement of tissue in the head region. Colours of cell layers are as follows: blue = head ectoderm, green = neural ectoderm, red = axial mesoderm, yellow = endoderm.

B- J Sagittal cryostat sections of embryos with patches of endoderm cells labelled using DiI at stage 4+ to 6; anterior to the right. **B** 3 somite embryo; two patches of cells were labelled at stage 5. Cells on the anterior edge of the *area pellucida* end up in extra-embryonic endoderm, while cells in the prechordal region end up within the headfold pocket. **C, D** Cells labelled half way between the prechordal fan and the anterior margin of the *area pellucida* at stage 5 are within the headfold pocket at the 3 somite stage (**C**), but end up in ventral head ectoderm and extra-embryonic endoderm at the 12 somite stage (**D**). **E, F** Fluorescent and light field images again showing labelled cells in the ventral, extra-embryonic endoderm of a 12 somite embryo, following pulse of DiI half way between the prechordal fan and the anterior margin of the *area pellucida* at stage 5. **G** 12 somite embryo; labelled cells primarily in the foregut and also ventral head ectoderm following pulse of DiI over prechordal plate at stage 5. **H** 12 somite embryo; cells labelled half way between the prechordal fan and anterior *area pellucida* margin at stage 5- end up in the ventral foregut, just inside the boundary between embryonic and extra-embryonic tissue. **I, J** Fluorescent and light field images showing labelled cells within the foregut of a 12 somite embryo, following pulse of DiI over prechordal plate at stage 6. Scale bar = 200μm throughout.

Panel **A** is modified from Bellairs and Osmond, 1998.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Stomodaeal plate

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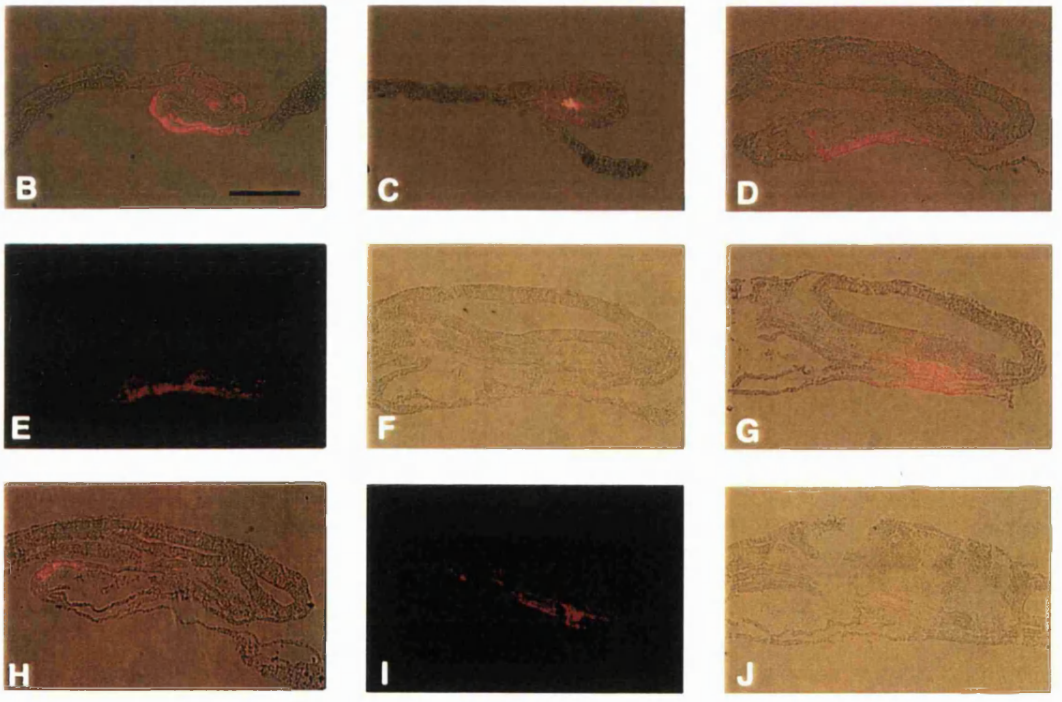
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the stage 4+ to 6 lower layer, allowing me to assess the extent to which the hypoblast had been replaced by definitive endoderm, and to draw an approximate boundary between the location of these two cell populations.

Lower layer cells at the anterior periphery of the *area pellucida*, following labelling at all stages examined (st.4+ to 6; 8 embryos) were later found in the extra-embryonic yolk sac endoderm (Fig5.2B). Cells labelled half way between the prechordal mesoderm fan and the anterior *area pellucida* margin at stage 5, were observed within the headfold pocket at the 3 somite stage (Fig5.2C), but also tended to end up in the yolk sac endoderm by the 12 somite stage (9 embryos; Fig5.2D-F). In these embryos, labelled cells were found stretching along the midline of the yolk sac endoderm, right back to the anterior intestinal portal; they were therefore very close to the junction of extra-embryonic and embryonic domains. Interestingly, cells labelled half way between the prechordal mesoderm fan and the anterior *area pellucida* margin at stage 5- were later observed in the posterior foregut floor (2 embryos), therefore just inside the boundary between embryonic and extra-embryonic domains (Fig5.2H). However, cells labelled half way between the node and the anterior margin at stage 4+ were already in the extra-embryonic endoderm peripheral to the headfold by the 3 somite stage. Finally, cells labelled directly over the prechordal mesoderm fan at all stages examined (st.4+ to 6) were observed within the headfold pocket at the 3 somite stage (Fig5.2C), and within the foregut at the 12 somite stage (4 embryos; Fig5.2G,I,J). As with all embryos, the population of labelled cells had expanded considerably along the AP axis of the head. Any labelled cells identified in the ventral head ectoderm after

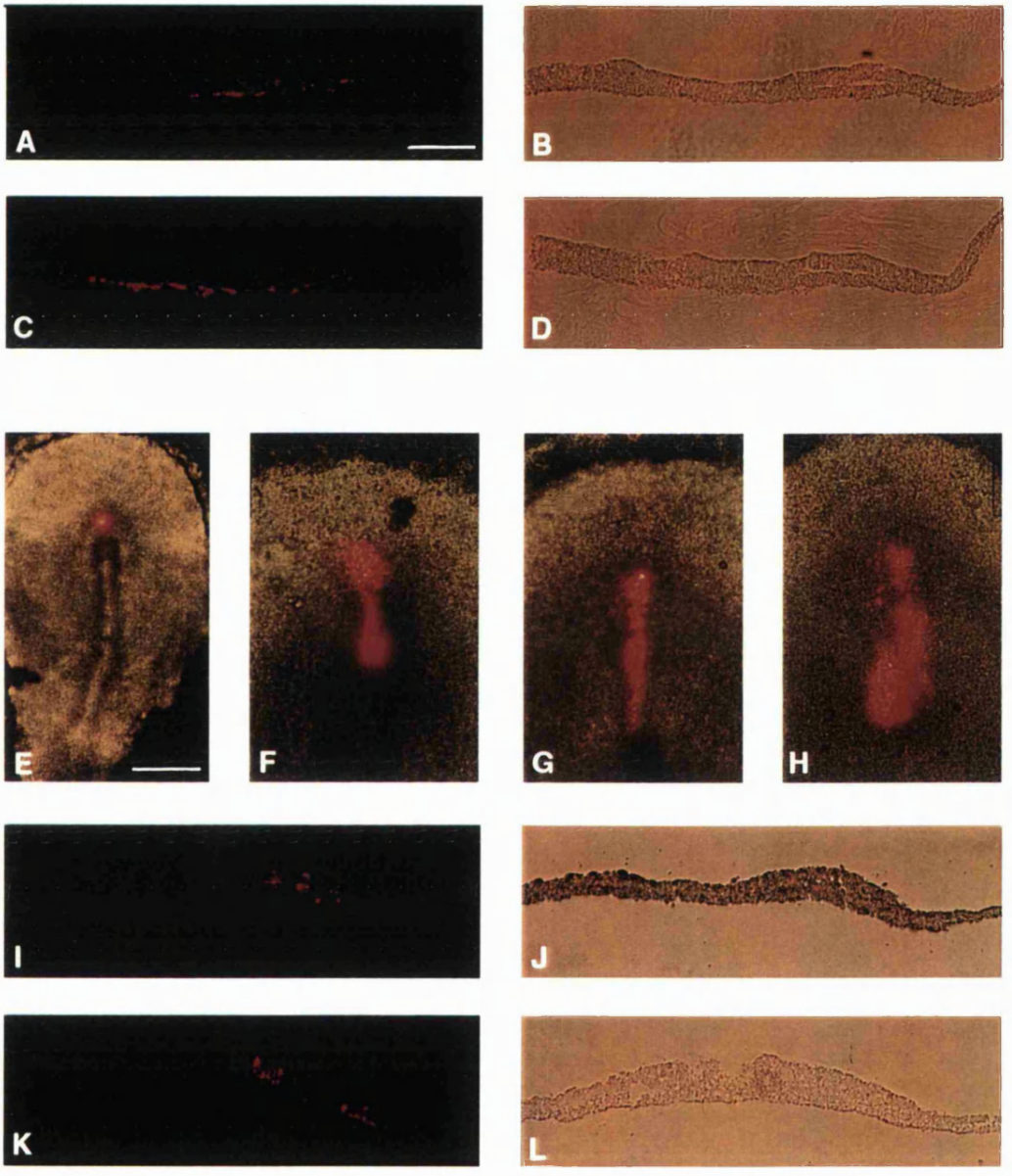
sectioning, were probably due to penetration of the DiI pulse through the lower layer, and directly onto the ectoderm anterior to the neural plate (Fig5.2G).

5.4 Mixing of cells between endoderm and axial mesoderm

As already described, cells labelled over the node at stage 4 were later found closely associated with the midline in stage 5 embryos, restricted to the region overlying the prechordal mesoderm and head process (5.2.1). Cells in the midline endoderm and mesoderm layers therefore appear to show similar patterns of movement at these stages. Initial analysis indicated that some labelled cells were also found in the mesoderm layer, and suggested that this was more prevalent towards the anterior of the embryo. Additional embryos were therefore labelled at the node at stage 4, and sagittal sections of the midline region cut at stage 5. In some embryos, labelled cells were found in both endoderm and mesoderm throughout the axis. These embryos were therefore discounted as it was thought that the initial pulse of DiI must have penetrated into both layers at the outset. However, in the remaining embryos, DiI was obviously confined predominantly to the endoderm layer. In these cases, labelled cells were found in the middle layer, specifically in the prechordal region, at the anterior limit of the axial mesoderm (Fig5.3A-D). These anterior cells therefore appear to have moved from the endoderm and integrated into the prechordal mesoderm, suggesting that some mixing of cells between these closely apposed layers can occur as they extend.

Figure 5.3 Cell mixing and relative movement of endoderm and axial mesoderm

A - D Fluorescent and corresponding bright field images of two stage 5 embryos; sagittal sections with anterior to the right. Endoderm cells in the node region were labelled at stage 4, and the embryos cultured further while the head process extended. Labelled cells can now be seen in the mesoderm layer specifically in the prechordal region, but are confined to endoderm in the rest of the axis. **E** Wholemount ventral view of stage 4+ embryo, showing patch of DiI applied to all cell layers just ahead of the node (fixed immediately after labelling). **F- G** Wholemount ventral views showing location of labelled cells after 3 hours further culture. A patch of more superficial cells (in focus) can be seen anterior to the rest of the deeper labelling. **I- L** Fluorescent and corresponding light field images of two of the stage 5 embryos shown in **F- H** ; sagittal sections with anterior to the right. Sections reveal that DiI had actually penetrated all three embryonic layers. Labelled cells in the endomesoderm are always found anterior to those in the ectoderm. However, no obvious difference can be distinguished between the location of labelling in endoderm and mesoderm layers. Scale bar in **A** = 150μm for **A- D, I- L**. Scale bar in **E** = 600μm in **E** and 250μm for **F- H**.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5.5 Relative movement of endoderm and axial mesoderm layers

DiI labelling was also used to investigate whether the axial mesoderm moves over a stationary endoderm layer as it extends, or whether the two layers move in register away from the node, with cell relationships remaining relatively constant. Thus, both endoderm and mesoderm layers were labelled at the same AP position. To do this, the injection procedure was altered slightly; the needle was moved to the vertical position by bending the glass in a Bunsen flame, and the pressure increased so that label was deliberately pushed through the endoderm and into the mesoderm. The region just ahead of the node at stage 4+ was labelled (Fig5.3E), and embryos were fixed after about 3 hours further incubation, when the head process had elongated. Sagittal sections of the midline region were then cut with a cryostat and the relative position of labelled cells in the two layers recorded. Upon observation of the inverted wholemounts, a patch of more superficial labelled cells could be seen anterior to the rest of the deeper labelling (Fig5.3F-H). However, sections revealed that in fact the DiI had penetrated all three embryonic layers. In every case, labelled cells in the endomesoderm were anterior to those in the ectoderm (Fig5.3I-L). However, no difference in the location of labelling could be observed between the endoderm and mesoderm, suggesting that these two layers move in concert in the midline during these stages.

5.6 Analysis and discussion of results

As presented in this chapter, focal injections of DiI provide very useful information on cell movements in the early embryo, and allow us to gain insight into possible interactions between the definitive embryonic layers at this time. During stage 4 and 4+, endoderm cells in the node region are seen to move anteriorly and spread out, mixing with other unlabelled cells. This suggests that definitive endoderm cells are still leaving the node at this stage and integrating into the lower layer, pushing the hypoblast right to the periphery of the *area pellucida.* Studies using homotopic grafts of thymidine-labelled cells also indicate that, although significant movements of streak-derived cells into the endoderm layer start as early as stage 2, cells still appear to be moving into the endoderm at late stage 4 (Rosenquist, 1972). The anterior movement of cells from the node region during streak stages is thought to be part of an ordered migration of endoderm cells in a radial direction away from a centre in the primitive streak, located just behind the node (Stern and Ireland, 1981).

Despite the continued emigration of node cells into the endoderm, my results show that the pattern of cell movements in the endoderm layer changes after stage 4+. Labelled cells are no longer seen to spread out, but remain restricted to the midline. This change in cell behaviour correlates with the emergence of axial mesoderm and the onset of node regression. It could therefore be that as prechordal mesoderm and head process cells leave the node, a population of midline endodermal cells also leave the node and move in concert with the mesoderm. Alternatively, the elongation of the labelled endodermal population along the AP axis could be due to the convergent

extension movements, which re-shape the entire embryo at this stage. However, it does appear that cells in the endodermal midline exhibit particular behaviour distinct from the rest of the endodermal population. Cells labelled posterior to the node at stage 4 are found in anterolateral positions as the node starts to regress, forming diagonal lines or bull's horns shapes; these cells are not found in the midline anterior to the node.

In addition, the experiments described here that address the relative movement of the embryonic layers, suggest that the endoderm and axial mesoderm move in concert. No difference in the location of labelled cells could be observed between the midline mesoderm and endoderm, as they extended away from the node during stages 4+ and 5. However, in all cases, the mesendoderm labelling was found to be anterior to that in the ectoderm, suggesting that the lower tissues slide together under the ectoderm during these stages. These results are in agreement with Dale and colleagues, who find that the prechordal mesoderm migrates in advance of the forebrain ectoderm, reaching its rostral-most location by stage 6 (Dale *et al.*, 1999). In contrast, neuroectoderm cells do not reach their rostral position until later, therefore passing over the prechordal mesoderm between stages 5 and 8. The marked disparity between mesendoderm and ectoderm labelling observed in my experiments at stage 5 may therefore have been reduced again by stage 8.

In the mouse embryo, the definitive endoderm and axial mesoderm are thought to emerge from the node as a mixed population of cells, only later segregating into separate layers (Beddington, 1994; Tam and Behringer, 1997). In the chick, the

endoderm and axial mesoderm appear to form defined layers as they first emerge from the node (Chapter VI; Sanders *et al.*, 1978; Stern and Ireland, 1981). However, as shown here, it does appear that cells from the endoderm can subsequently become incorporated into the central layer, specifically in the prechordal region. This suggests that transfer of information between these closely apposed anterior tissues may be by cell movement, as well as by local secretion of signalling molecules. It is interesting that cell integration was only observed in the prechordal region, and not further posteriorly. This could be because the endoderm and mesoderm are more intimately associated in the prechordal region, as observed when removing the endoderm layer (see Chapter VI). Alternatively, the prechordal mesoderm cells display different characteristics from the head process; the looser, spreading behaviour of the prechordal mesoderm may allow integration of endoderm cells, unlike the tighter, rod-like arrangement of the head process.

The structure of the prechordal region has been studied in detail, using electron microscopy (Seifert *et al.*, 1993). It is proposed that the prechordal mesoderm is formed as part of the mesodermal layer during gastrulation. However, just anterior to this, the thin epithelial cells of the endoderm layer become columnar, and stretch right up to the ventral surface of the neural plate. This region is defined by Seifert as the prechordal plate, and these columnar cells will later form the thickened endoderm at the very anterior of the foregut. It is therefore possible (although unlikely) that the proposed cell mixing I observe is in fact labelling of these thickened, columnar endodermal cells, just anterior to the limit of the prechordal mesoderm.

The fate mapping studies of the endoderm at stage 4+ to 6 presented here, show that the more central region of endoderm, closely surrounding the prechordal mesoderm and head process, contributes to the foregut. In contrast, the more anterior, peripheral area ends up in the extra-embryonic yolk sac endoderm at the 12 somite stage. These results agree with other detailed fate-mapping studies of the endoderm, previously carried out using carbon markings (Bellairs, 1953a). Bellairs found that at stage 4, the region of prospective foregut endoderm is centred around the node. By stage 5, the foregut endoderm domain appears to extend further anteriorly, but is still a comparable region in that it roughly corresponds with the anterior limit of invaginated mesoderm. My results suggest that the middle of the region between the prechordal mesoderm and the anterior margin, is very close to the boundary between embryonic (foregut) and extra-embryonic (yolk sac) endoderm populations. Cells labelled at this mid-point at stage 5- end up on the foregut side of the boundary, whereas those labelled at stage 5 and 5+ end up on the extra-embryonic side. This is likely to be due to the fact that a slightly more central region is labelled at stage 5-; by stage 5 the head process is longer and the prechordal mesoderm is closer to the anterior end of the embryo, because of the convergent-extension movements.

Bellairs also used carbon markings on embryos in culture to document the morphogenetic movements of the endoderm in the early stages of foregut formation (Bellairs, 1953b). The most anterior tip of the foregut is believed to be formed as a pocket between two sets of opposing movements in the endoderm. The endoderm beneath the head process is thought to move anteriorly, resulting in the accumulation of more endoderm at the anterior end of the head process than can be accommodated

in a single flattened sheet. This therefore contributes to formation of the headfold, bending ventrally at the anterior end of the embryo. The thin medial endoderm, closely associated with the axial mesoderm, remains dorsal in the foregut, while the thickened endoderm makes up the most anterior end of the gut. More lateral anterior endoderm is also thickened; these cells swing in an obliquely backward and downward movement to form the ventral floor of the foregut. These latter movements also result in the formation of folds at right angles to their direction, so that the horse-shoe shaped ridge of the anterior intestinal portal forms, gradually spreading backwards to enclose the anterior foregut.

It has been shown that cells do not roll in medially over the ridges of the anterior intestinal portal. Instead, the lateral ridges move as a whole toward the midline, where they fuse to form the foregut floor. This becomes separated from the extra-embryonic *area pellucida* endoderm, which also becomes continuous and forms the yolk sac endoderm, ventral to the head (Bellairs, 1953b). In this way, two initially continuous cell populations become divided into two entirely separate cell layers. In the results presented here, two distinct groups of labelled endoderm cells can be seen at the 12 somite stage, after applying DiI close to the boundary of the prospective foregut endoderm at stage 5. This segregation is therefore likely to be caused by the morphogenetic movements described above. The presence of labelled cells in the ventral head ectoderm is probably due to initial penetration of the DiI pulse through the thin endoderm layer and directly onto the non-neural ectoderm anterior to the neural plate. However, the observation that more labelled endoderm cells tend to end up in extra-embryonic tissues suggests that the endoderm moves anteriorly in relation

to the ectoderm shortly after labelling (also described above; Bellairs, 1953b). This further supported by the observation that endoderm cells initially labelled in the same position can end up within the headfold pocket at 3 somite stages, but in extra-embryonic yolk sac endoderm at 12 somites.

CHAPTER VI

LACK OF FOREBRAIN REGIONALISATION FOLLOWING REMOVAL OF ANTERIOR DEFINITIVE ENDODERM

6.1 Introduction

As already discussed in Chapter IV of this thesis, differences in the location of anterior brain patterning signals are emerging between mouse and chick. The chick node can induce a nervous system with complete pattern, whereas the mouse node is unable to do so, and produces a nervous system lacking forebrain and midbrain structures. Furthermore, a separate signalling centre, the AVE, has been identified in mouse that is proposed to be necessary for generating the most anterior parts of neural pattern (reviewed in Beddington and Robertson, 1998). Studies involving removal of the equivalent tissue in chick, the anterior hypoblast, suggest that it does not play a role similar to the AVE (Knoetgen *et al.*, 1999; S.Withington *et al.* in preparation).

However, node-derived tissues are essential in both species for complete anterior development. Analysis of chimaeric mouse embryos shows that both *lim1* and *otx2* are sequentially required in extra-embryonic and embryonic tissues to achieve normal anterior development (Rhinn *et al.*, 1998; Shawlot *et al.*, 1999). In addition, *noggin/chordin* double mutant embryos have been found to lack extensive forebrain regions (Bachiller *et al.*, 2000). These organiser factors are not expressed in the AVE, further highlighting the necessity for node-derived signalling in full anterior patterning. In chick, prechordal mesendoderm clearly displays anterior patterning signals, and can induce forebrain markers when grafted adjacent to the presumptive hindbrain of a host embryo (Foley *et al.*, 1997). It therefore appears that multiple signalling/ organising centres may work in combination to produce and refine a complete neural pattern. Even in the mouse, node-derived tissues seem essential to maintain and reinforce an initial anterior identity established by the AVE (Thomas and Beddington, 1996; Shawlot *et al.*, 1999).

The definitive embryonic endoderm is formed by node and anterior streak-derived cells inserting themselves into the hypoblast and gradually pushing it out of the way, towards the edge of the *area pellucida*. Given the geographical location of such cells, it is clearly possible for the endoderm to signal directly to the overlying neural ectoderm and/or the axial mesoderm as it is laid down. However, few studies have examined in detail the role of the definitive endoderm in anterior neural patterning during this time period. Recent analysis of the *hex* mutant mouse has provided compelling evidence for a role of the definitive endoderm in anterior brain formation (Martinez-Barbera *et al.*, 2000a). Here, I have investigated the role of the definitive endoderm by removing a defined sector of it from stage 4 (full length streak) through to stage 6 embryos, while leaving axial mesoderm intact. This chapter discusses the effects of such removals, in particular those concerning anterior brain patterning.

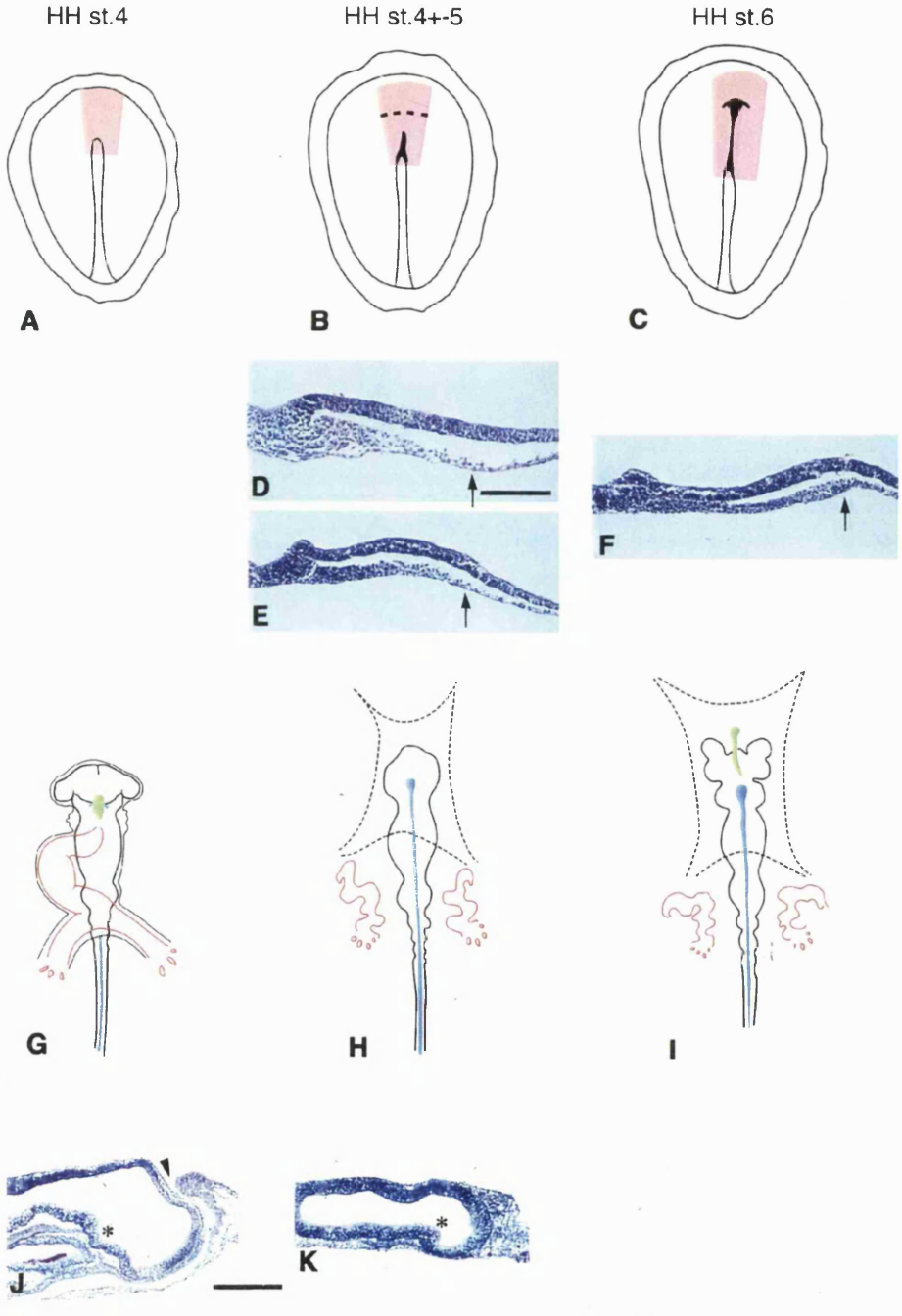
6.2 Experimental design for lower layer removal and overview of syndrome observed

It was possible to remove just endoderm cells, while leaving all axial and paraxial mesoderm intact, since the embryos were set up in ring culture. In this way, the tissues of the embryo were reversed, leaving endoderm on the upper surface and directly accessible to manipulation. The endodermal layer was gradually loosened and peeled back using needles. The embryos were then cultured further, until appropriate stages when brain morphology could be assayed by *in situ* hybridisation for various marker genes or candidate patterning molecules.

Figure 6.1 shows the area of tissue removed at each stage. This covered a region extending from the anterior *area opaca/ area pellucida* boundary back, over all axial mesoderm that had already emerged, to the node. The area removed therefore comprises mainly definitive endoderm of the future foregut (see Chapter V). Anterior to this, it also

Figure 6.1 Endoderm removal operation and stage specificity of effects

A- **C** Schematic views of operation, viewed from the ventral aspect as set up in ring culture. Area of lower layer removed is indicated in pink in stage 4 (**A**), stage 4+ to 5 (**B**) and stage 6 (**C**) embryos. Dashed line in **B** indicates outer limit for a subset of more restricted removals performed in a subset of embryos. **D**- **F** Wax sagittal sections of head process, anterior to right and regressing node on the left. Arrows mark the anterior limit of prechordal mesoderm. **D**, **E** Continuous lower layer can clearly be seen and can be removed intact at these stages (4+ to 5). **F** Closer apposition of endoderm and mesoderm layers, and a continuous endoderm layer is no longer clearly resolvable at this stage, especially anteriorly (stage 6). **G**- **I** Schematic ventral views of 12 somite embryos, following operations at stage 4, 4+ to 5 and 6, respectively. Embryo shown in **G** is normal. Solid black line = outline of brain, showing simple expanded prosencephalon in **H**, but forebrain subdivisions and eye cups present in **I**. Dashed line = remaining window of absent lower layer, including foregut endoderm. Red = heart rudiments, which remain laterally and separated in **H** and **I**. Green = stomodaeal ectoderm, absent from **H** and forming a pipe extending from an anterior position back beneath the brain in **I**. Dark blue = prechordal mesoderm and notochord. Pale blue = somites. **J** and **K** Wax sagittal sections of 12 somite embryos after stage 4 and 4+ to 5 endoderm removals, respectively. Asterisk indicates ventral depression in infundibular region. Arrowhead marks morphological division between diencephalic and telencephalic rudiments. **K** shows considerable loss of dorsal forebrain patterning and expansion, absence of foregut and head ectoderm does not bend ventrally, but remains extended ahead of the brain. Prechordal mesoderm is still present. Scale bar in **D** = 175μm in **D**- **F**. Scale bar in **J** = 350μm in **J**, **K**.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includes the region over which heart mesoderm migrates towards the midline. In addition, the hepatogenic endoderm maps to the extreme of this region at this stage, eventually coming to lie more posteriorly and ventrally following headfold formation. It is likely that, particularly in the stage 4 hosts, the area removed also included some anterior hypoblast and extra-embryonic endoderm at the periphery.

I found it possible to remove the endoderm as a complete layer up until stage 5+. After this point, it became increasingly more difficult to identify separable endodermal and mesodermal layers as the tissues were so closely integrated, particularly in the prechordal region. This can clearly be seen in wax saggital sections of embryos at these various stages (Fig6.1D-F; arrows indicate anterior limit of prechordal mesoderm). In addition, as soon as the headfold started to form, it was impossible to create the tension in the embryo necessary for clean separation of the layers.

Removal of such a sector of endoderm produced three main effects. Firstly, no foregut tunnel was formed, since these cells were physically removed by the manipulation. Secondly, the precardiac mesoderm was unable to migrate towards the midline and remained laterally as two separate regions, developing to eventually form two beating structures. As a result, the normal ventral structures of the embryo were unable to form and the embryo remained opened out, with the notochord still exposed on the ventral side. The brain was also abnormally flattened out, due to lack of headfold formation and therefore subsequent flexion. Finally, but most importantly for the purposes of this study, these embryos showed pattern deletion within the forebrain. There was frequently loss of telencephalon and eye cups, with formation instead of a basic prosencephalon that lacked any subregionalisation. This syndrome is described in more detail in the remainder of this chapter.

6.3 Stage specificity for producing effects on anterior neural pattern

After performing significant numbers of lower layer removals, it became apparent that the stage of the embryo was critical for producing the effects outlined above (see Fig6.1). If the lower layer was removed at stage 4, no apparent effect was observed, and the majority of embryos (166/221; 75%) developed normally. However, endoderm removal between stage 4+ and 5, therefore just as the first axial mesoderm cells were being laid down and extending into a full head process, produced all aspects of the syndrome in the majority of cases (171/235; 73%). Interestingly, in the cases where endoderm was successfully removed cleanly between stage 5+ and 6, forebrain pattern appeared to be complete, even though the embryos still lacked a foregut tunnel and the heart remained laterally. The forebrain patterning defects seen after stage 4+ and 5 removals are therefore not just disrupted morphology due to the brain remaining flattened out. In essence, the period during which endoderm removal causes defects in subsequent forebrain regionalisation is restricted to stages 4+ and 5.

6.3.1 Immediate response to lower layer removals

In order to examine why most embryos developed normally following removal at stage 4, embryos were fixed either immediately or a very short time after operations (Fig6.2). As shown in Fig6.2A-D, within about 6 hours of further culture, the hole in the endoderm layer had been reduced to almost nothing. The exposed area appeared to be filled in from the posterior end (node region) first, moving anteriorly so that before any prechordal mesoderm had left the node, the neural ectoderm was once again covered by endoderm. This was not the case following a stage 5 removal, however (Fig6.2E-G). The hole in the endoderm layer did not close up to any great extent (although it did change shape due to convergent extension movements of the embryo), so that any axial mesoderm that had left the node prior to the operation remained permanently exposed.

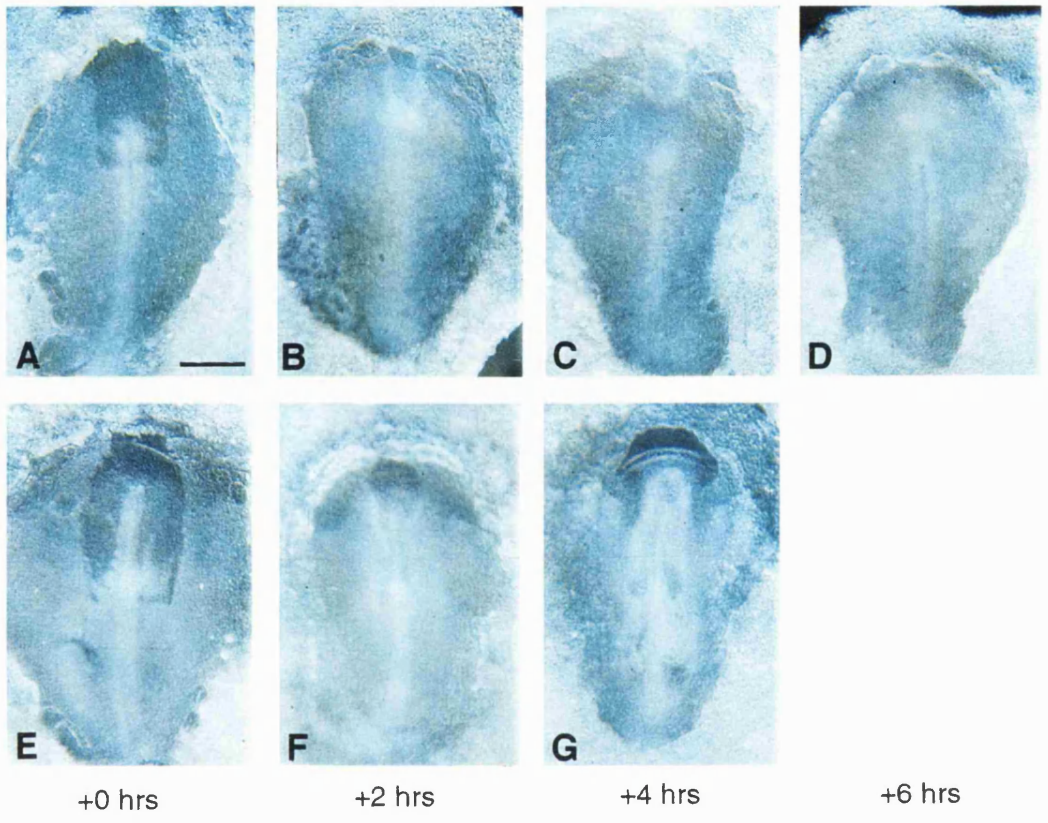


Figure 6.2 Behaviour of lower layer after operations

Dark field views of embryos from the ventral aspect, as set up in ring culture, fixed shortly after lower later removals. **A- D** Embryos fixed at successive timepoints following removal of lower layer at stage 4. Window of absent lower layer quickly closes up, from the posterior end (node region), so that by the time any prechordal mesoderm emerges, the lower layer is again almost complete (**D**). **E- G** Embryos fixed at successive timepoints following removal of lower layer at stage 5. The window of absent lower layer remains, so that all axial mesoderm emerged prior to the operation remains abnormally exposed and lacks contact with foregut endoderm. Scale bar = 650μm throughout.

As the node continued to regress, it moved back under the remaining endoderm, so the more posterior mesoderm laid down later was closely apposed by endoderm as normal.

6.3.2 Cell movements confirmed by DiI labelling

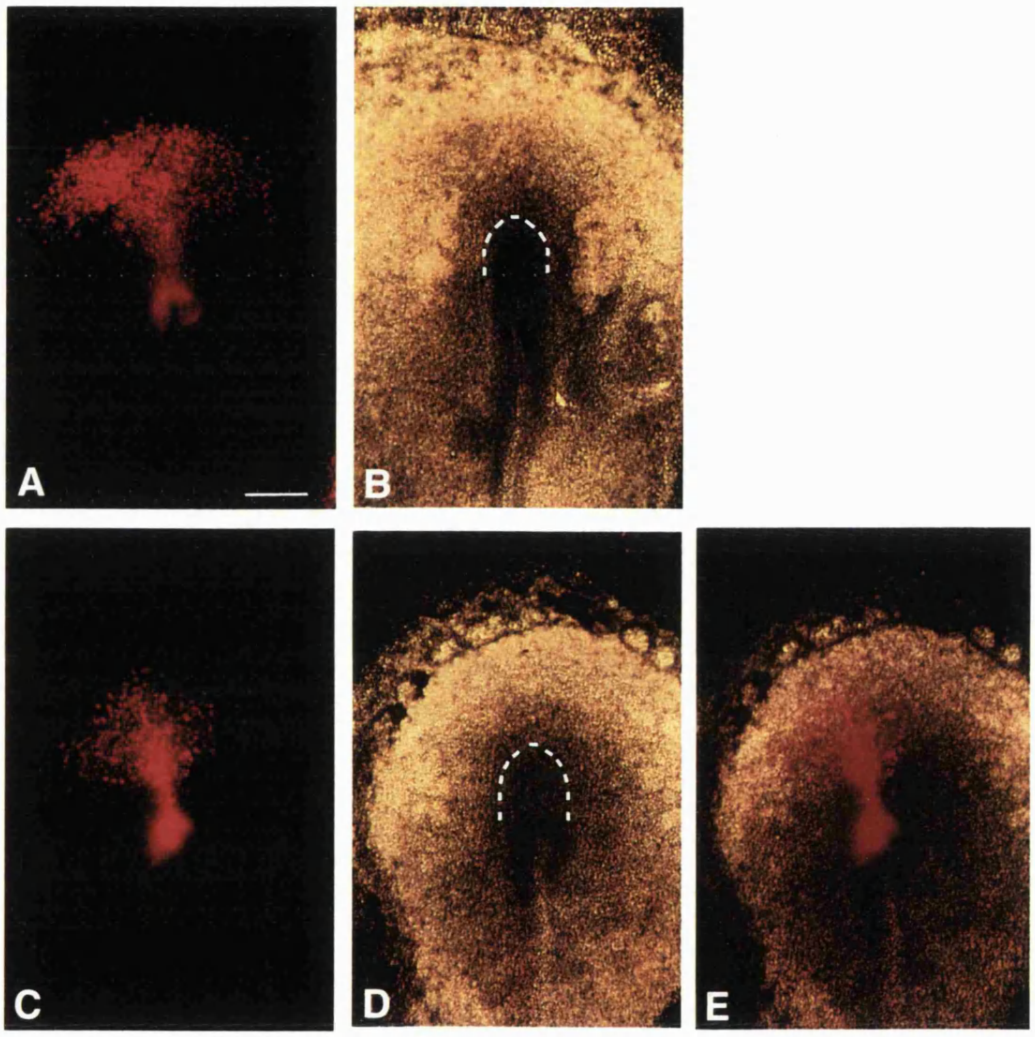
The origin of cells that move to fill in the gap of endoderm following a stage 4 removal, was examined further using DiI labelling. Lower layer was removed as usual and DiI was then injected onto the exposed 'mesodermal' cells of the node. The embryos were subsequently cultured for about 6 hours, until the lower layer was again complete. As shown in Fig6.3, labelled cells were now found in the endoderm anterior to the node, *i.e.* part of the region that had filled in the area removed. This suggests that cells were continuing to move down through the node into the endoderm layer after the operation was performed, and so healing of the exposed area was observed. My labelling studies on normal embryos support the hypothesis that node cells are normally still entering the endoderm at this stage (see Fig5.1); however, response to the removal operation may also be a contributory factor.

6.3.3 Gene expressions in definitive endoderm

Endodermal gene expressions were examined a short time after lower layer removal, to assess for restoration of normal patterns. *Hex* is a homeobox gene that plays an important role in anterior brain patterning in mouse. It is first expressed in the mouse AVE, but chimaeric analysis shows that the essential requirement for *hex* function in forebrain patterning resides in the definitive endoderm (Martinez-Barbera *et al.*, 2000a). In the chick, *hex* is also seen in the hypoblast at early stages, being swept to the periphery as the cells are moved by intercalation of definitive endoderm (Yatskievych *et al.*, 1999). From stage 5 onwards, expression is seen in a restricted patch of endoderm directly underlying the prechordal mesoderm, and an anterior crescent of pharyngeal endoderm that later becomes associated with the forming heart tube (Fig6.4A). *Hex* expression in the prechordal region may also extend to mesoderm cells, although this

Figure 6.3 Cell movements immediately after lower layer removal

Immediately after lower layer removal at stage 4, the deeper 'mesodermal' layer of the node was labelled with DiI. Embryos were cultured further for 6 hours, during which time healing of the window in absent lower layer was mostly completed. **A, B** Fluorescent and dark field images of the same embryo (now stage 4+). **C - E** Fluorescent, dark field and combined images of the same embryo (also now sage 4+). Labelled cells now populate the now intact lower layer around and ahead of the node. This area is much more extensive than that occupied by the newly emerging prechordal mesoderm (indicated by dotted white lines on **B** and **D**). Scale bar = 300μm throughout.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has been difficult to record decisively on saggital sections (see also Yatskievych *et al.*, 1999). Following lower layer removal at stage 4, *hex* expression is fully restored by headfold stages (Fig6.4B). However, endoderm removal between stages 4+ and 5 leads to permanent loss of *hex* expression in 8/10 cases (Fig6.4C,H). In addition, the complete absence of prechordal expression in these embryos suggests that any normal mesodermal *hex* expression, whether imported by cell intercalation or induced by underlying endoderm, has also been prevented. Therefore, conditions of endoderm removal that lead to forebrain pattern loss clearly result in failure to recover *hex* expression.

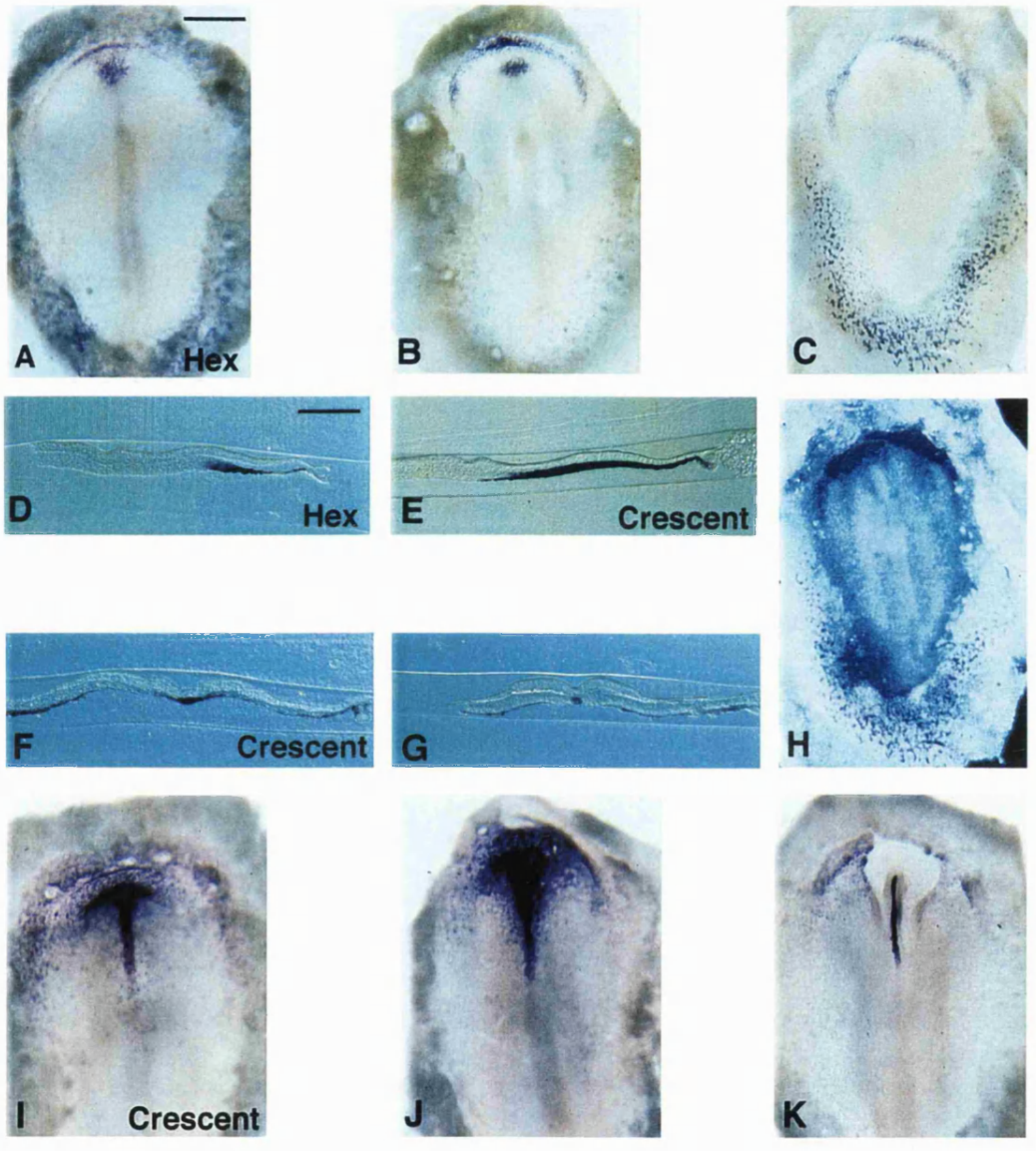
Crescent is a chick *Frzb* homologue strongly expressed in the hypoblast before gastrulation, and later in anterior definitive endoderm and head process mesoderm (Fig6.4I,F; Pfeffer *et al.*, 1997). *Frzb* family members are secreted proteins thought to play a role in head formation, since they can act as local antagonists of the *Wnt* signalling pathway (Leyns *et al.*, 1997; Wang *et al.*, 1997; Niehrs, 1999). *Crescent* is expressed in a broad, intense domain in the definitive endoderm, centred on but more widespread than the site of *hex* expression. Seen in plan view, the area of *crescent* expression at stage 4+ and 5 closely correlates with the region of endoderm that causes forebrain pattern deletions when removed. Indeed, I find that when removals are carried out at stage 4, normal *crescent* expression is restored in the endoderm by headfold stages (Fig6.4J). However, following a stage 4+ to 5 removal, the great majority of endodermal *crescent* expression is absent, and only axial mesodermal expression remains (8/8 embryos; Fig6.4G,K).

6.4 Loss of forebrain gene expression following endoderm removal

Following removal operations, embryos were cultured until appropriate stages when brain morphology and pattern could be assayed by *in situ* hybridisation for various

Figure 6.4 Gene expressions in headfold stage definitive endoderm

All wholemounts are viewed form the ventral aspect, anterior to the top. **A** Normal *hex* expression at stage 5+. **B** Full restoration of *hex* expression by stage 5+, following lower layer removal at stage 4. **C** Central domain of *hex* expression in prechordal region is completely absent, several hours after endoderm removal at stage 4+ to 5. **D, E** Sagittal sections along the midline of a normal stage 5 embryo, anterior to the right and node on the left, showing *hex* and *crescent* expression, respectively. *Hex* expression is seen in the very anterior endoderm, and also in the endoderm and probably mesoderm (see text) of the prechordal region (**D**). *Crescent* expression is seen in all emerged axial mesoderm, and in endoderm stretching right to the periphery of the *area pellucida* (**E**). **F** and **G** Transverse sections showing *crescent* expression in endoderm and head process of a normal embryo (**F**) and an embryo following endoderm removal at stage 4+ to 5 (**G**; level of section shown in **K**). **H** Dark field image of embryo in **C**, showing that prechordal mesoderm structure is intact, even though *hex* expression is absent in this region. **I** Normal *crescent* expression at stage 6. **J** Full restoration of *crescent* expression by stage 6, following lower layer removal at stage 4. **K** Large area of *crecent*-expressing endoderm remains absent several hours after endoderm removal at stage 4+ to 5. Scale bar in **A** = 500μm in **A- C, H, K**; 350μm in **I, J**. Scale bar in **D** = 250μm in **D- G**.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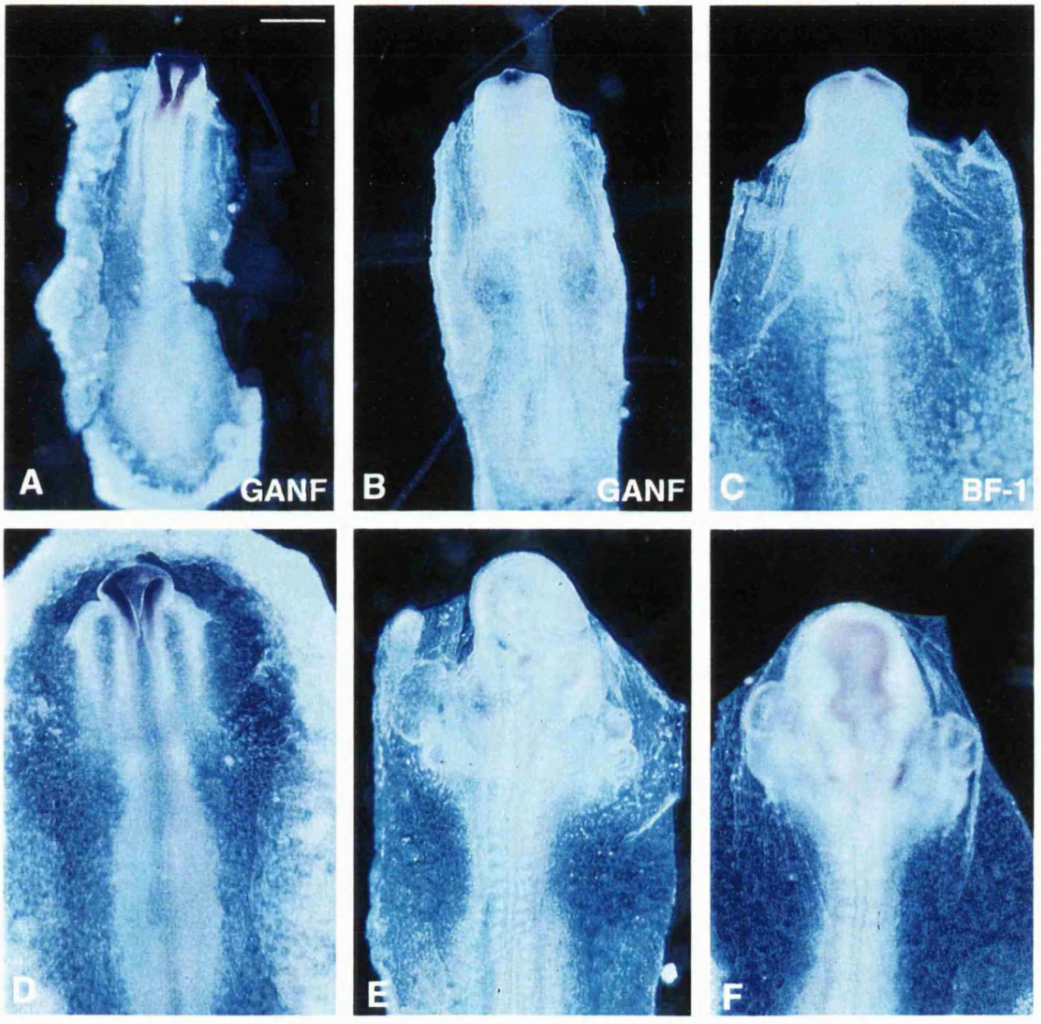
marker genes or candidate signalling molecules. Analysis at the 12 somite stage was chosen as an informative end-point, since at this stage the forebrain normally shows a distinctive T-shaped morphology due to lateral expansion of the telencephalon and eye cups. Analysis of *otx 2* expression at the 12 somite stage, a gene expressed throughout the forebrain and midbrain (Fig3.2), highlights the loss of anterior brain structure following stage 4+ to 5 removals (Fig6.7E-I). However, the anteroposterior extent of *otx 2* expression remained pretty normal following all operations. More specific forebrain markers were therefore also examined.

GANF, the chick homologue of the mouse homeobox gene *hesx 1,* is first expressed in the anterior neural plate at stage 4+. By the 12 somite stage, expression is normally observed in the telencephalon and in ventral head ectoderm (see normal expression chapter; Fig3.3F,G,K). Following lower layer removal at stage 4, this normal pattern of *GANF* expression was seen (Fig6.5B). However, *GANF* expression was not maintained in those embryos showing patterning defects; by the 12 somite stage, neural *GANF* expression was completely lost or reduced in 88% of embryos (22/25) following stage 4+ to 5 endoderm removal (Fig6.5E).

The winged helix transcription factor gene *BF-1* shows normal early expression restricted to telencephalon and medial eye cup regions (Alvarez *et al.*, 1998), and is known to be essential for regulating cell proliferation and formation of forebrain architecture (Dou *et al.*, 1999). Normal *BF-1* expression was seen in the majority of embryos following lower layer removal at stage 4 (Fig6.5C; compare with normal expression Fig3.5E,F,I). However, in a manner similar to *GANF*, *BF-1* expression was either completely lost or reduced in 87% of cases (13/15) following removal between stage 4+ and 5 (Fig6.5F).

Figure 6.5 ***GANF and BF-1*** **expression following endoderm removal**

Dorsal views of wholemount embryos, anterior to the top. **A**, **D** 4 somite embryos showing normal *GANF* expression after endoderm removal at stage 4 (**A**) and recapitulation of this normal pattern following endoderm removal at stage 4+ to 5 (**D**). **B**, **E** 12 somite embryos showing normal *GANF* expression after stage 4 endoderm removal (**B**) and complete absence of *GANF* expression following endoderm removal at stage 4+ to 5 (**E**). **C**, **F** 12 somite embryos showing normal *BF-1* expression after endoderm removal at stage 4 (**C**) and complete absence of *BF-1* expression following endoderm removal at stage 4+ to 5 (**F**). Scale bar in **A** = 450μm for **A**, **B** and 300μm for **C**- **F**.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The gene encoding the secreted growth factor *FGF 8* also shows a restricted patch of expression in the telencephalon at the 12 somite stage (see Fig3.4E,F). Normal telencephalic expression of *FGF 8* was observed in the majority of embryos following endoderm removal at stage 4 (Fig6.6A). Once again, however, this expression was specifically lost or reduced in size in most embryos (75%; 12/16) after stage 4+ to 5 endoderm removals (Fig6.6B). Note that all other aspects of *FGF 8* expression, in the midbrain/ hindbrain boundary and neural crest, remained unaffected.

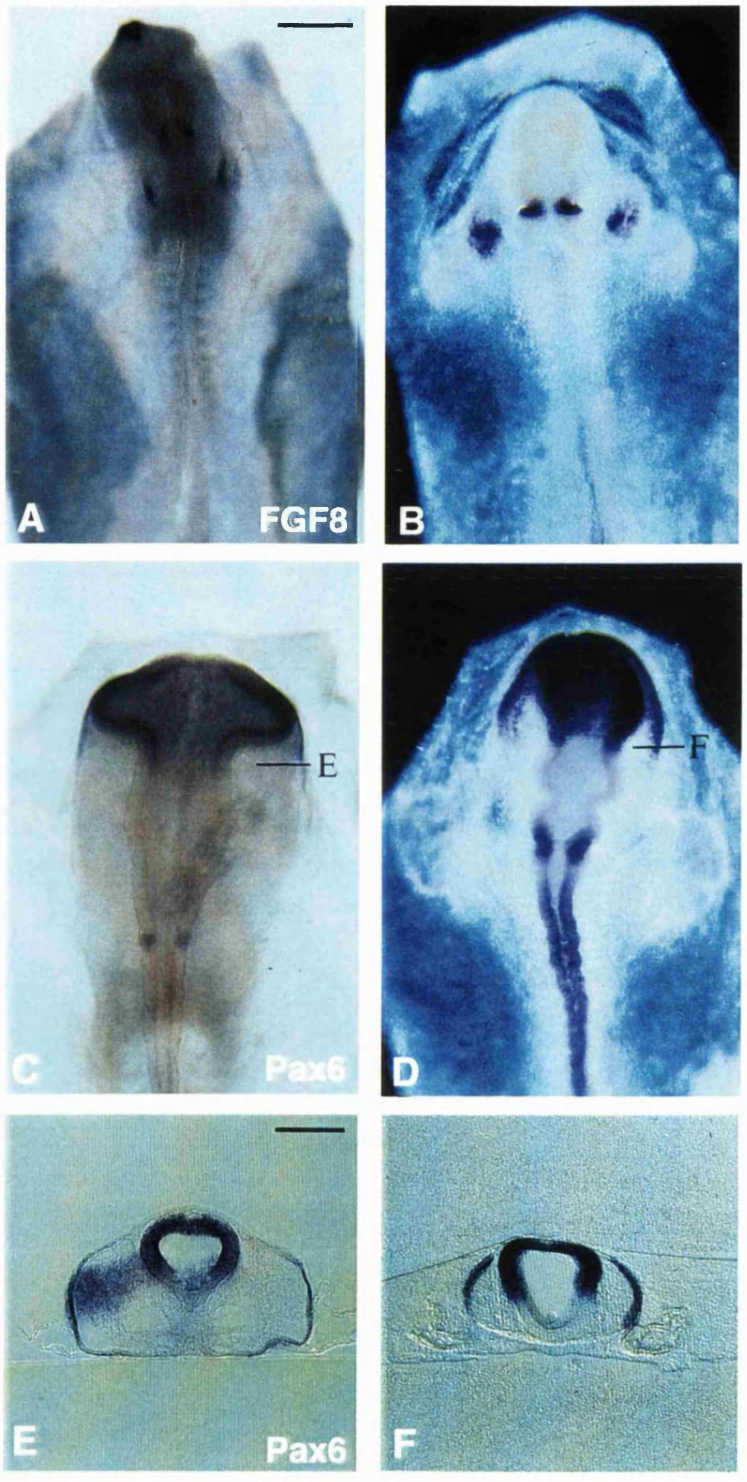
The homeobox gene *pax 6* is initially expressed throughout the dorsal aspect of the prosencephalon, but later becomes progressively stronger laterally as the telencephalon and eye cups expand, and is down-regulated at the dorsal midline (see Fig3.6). Interestingly, by the 12 somite stage, *pax 6* has an expression pattern complementary to *BF-1* and *FGF 8*, since it is excluded from the anterior neural folds of the telencephalon but is expressed throughout the rest of the prosencephalon. Following a stage 4 lower layer removal, normal *pax 6* expression was observed at the 12 somite stage (Fig6.6C). However, after removing endoderm at stage 4+ to 5, *pax 6* expression was seen throughout the basic forebrain vesicle and was not down-regulated in the dorso-anterior midline (11/11 embryos; Fig6.6D). *Pax 6* expression was not expanded ventrally, however, consistent with the presence of normal inhibitory signals from ventral midline (Fig6.6E,F).

6.5 Early patterning of the anterior neural plate after endoderm removal

It was necessary to determine whether the loss of anterior brain regions following endoderm removal resulted from an overall reduction in neural induction, or from failure to correctly pattern a normal-sized neural plate. Therefore, gene expressions were also analysed at stage 7, just a few hours after operations, and at 5 somites, a stage when the anterior neural ridge is thought to play an important role in forebrain formation

Figure 6.6 ***FGF 8* and *Pax 6* expression after endoderm removal**

Dorsal views of wholemount embryos, anterior to the top. **A**, **B** 12 somite embryos showing normal *FGF 8* expression after endoderm removal at stage 4 (**A**) and specific loss of telencephalic *FGF 8* expression following endoderm removal at stage 4+ to 5 (**B**). Note that all other aspects of *FGF 8* expression remain normal in these embryos. **C**, **D** 12 somite embryos showing normal *pax 6* expression after stage 4 endoderm removal, with expression excluded from the most anterior neural folds (**C**). Following endoderm removal at stage 4+ to 5, *pax 6* expression is seen throughout the basic forebrain vesicle and is not down-regulated in the dorsal midline of anterior neural folds (**D**). **E**, **F** Transverse sections through 12 somite embryos, at the levels shown in **C** and **D**. *Pax 6* expression is not expanded ventrally following endoderm removal at stage 4+ to 5 (**F**) and remains absent from the ventral midline, as normal (**E**). Scale bar in **A** = 300μm for **A**, **B**, **D** and 150μm for **C**. Scale bar in **E** = 150μm for **E** and 200μm for **F**.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F

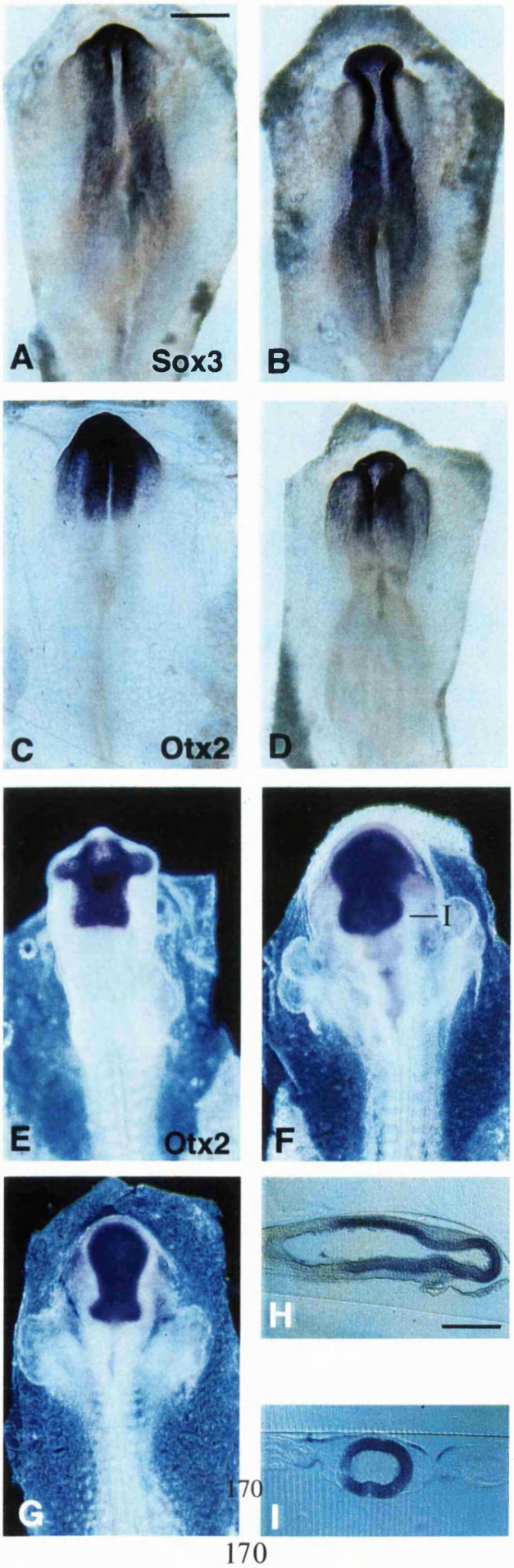
(Shimamura and Rubenstein, 1997). The extent of expression of the pan-neural gene *sox 3* was found to be normal at stage 7, whether the removal was performed at stage 4 or between stage 4+ and 5 (Fig6.7A,B; compare with normal exp. Fig3.1). Thus initial neural induction, a process likely to be well underway by late stage 4, was not affected by the removal operations.

Otx 2 is expressed in anterior neuroectoderm from very early stages, and its continued expression is essential for development of forebrain and midbrain structures (Fig3.2). At stage 7, the extent of *otx 2* expression in the anterior neural plate was normal, following removals at all stages (Fig6.7C,D). *GANF* expression is first observed in the anterior neural plate at stage 4+, therefore also at a stage just prior to those of effective endoderm removals. At stage 7, *GANF* expression was normal following endoderm removals both at stage 4 or later (Fig6.5A,D). Following endoderm removal at stage 4+ to 5, 82% of embryos (9/11) showed normal *GANF* expression at stage 7, even though *GANF* expression was absent by 12 somites (Fig6.5E). It therefore appears that the prospective forebrain region is induced as normal following endoderm removal, but this character is not properly maintained and does not become subregionalised.

FGF 8 is first expressed in chick at the 5 somite stage, and its expression in the anterior neural ridge (ANR) about this time in the mouse has been implicated in induction and maintenance of *BF-1*, therefore acting as an important signalling centre for telencephalon formation (Shimamura and Rubenstein, 1997). I found that early *FGF 8* expression in the ANR was reduced or completely absent at the 5 somite stage in 73% of cases (16/22), following endoderm removal at stage 4+ to 5. The deficits in forebrain pattern observed here therefore correlate with a loss of *FGF 8* signalling from the ANR.

Figure 6.7 ***Sox 3*** **and** ***Otx 2*** **expression following endoderm removal**

Dorsal views of wholemount embryos, anterior to the top. **A**, **B** Stage 7 embryos showing the normal extent of *sox 3* expression after endoderm removal at stage 4 (**A**) and normal development of this expression pattern following endoderm removal at stage 4+ to 5 (**B**). **C**, **D** Stage 7 embryos showing the normal extent of *otx 2* expression (**C**) and development of this normal expression following endoderm removal at stage 4+ to 5 (**D**). **E**, **F**, **G** 12 somite embryos showing normal *otx 2* expression in forebrain and midbrain following endoderm removal at stage 4 (**E**) and maintenance of the normal extent of expression after stage 4+ to 5 endoderm removal (**F**, **G**). Anterior brain structure is obviously incomplete, however, in these latter embryos. **H**, **I** Sagittal and transverse sections through 12 somite embryos, following endoderm removal at stage 4+ to 5. *Otx 2* expression is not affected by the removal operation, although brain shape appears abnormally flattened out. Scale bar in **A** = 700μm for **A**- **D** and 300μm for **E**- **G**. Scale bar in **H** = 300μm for **H** and **I**.



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6.6 Autonomous specification of stomodaeal ectoderm

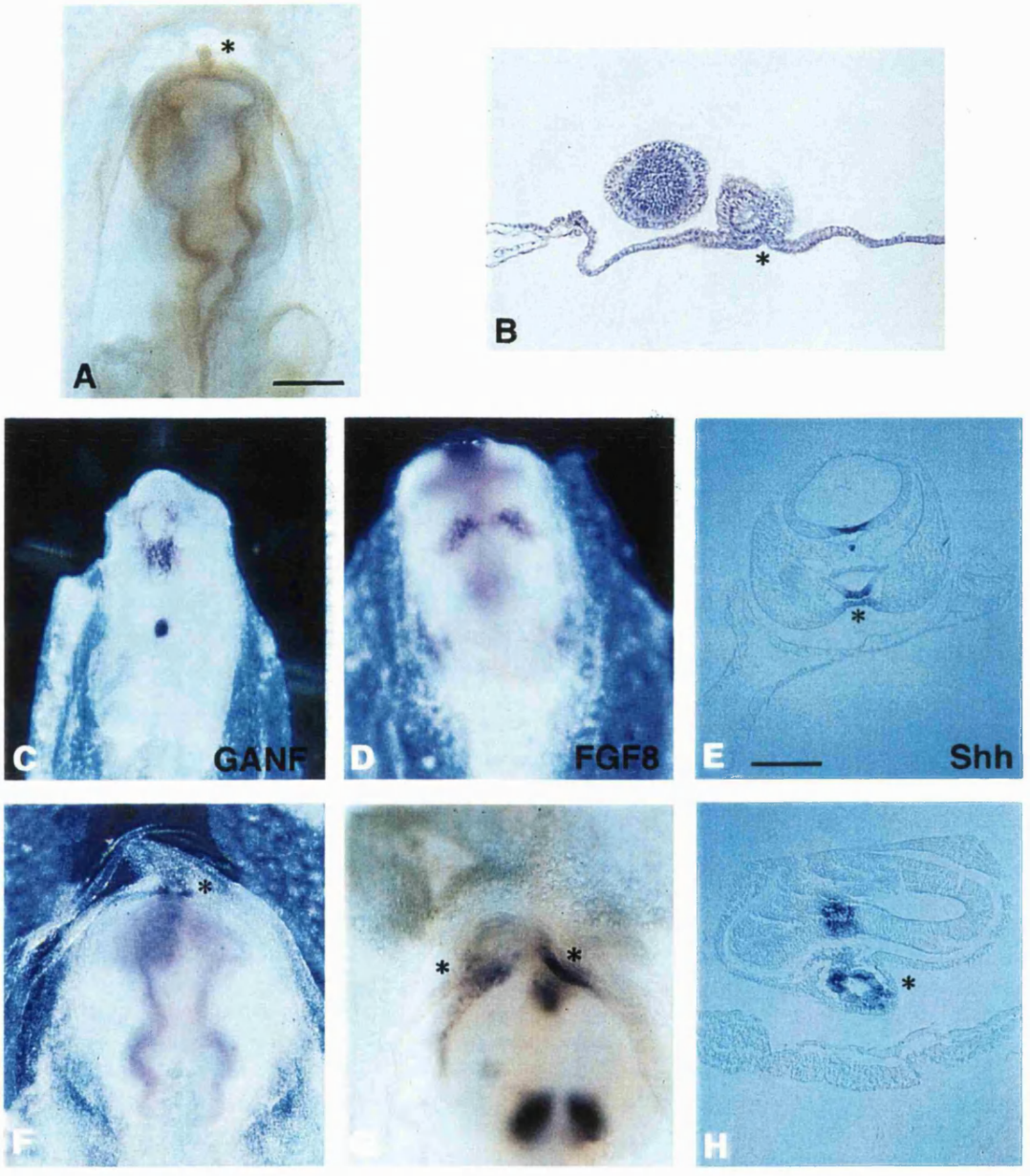
Following stage 5+ to 6 endoderm removals, a very interesting structure was frequently observed. A hollow tube of epithelium was seen stretching from way ahead of the embryo, diving through the ectoderm and extending back towards the base of the forebrain, where it ended blindly (Fig6.8A,B). This tube is never seen in normal embryos, but its identity as stomodaeal ectoderm was ascertained by using gene markers. The stomodaeal plate is the region where ventral head ectoderm fuses with endoderm at the tip of the foregut. Rathke's pouch is formed at the anterior end of this plate, bringing stomodaeal ectoderm into direct contact with the floor of the diencephalon and subsequently forming the anterior pituitary. *GANF* and *Shh* are both normally expressed in stomodaeal ectoderm (Fig6.8C,E), and are both observed in the hollow epithelial pipe (Fig6.8F,H). The adjacent ectodermal pattern of *FGF 8* expression (Fig6.8, compare G and D) further suggests that this tube is stomodaeal ectoderm. Due to lack of headfold and foregut tunnel formation following endoderm removal, experimental embryos remain flattened out and the facial ectoderm that would normally bend back round the ventral side of the head remains stretched out anterior to the CNS. It therefore appears that stomodaeal ectoderm is specified as normal in its proper fate map location in anterior ectoderm, but is unable to achieve its normal ventral position. Formation of the tube suggests that this ectoderm actively seeks cellular association with ventral diencephalon and foregut, necessary for subsequent development of Rathke's pouch and its other normal derivatives.

6.7 Analysis and discussion of results

The results presented in this chapter demonstrate an early role for the definitive foregut endoderm in patterning the anterior brain. Removal of this tissue during stage 4+ and 5 produces embryos with a simple, expanded forebrain vesicle. Morphological

Figure 6.8 Specification of stomodaeal ectoderm without foregut formation

Embryos were fixed at the 12 somite stage. Endoderm removal operations were performed at stage 5+ to 6; that is, when brain pattern produced is apparently complete, but the embryos still lack a foregut tunnel. **A** Light field image showing the presence of an abnormal pipe or tube extending ahead of the brain (asterisk); the tube has shrunk somewhat upon fixation. **B** Transverse section through the hollow tube (asterisk; other structure is skimming section of forebrain), showing its epithelial structure and its location beneath the anterior surface ectoderm. **C, F** Control embryo shows normal *GANF* expression in the stomodaeal ectoderm on the ventral side of the head (**C**); *GANF* also marks the anterior pipe seen in experimental embryos (asterisk in **F**; dorsal view). **D, G** Control embryo shows two chevrons of *FGF 8* on the ventral side of the head, flanking the stomodaeal region (**D**); in the experimental embryo, these patches of *FGF 8* expression instead lie on the lip of a very shallow foregut-type pocket, flanking the position of the abnormal pipe (asterisks in **G**; ventral view). Other patches of *FGF 8* expression visible in this panel are in telencephalon and isthmus, as normal. **E, H** Transverse sections at level of anterior foregut, showing normal *Shh* expression in stomodaeal ectoderm and the closely apposed ventral foregut (asterisk in **E**); following endoderm removal, *Shh* expression is seen in the anterior pipe as it extends back beneath the forebrain. Scale bar in **A** = 200μm for **A**, **C**, **D**, **F**, **G** and 50μm in **B**. Scale bar in **E** = 150μm for **E**, **H**.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specialisations such as eye cups are not formed. In addition, telencephalic expression of genes such as *BF-1* and *FGF 8*, known to be important in regulating the programmes of cell growth and differentiation in the forebrain, are missing.

The forebrain defects observed here are not caused by a reduction in the overall area of neural induction, nor in the initial allocation of anterior neural tissue. At neural plate and early headfold stages, expression of both pan-neural and anterior neural genes is normal. This is not surprising, since neural induction is thought to be well underway by the full streak stage (stage 4) of normal development, with initial cellular changes and gene expressions in the ectoderm already evident (reviewed in Smith and Schoenwolf, 1998). Therefore, at the stages when the head process is developing, anterior regionalising signals *in vivo* would be expected to act by locally modulating this already established state of general neural induction. Head process mesendoderm emerged from the newly regressing chick node is reported to be a poor neural inducer while being an effective anterioriser (Foley *et al.*, 1997). My results suggest that the definitive endoderm may also play an important role in maintaining and embellishing an initial broad pattern induced by the node. Upon removal of this tissue, initial allocation of anterior neural tissue occurs as normal, but patterning or subdivision of this forebrain field is not continued during development. A similar hypothesis has been suggested from recent results in mouse (Martinez-Barbera *et al.*, 2000a).

Loss of forebrain markers is first observed at the 5 somite stage. This is just the time when *FGF 8* expression is initiated in the anterior neural ridge (ANR), at the boundary of the anterior neural plate and the non-neural ectoderm ahead of it. The ANR has recently been shown in the mouse to be an important signalling centre for forebrain development, and is thought to be necessary for induction and/or maintenance of *BF-1* expression. In addition, recombinant FGF 8 protein is capable of inducing *BF-1* (Shimamura and Rubenstein, 1997). Data presented here show that *FGF 8* expression in

the ANR fails to be induced normally following endoderm removal at stage 4+ to 5, and down-regulation of *GANF* expression follows shortly after this (around the 7 somite stage). The extent and timing of the forebrain defects observed here therefore fit with a loss of *FGF 8* expression in the ANR. Furthermore, these experiments suggest that the definitive endoderm normally acts to establish this secondary organiser in the anterior neural plate, thereby allowing development of the complete forebrain pattern.

The importance of node-derived signals in complete anterior development has been highlighted in mouse, by analysis of *hex* chimaeric embryos (Martinez-Barbera *et al.*, 2000a), as well as *otx2* and *lim1* mutants (Rhinn *et al.*, 1998; Shawlot *et al.*, 1999). All of these molecules are first expressed in the AVE; a separate, extra-embryonic signalling centre known to be required for head formation in mouse (Beddington and Robertson, 1998). Normal anterior development therefore appears to require at least two essential signalling steps. The AVE, in synergy with the early node, initiates the induction and patterning of the forebrain; then axial mesoderm and endoderm emanating from the node are required to maintain and refine this initial pattern. The allocation of the same signalling molecules in both the AVE and definitive mesendoderm would allow the anterior ectoderm to remain in contact with inducing signals for a prolonged period, after the initial tissue has been replaced during gastrulation. In addition, there is evidence to suggest that, at least at early somite stages, the most anterior foregut still contains descendants of the visceral endoderm lineage (Lawson and Pedersen, 1987). Embryos homozygous for a targeted mutation in *Gata4*, expressed in the AVE and later in cardiac mesoderm (Heikinheimo *et al.*, 1994), fail to form a normal foregut and the myocardial primordia do not fuse in the ventral midline (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). By contrast, heart development progresses apparently normally in chimaeras where the extraembryonic lineages are wild type and the embryonic tissues largely mutant (Narita *et al.*, 1997). Thus, the anterior patterning role of the AVE may persist due to the

retention of some cells in the foregut, in close proximity to the cardiac region and developing forebrain.

In the chick, *hex* is expressed in anterior hypoblast at early stages and later in definitive foregut endoderm. Expression in the definitive endoderm is restricted to two domains; a central patch in the endoderm directly under the prechordal mesoderm (and probably also in prechordal mesoderm) and a more peripheral arc in pharyngeal endoderm that later becomes associated with the forming heart tube. I find that failure to restore all midline *hex* expression at headfold stages correlates with conditions of endoderm removal that cause anterior neural pattern loss. In mice, chimaera experiments using the *hex* null mutant have established that its crucial role for forebrain patterning is situated in rostral definitive endoderm, with the neural phenotype closely resembling the deficit seen in my endoderm-extirpated chick embryos (Martinez-Barbera *et al.*, 2000a). It is therefore a likely possibility that *hex* confers properties to definitive endoderm cells which support production of the inter-layer signals, from endoderm to overlying structures, that my experiments reveal.

Crescent, a chick *frzb* homologue is also strongly expressed in the anterior endoderm at the time when its removal produces forebrain defects. Members of the *frzb* family are secreted proteins with very similar structure to the ligand binding domain of the *Frizzled* family of *Wnt* receptors (reviewed in Zorn, 1997). *Frzbs* are therefore thought to act as antagonists of *Wnt* signalling, binding to Wnt proteins in the extracellular space and preventing them from interacting with their receptors (Leyns *et al.*, 1997; Wang *et al.*, 1997). Head induction is proposed to rely on the combined down-regulation of Wnt and certain subclasses of TGFβ signalling (Glinka et al., 1997; Niehrs, 1999). Therefore, the expression of *crescent* in the anterior hypoblast and then definitive endoderm suggests that it could play a role in producing an environment in the anterior embryo that

favours head development, throughout the period of gastrulation. As shown here, the domain of *crescent* expression in definitive foregut endoderm at headfold stages correlates strikingly with the area required for anterior pattern completion in my experiments. After endoderm removal so timed as to result in later pattern truncation, this domain of *crescent* expression is not restored. I cannot claim that *crescent* itself provides the crucial endoderm-derived anteriorising signal revealed by my embryological results; numerous *frzb* family members and other molecules exhibiting similar antagonist functions have been identified, and are expressed in similar appropriate distributions in various vertebrates, suggesting they may act redundantly (Leyns *et al.*, 1997; Wang *et al.*, 1997; Glinka *et al.*, 1998; Hoang *et al.*, 1998; Shawlot *et al.*, 1998; Piccolo *et al.*, 1999; Pearce *et al.*, 1999; Simpson *et al.*, 1999). Nevertheless, my results suggest that Crescent protein and others functionally parallel with it are candidates for such signals.

These experiments have also uncovered an unexpected role of the definitive foregut endoderm in induction of the stomodaeal ectoderm. The stomodaeal plate is the region where ventral head ectoderm fuses with endoderm at the tip of the foregut, with no mesoderm intervening. This stomodaeal plate later breaks down in the chick as the oral cavity is formed, and the lumen of the foregut comes into contact with the amniotic cavity (Bellairs and Osmond, 1998). Rathke's pouch forms at the anterior end of the stomodaeal plate, bringing the stomodaeal ectoderm into direct contact with the ventral diencephalon. Prolonged interaction of Rathke's pouch with the ventral diencephalon is necessary for normal development of the anterior pituitary (Gleiberman *et al.*, 1999). However, it is thought that the fate of the anterior pituitary progenitors is determined earlier during formation of the anterior neural plate, before the movements and flexion of headfold formation bring this tissue onto the ventral side of the head. Fate map studies performed on chick-quail chimeras show that the anterior pituitary develops from the anterior neural ridge (ANR) of a 3-4 somite embryo (Couly and Le Douarin, 1988).

Furthermore, removal of the central part of the anterior neural ridge of a 3-4 somite embryo completely prevents Rathke's pouch formation, and thus the entire anterior pituitary (elAmraoui and Dubois, 1993). *Rpx/Hesx1* (Hermesz *et al.*, 1996) is expressed in the anterior neural plate and ANR. It is also later expressed in the oral ectoderm and Rathke's pouch, and *hesx1* -/- embryos show defects in pituitary morphogenesis (Dattani *et al.*, 1998).

Results presented in this chapter strongly suggest that the definitive endoderm induces the stomodaeal ectoderm at its initial anterior position. Endoderm removal at stage 5+ and 6 produces a pipe-like structure extending from the anterior ectoderm back towards the ventral forebrain. This tube expresses *GANF* and *Shh*, both of which are normally expressed in stomodaeal ectoderm and early Rathke's pouch. The formation of a pipe suggests that this ectoderm actively seeks cellular association with ventral diencephalon and foregut, necessary for subsequent development of its normal derivatives. As mentioned above, it is highly unlikely that any further development of Rathke's pouch would be able to proceed, in the absence of correct interaction with the diencephalon. Indeed, *Shh* is normally down-regulated as Rathke's pouch starts to form (Ericson *et al.*, 1998). Nevertheless, my data identify an earlier step, prior to these interactions, for induction of the stomodaeal ectoderm by the anterior endoderm. This tube is only produced after stage 5+ and 6 removals, suggesting that the inducing role of the endoderm is complete by stage 5+, but not earlier.

The embryos produced following endoderm removal at stage 4+ to 5 often lacked eye cups. Normally, the first morphological sign of eye development is a bulging at the lateral sides of the prosencephalon. These are the rudiments of the optic vesicles, which continue to grow laterally from the neural tube, remaining connected to the brain by the optic stalks. When these vesicles contact the overlying head ectoderm, the ectoderm thickens to form the lens placodes. Close contact between optic vesicle and head

ectoderm is necessary for thickening of the lens placode, and subsequent growth and differentiation of a complete optic cup. However, induction of the lens begins well before formation of the optic vesicles. Lens formation is thought to involve a series of tissue interactions beginning right back at gastrulation (reviewed in Grainger, 1992), when early lens inductive signals are transmitted in a planar manner from the anterior neural plate to the adjacent ectoderm (Henry and Grainger, 1990). These signals establish a bias, or predisposition, throughout the head ectoderm to form lens. This area of facial ectoderm is larger than that which will actually form the lens, and is refined by continued signalling during neurula stages.

The *pax 6* gene (Walther and Gruss, 1991) encodes a DNA-binding transcription factor essential for normal eye development (Hogan *et al.*, 1986; Hill *et al.*, 1991). It is expressed in several eye tissues throughout development and is probably required at several stages (Collinson *et al.*, 2000). However, *pax 6* is first expressed in the chick at stage 6, in a band of non-neural ectoderm, close to the anterior margin of the neural plate (Li *et al.*, 1994). This tissue is fated to become facial and lens ectoderm (Couly and Le Douarin, 1990), and closely correlates with the time and position that early lens determination takes place in *Xenopus* (Henry and Grainger, 1990). Widespread expression of *pax 6* is maintained throughout the head ectoderm, prior to formation of the optic vesicles, suggesting that an early role of *pax 6* is to maintain the competence of the head ectoderm to form lens (Li *et al.*, 1994; Collinson *et al.*, 2000).

In the experimental embryos presented here, *pax 6* expression appears to be induced in both forebrain and head ectoderm regions as normal. However, specification of forebrain territory that undergoes the characteristic growth programme of optic vesicle formation does not appear to occur. As a result, *pax 6* expression remains widespread throughout the simple prosencephalic vesicle and the overlying head ectoderm. Presumably, the head ectoderm remains in a preliminary state of lens formation, since it

receives no optic vesicle signal. Indeed, no ectodermal thickening characteristic of the developing lens placode could be found in sections of experimental embryos cultured on to 18-20 somites (n=6; data not shown).

Finally, endoderm removal during stage 4+ to 5 results in the formation of two separate lateral heart structures. Cells destined to form the heart normally ingress through most of the length of the streak during stage 4 (Schoenwolf and Garcia-Martinez, 1995), and then move to form two heart-forming regions (HFR) in the mesoderm just lateral to the node (Garcia-Martinez and Schoenwolf, 1993). As the node starts to regress, precardiac mesoderm cells migrate in an arc towards the midline, where they fuse to form a crescent. These cells cease to move when they arrive at the anterior intestinal portal and, as the head region forms, the two heart rudiments zip together to form a linear heart tube along the ventral side of the embryo. The developmental fate of precardiac mesoderm cells is not fixed during early gastrula stages, and signals from underlying definitive endoderm are required to complete development of beating heart structures (Sugi and Lough, 1994; Nascone and Mercola, 1995; Schultheiss *et al.*, 1995). In the experimental embryos presented here, more lateral definitive endoderm is left in place. Therefore, cardiomyogenic signals from this endoderm can be transmitted as normal, in order to commit most of the overlying mesoderm to a cardiac fate. However, the substrate for migration of these precardiac mesoderm cells to the anterior midline (the central anterior endoderm) has been removed, and so two separate beating heart structures form that remain lateral to the head region. This *cardia bifida* is also produced by implanting a barrier to prevent the merger of lateral plate mesoderm (DeHaan, 1959).

Many aspects of the syndrome observed following endoderm removal can therefore be explained by considering the normal signalling processes and interactions occuring in the embryo at that time. The stage specificity for producing these effects is remarkable, but why is it that signalling from the endoderm should be required for forebrain

patterning between the particular stages 4+ and 5? As discussed in Chapter V, fate mapping studies show that at stage 4, the region of prospective foregut endoderm is centred around the node. It is only during stage 4+ and 5 that this foregut domain appears to extend to lie under a broader region of neural plate, in correlation with the invaginating mesoderm (Bellairs, 1953a). Therefore, it may be that the signalling capacity of the endoderm is only operational once the domain of prospective foregut has enlarged under the neural plate. In addition, the loss of gene expressions observed here following endoderm removals, are consistent with a role for the endoderm in directly establishing the forebrain signalling centre at the boundary of the neural plate and the non-neural ectoderm anterior to it (the ANR). DiI labelling studies have shown that the mesendoderm migrates in advance of the forebrain ectoderm, reaching its rostral-most location by stage 6 (Chapter V; Dale *et al.*, 1999). In contrast, neuroectoderm cells do not reach their rostral position until later, therefore passing over the prechordal mesoderm between stages 5 and 8. This indicates that the correct apposition of foregut endoderm and the ANR may therefore only be achieved during stages 4+ and 5, as the endoderm is extending anteriorly. After this stage, the rostral end of the neural plate slides further anterior, and so tissue relationships change once again.

CHAPTER VII

FURTHER ANALYSIS OF ENDODERM SIGNALLING AND ITS INTERACTION WITH MESODERM TO PATTERN THE CNS

7.1 Introduction

Having examined in detail the syndrome produced after removal of a defined sector of anterior endoderm, as described in Chapter VI, it was necessary to explore how these effects on forebrain patterning were achieved. In order to demonstrate an active signalling role for this region of endoderm, and confirm its patterning ability, it was grafted adjacent to prospective posterior neural plate and the induction of anterior neural markers examined. It was also ascertained whether other regions of endoderm could substitute for the removed area, when used as a replacement.

It was of particular importance to establish whether endoderm removal had any effect on signalling capacities of the axial mesoderm, since the role of this tissue in both anteroposterior and dorsoventral patterning of the neural tube has been well documented (see introduction chapter). To this end, I examined the expression of genes in the axial mesoderm following endoderm removal; both those expressed throughout the AP axis and those expressed specifically anteriorly. The morphology of the brain was also studied following endoderm removal, to assess for ventral midline character and DV patterning. Finally, the effects of removing endoderm alone were compared with those of removing endoderm and mesoderm together. Unfortunately, it was not technically possible to study the effects of removing mesoderm alone, while leaving endoderm in place.

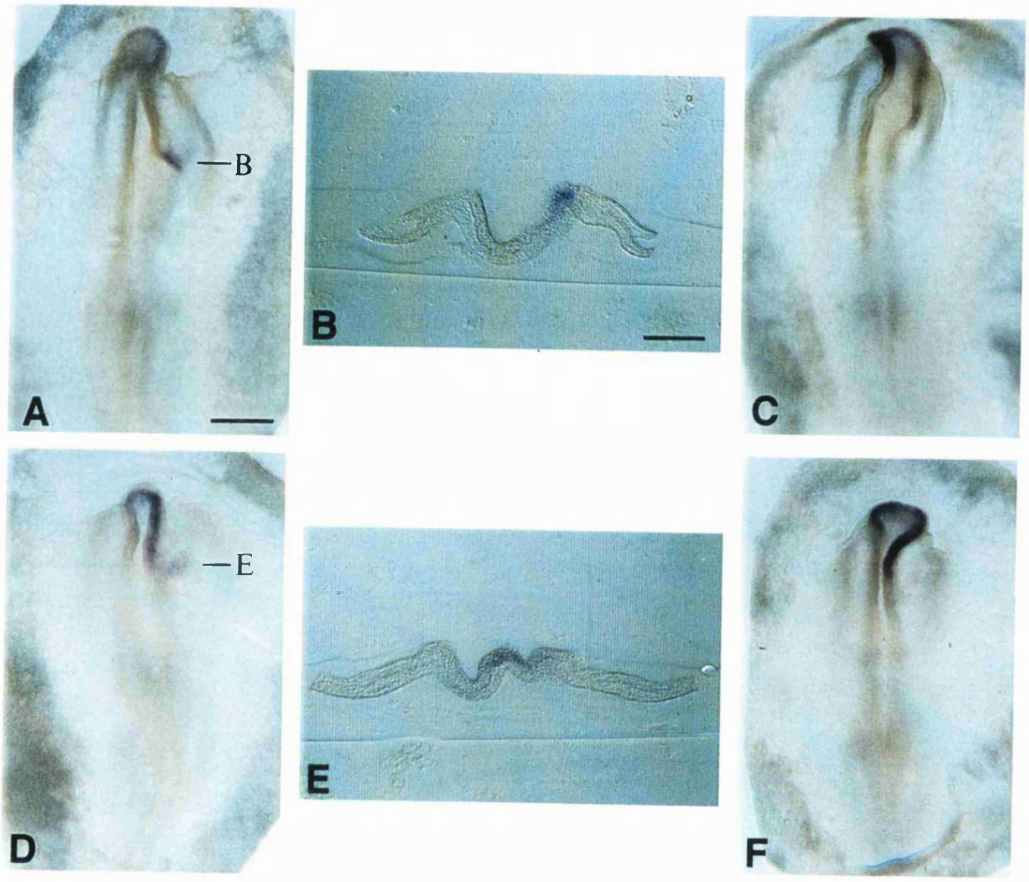
7.2 Further analysis of anterior endoderm signalling

7.2.1 Ability of endoderm to induce anterior markers in posterior neural plate

Thus far, all my evidence for a role of the anterior endoderm in patterning the forebrain has originated from studying the effects of removing this tissue. I therefore wanted to demonstrate an active signalling role for this region of endoderm, and confirm its patterning ability. Pieces of endoderm from over and just ahead of the stage 5 prechordal mesoderm were very carefully removed and re-inserted, between endoderm and presumptive posterior neural plate, on one side of the node in the same embryo. Foley *et al.* (1997) had previously shown by DiI labelling that this area mapped to future hindbrain territory. Seven hours after operations, embryos were fixed and assayed for induction of the forebrain marker *GANF*. In 8/15 cases, asymmetric *GANF* expression was seen, biassed towards the operated side of the embryo, as an extension of the normal expression domain (Fig7.1D,F). In some cases, a completely separate patch of expression was seen at hindbrain level, associated with the graft (Fig7.1A,C). Additional neural structure was also often associated with the endoderm graft (Fig7.1B,E). More peripheral endoderm, taken from the *area pellucida/ area opaca* boundary, was also tested in this manner. In no case (0/6) was asymmetric or ectopic *GANF* expression observed in the host, suggesting that there is positional specificity within the anterior endoderm and not all regions possess the same signalling properties.

Figure 7.1 Induction of *GANF* expression following endoderm grafts

Stage 8 embryos viewed from the dorsal aspect. Pieces of definitive endoderm from over and just ahead of the prechordal mesoderm were excised at stage 5 and re-inserted, between endoderm and presumptive posterior neural plate, on one side of the node in the same embryo. **A**, **C** A separate patch of ectopic *GANF* expression is induced at hindbrain level on the operated side. **B** Transverse section through embryo in **A**, showing *GANF* expression in neural ectoderm. **D**, **F** *GANF* expression is asymmetric, due to extension of the normal domain on the operated side. **E** Transverse section through embryo in **D**, showing *GANF* expression present in ectopic neural structure. Scale bar in **A** = 700μm in **A**, **C**, **D**, **F**. Scale bar in **B** = 400μm for **B**, **E**.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7.2.2 Reducing the size of territory removed in order to localise vital area

The area of endoderm removed in the great majority of experiments stretched from the node to the anterior edge of the *area pellucida*, as shown in Fig6.1. However, a further series of removal operations were performed where the territory removed was reduced, in order to pinpoint the region responsible for causing the syndrome (data not shown). The standard area removed was split into two, such that either only the very anterior portion of endoderm, or the more central portion around and just ahead of the prechordal mesoderm, was removed (Fig6.1B). Subsequent effects of just removing the central portion were morphologically very similar to those after removing the entire area, suggesting that the forebrain patterning signals studied here reside in the central portion rather than more peripherally.

7.2.3 Ability of replacement endoderm to recover the syndrome

Lower layer from the pre-nodal sector of stage 4 donors (largely hypoblast and presumptive extra-embryonic endoderm) or the perinodal and posterior head process level of stage 6 or 6+ donors (more posterior definitive endoderm) were tested for their ability to substitute for anterior foregut endoderm. Large pieces of these tissues were grafted into the exposed area of a stage 4+ or 5 host, following the standard operation of endoderm removal. This procedure proved particularly difficult, and the lower layer contracted very quickly so that it was often hard to entirely cover the exposed 'window' of the host. Nevertheless, preliminary analysis at the 12 somite stage, assessing morphology only, suggested that the graft had not rescued the forebrain patterning defects (14 embryos examined). Migration of precardiac mesoderm across the grafted endoderm also remained abnormal; there was some fusion of cardiac tissue

at the midline, but most tissue remained laterally and two largely separate heart regions were still formed. There was also little evidence for restoration of normal foregut formation.

7.3 Expression of axial mesoderm markers after endoderm removal

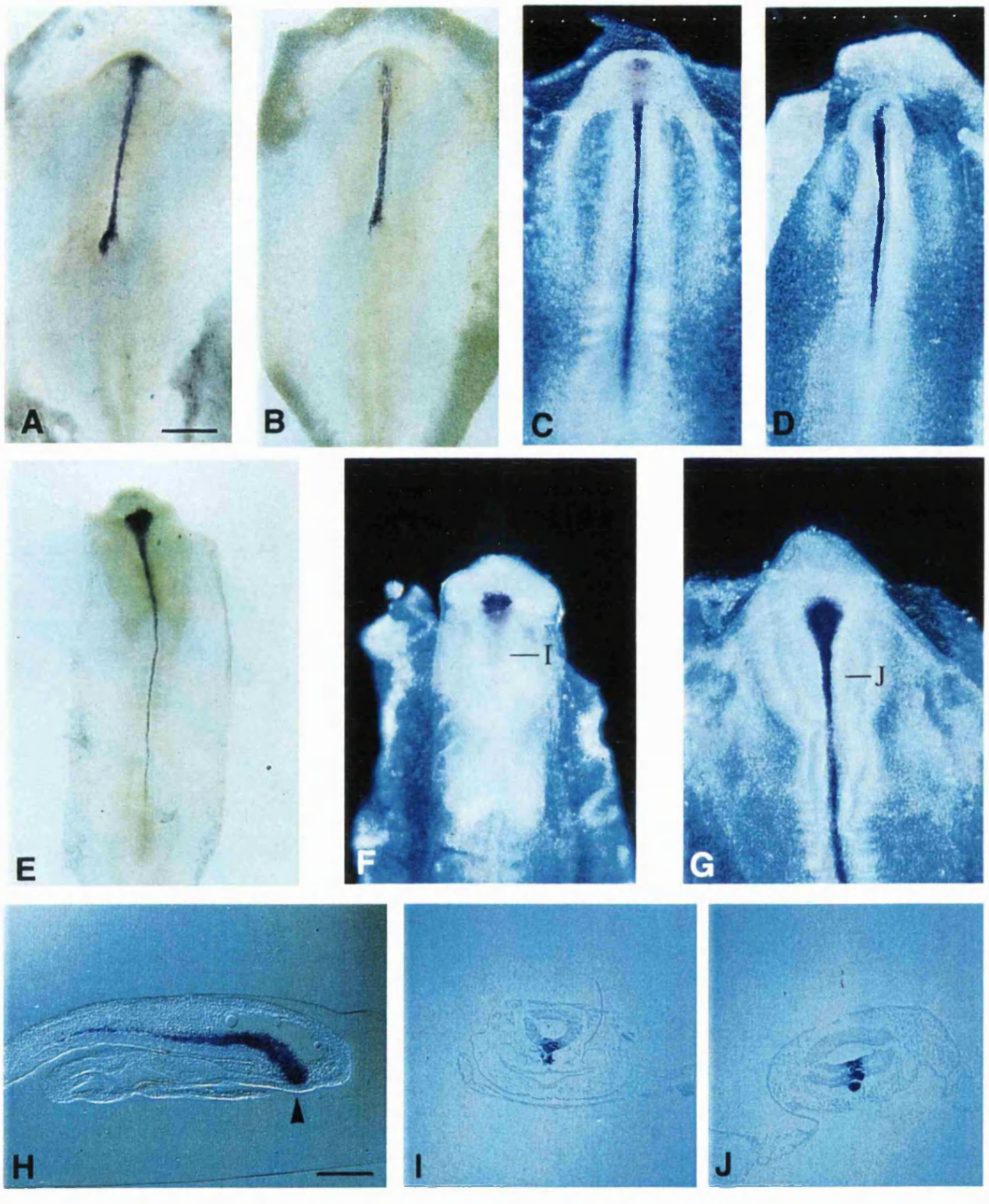
In order to determine whether removal of endoderm affected the signalling capacities of the axial mesoderm in any way, gene expressions in this tissue were examined. Genes expressed along the length of the axial mesoderm, such as *sonic hedgehog* and *chordin*, were used, as well as those restricted to prechordal mesoderm, such as *BMP7* and *goosecoid.*

7.3.1 *Sonic hedgehog* and *chordin* expression

Sonic hedgehog (*shh*) normally begins to be expressed at stage 5-, throughout the entire length of emerged axial mesoderm back to the node, where it exhibits characteristic left/ right asymmetry (Fig7.2A). It continues to be expressed right along the length of prechordal mesoderm and notochord until very late stages. Following endoderm removal, the extent of *shh* expression in prechordal mesoderm and notochord at the 5 somite stage was normal, regardless of whether the removal was performed at stage 4 or stage 4+ to 5 (Fig7.2A-D). The same was true at the 12 somite stage; the extent of *shh* expression in axial mesoderm was normal following all removals (19/19 embryos), still showing the characteristic fan shape at the base of the diencephalon (Fig7.2E-J).

Figure 7.2 ***Shh*** **expression in axial mesoderm after endoderm removal**

Ventral views of wholemount embryos, anterior to the top. **A, B** Matched stage 7 embryos showing the normal extent of *shh* expression, throughout emerged axial mesoderm (**A**) and maintenance of this normal expression pattern following endoderm removal at stage 4 (**B**). **C, D** Matched stage 8 embryos showing the normal extent of *shh* expression (**C**) and no effect on this normal expression following endoderm removal at stage 4+ to 5 (**D**). **E, F, G** 12 somite embryos showing normal *shh* expression throughout axial mesoderm, with the characteristic fan shape at the base of the diencephalon (**E**) and maintenance of the normal extent of expression after stage 4 (**F**) and stage 4+ to 5 (**G**) endoderm removals. **H** Sagittal section through 12 somite embryo, showing anterior limit of *shh* expression at the base of the diencephalon, in the infundibular region (arrowhead). **I, J** Transverse sections of 12 somite embryos at anterior foregut level, showing normal *shh* in ventral brain and notochord in both control embryos (**I**) and after endoderm removal at stage 4+ to 5 (**J**). Scale bar in **A** = 450μm for **A, B**; 550μm in **E** and 350μm for **C, D, F, G**. Scale bar in H = 250μm for **H- J**.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Chordin is expressed strongly in the node from stage 4 onwards, being maintained as the node starts to regress. Expression is also seen along the axial mesoderm as it is laid down. However, unlike *shh*, it does not extend right to the anterior limit of prechordal mesoderm and does not show the fan shape, remaining posterior to the headfold pocket at the 3 somite stage (unlike *shh*; see Fig7.2C). Following lower layer removal at either stage 4 or stage 4+ to 5, the extent of *chordin* expression in axial mesoderm was normal, at both the 5 and 12 somite stages (9/9 embryos; data not shown).

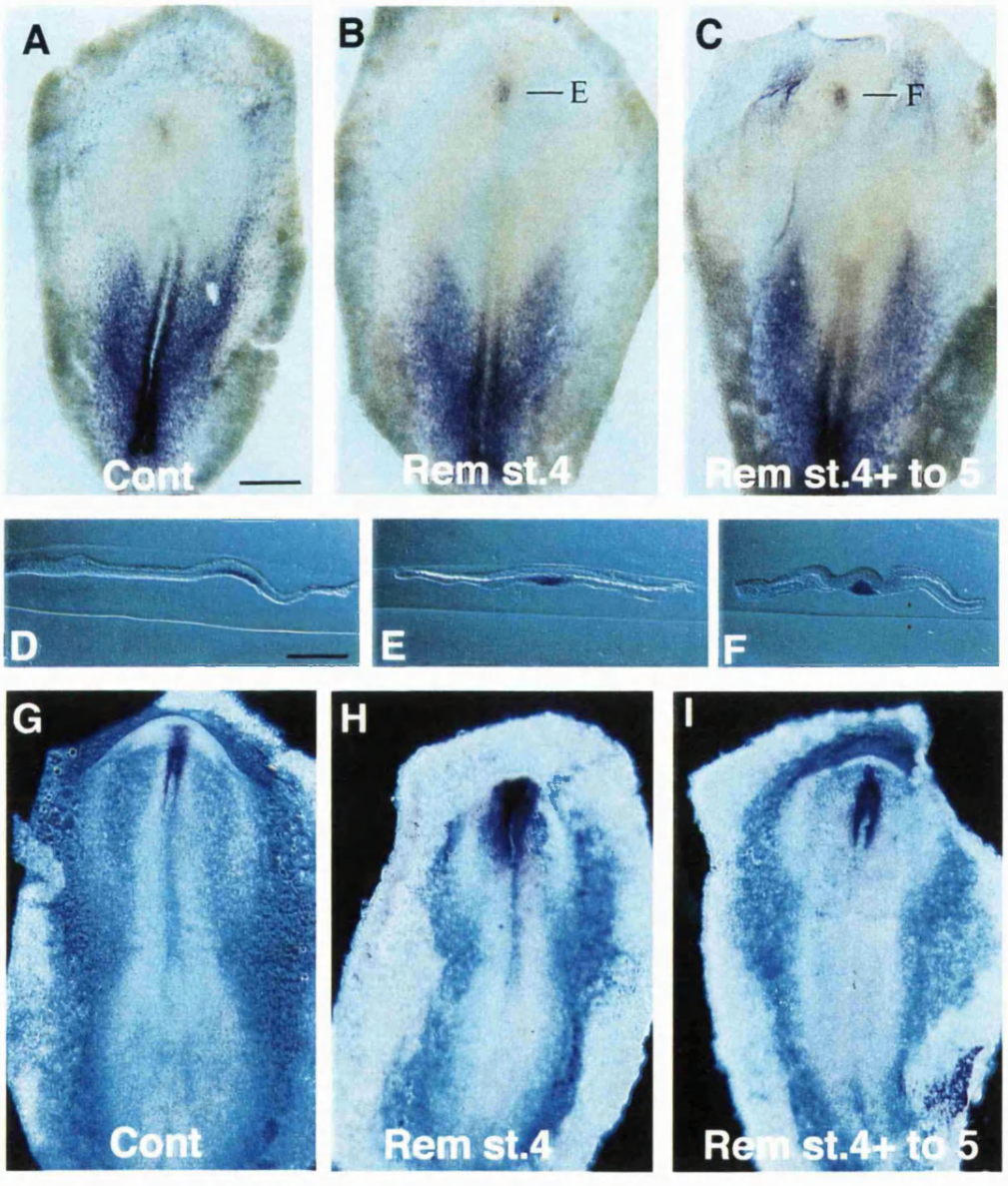
7.3.2 *BMP7* and *goosecoid* expression

Expression of *BMP7* specifically in the prechordal mesoderm is proposed to be important in governing the anterior patterning capabilities of this tissue (Dale *et al.*, 1997; Dale *et al.*, 1999). It was therefore important for me to examine whether or not *BMP7* expression was affected following endoderm removal. At headfold stages 6-7, *BMP7* expression in prechordal mesoderm remained normal following both stage 4 and stage 4+ to 5 (10/11 embryos) removals (Fig7.3A-F).

Goosecoid is expressed strongly in the node from stage 3, but is rapidly down-regulated at stage 4+, as the node starts to regress. Expression remains in the prechordal mesoderm cells that leave the node at this point, and stays confined to anterior cells in the fan-shaped tip of the head process and associated endoderm of the prechordal plate (Izpisua-Belmonte *et al.*, 1993). Following removal operations at both stage 4 and stage 4+ to 5 (8/8 embryos), *goosecoid* expression in the prechordal mesoderm at stage 7 was normal (Fig7.3G-I).

Figure 7.3 ***BMP 7*** **and** ***Goosecoid*** **expression after endoderm removal**

A- C Ventral views of stage 6 wholemount embryos, anterior to the top. The normal patch of *BMP 7* expression in prechordal mesoderm at this stage (**A**) is maintained following endoderm removal at stage 4 (**B**) and stage 4+ to 5 (**C**). **D** Sagittal section of control stage 6 embryo, confirming *BMP 7* expression in prechordal mesoderm. **E, F** Transverse sections of stage 6 embryos at prechordal level, showing normal *BMP 7* in control embryos (**E**) and maintenance of prechordal mesoderm integrity (and *BMP 7* expression) after stage 4+ to 5 endoderm removal (**F**). **G- I** Dorsal views of stage 7 wholemount embryos, anterior to the top. The normal pattern of *goosecoid* expression at this stage (**G**) is maintained following endoderm removal at stage 4 (**H**) and stage 4+ to 5 (**I**). Scale bar in **A** = 450μm for **A- C, G- I**. Scale bar in **D** = 250μm for **D- F**.


A
B
C
— E
— F
Cont
Rem st.4
Rem st.4+ to 5
D
E
F
G
H
I
Cont
Rem st.4
Rem st.4+ to 5

Thus, prechordal mesoderm and notochord were shown to be present and expressing their normal array of markers, even in embryos after endoderm removal at stage 4+ to 5, which would have therefore been likely to show pattern truncations in the forebrain.

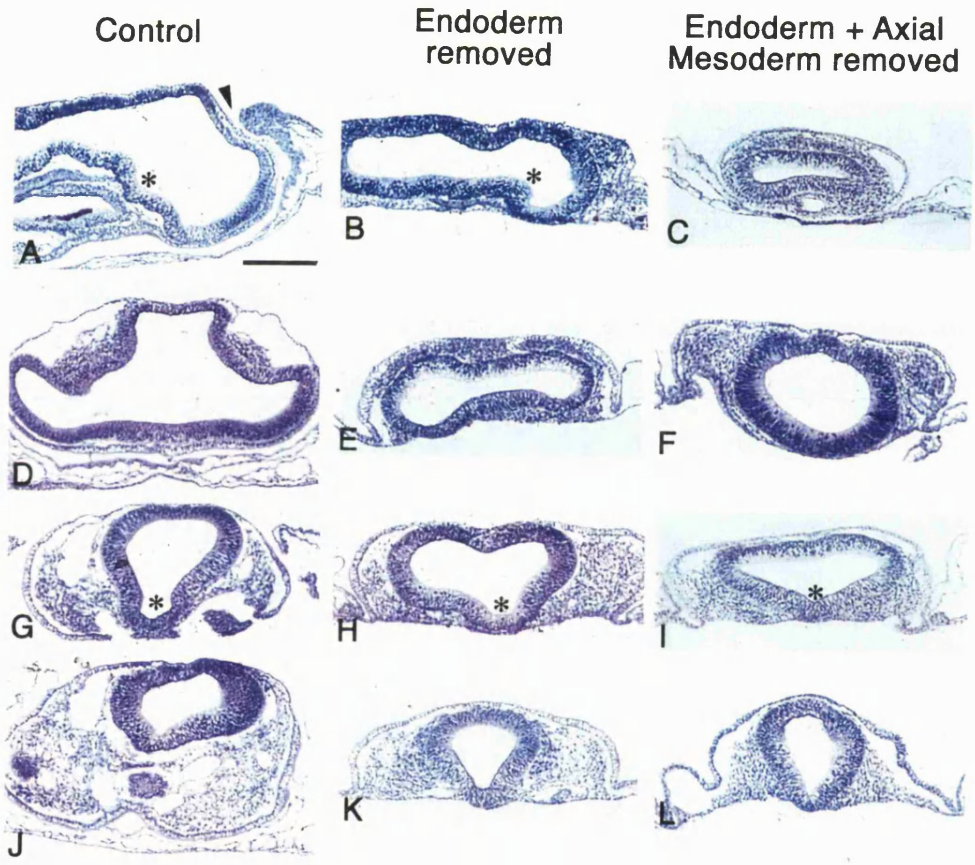
7.4 Dorso-ventral patterning after endoderm removal

As discussed in Chapter 6, endoderm removal between stage 4+ and 5 resulted in formation of a relatively unpatterned forebrain vesicle at the 12-15 somite stage. The dorsal forebrain territory failed to become sub-divided into telencephalon (anteriorly) and diencephalon associated with lateral eye cups. Failure of eyecup formation and forebrain expansion can be caused by loss of ventral midline- organised growth and patterning, following disruption of *Shh* gene function (holoprosencephaly; Chiang *et al.*, 1996; Towers *et al.*, 1999). However, DV patterning does not appear to be affected in the brains of the embryos presented here. The simple forebrain vesicle was bulbous and expanded (Fig7.4B,E), which is very different from the distinctive narrow proboscis-like structure associated with loss of *Shh* gene function. In addition, the early morphology of the infundibular region remained as a keel-shaped depression in the ventral midline associated with the prechordal mesoderm (Fig7.4B,H), whereas neural ventral midline morphology is suppressed following loss of *Shh* function. Finally, *Shh* was expressed throughout the neural ventral midline of endoderm-removed embryos, as it is in normal embryos (Fig7.2I,J) and expression of *pax6* was not expanded ventrally, consistent with the presence of normal inhibitory signals from ventral midline (Fig6.8E,F; Macdonald *et al.*, 1995).

Figure 7.4 Comparing endoderm only with endoderm + axial mesoderm removals

Wax sections of 12 somite embryos; **A**, **B** are sagittal sections, **C- L** are transverse sections. **A**, **D**, **G** and **J** are from control embryos. **B**, **E**, **H** and **K** are from embryos following endoderm removal at stage 5. **C**, **F**, **I** and **L** are from embryos after combined endoderm and axial mesoderm removal at stage 5.

A, **B** Sections comparing control embryo with embryo after endoderm removal. Asterisks mark future infundibular region of brain, which normally associates with prechordal mesoderm, tip of foregut and invaginated stomodaeal ectoderm. Arrowhead marks morphological division between telencephalic and diencephalic rudiments (**A**). Note relatively short forebrain with undivided morphology after endoderm removal, although infundibular depression is maintained (**B**). **C** Section at anterior level after combined endoderm and mesoderm removal, showing presence of stomodaeal pipe, despite absence of telencephalon patterning. **D- F** Sections at mid-optic vesicle level. Note lateral expansion in control embryo (**D**), but only a simple anterior brain vesicle formed after both types of removal operation (**E**, **F**). Flattened appearance, as shown in **E**, sometimes occurs due to ring culture. **G- I** Sections at infundibular level. Note keel-shaped depression (asterisk) in neural ventral midline of control embryo (**G**), which is also maintained following both types of removal operation (**H**, **I**). **J- L** Sections at a slightly more posterior level. In the control embryo, section grazes through anterior foregut, with prechordal mesoderm lying between foregut and ventral brain (**J**). After endoderm removal, the prechordal mesoderm is abnormally closely apposed to the ventral brain, due to lack of foregut tunnel (**K**). Following removal of axial mesoderm as well as endoderm, there is little further deficit in CNS ventral midline morphology or growth of brain vesicles (**L**). Scale bar = 125μm throughout.


Control
Endoderm removed
Endoderm + Axial Mesoderm removed
A
B
C
D
E
F
G
H
I
J
K
L

7.5 Comparison of endoderm only with combined endoderm + mesoderm removals

Most of these operations, together with analysis of subsequent brain morphology, were performed by J.Cooke. These preliminary studies suggested that removal of the entire axial mesoderm together with the definitive endoderm at stage 5 and 5+, produced negligible additional effects and resulted in embryos whose anterior CNS morphology closely resembled that resulting from endoderm removal alone. The simple forebrain vesicle still remained relatively expanded (although slightly less so than after endoderm alone removals) and the early features of ventral midline specialisation were still intact in the infundibular region (Fig7.4F,I,L). Similar morphology was also observed after endoderm plus mesoderm removals as late as stage 6 and 6+, suggesting that forebrain patterning defects may still be produced. In contrast, removal of endoderm alone by this stage did not appear to affect later forebrain patterning (Chapter VI).

It is also interesting to note that after stage 6 removals of endoderm plus mesoderm, the 'pipe' of stomodaeal ectoderm was still often seen ahead of the brain (Fig7.4C; see Chapter VI). These embryos appeared to lack forebrain regionalisation (unlike the endoderm-only removals that displayed this pipe), suggesting that induction of stomodaeal ectoderm does not depend on the presence of a completely specified forebrain, but involves direct vertical signalling from definitive endoderm to ectoderm during stage 5+.

7.6 Analysis and discussion of results

The experiments presented in this chapter examine the possible routes of endoderm signalling and explore the tissue relationships in the early embryo in more detail. My results strongly suggest that definitive endoderm can directly influence ectoderm pattern. Grafts of definitive endoderm are able to induce ectopic *GANF* expression in presumptive hindbrain territory. In addition, several known signalling properties of the axial mesoderm are unaffected by endoderm removal. In the anterior extreme of the embryo, endoderm and ectoderm lie directly next to one another, since mesoderm does not reach this far. Other recent evidence in mouse suggests that the endoderm may play a direct role in establishing a forebrain signalling centre, at the anterior border of neural plate and non-neural ectoderm (the ANR; Martinez-Barbera *et al.*, 2000a). My experiments support this hypothesis, and show that removal of definitive endoderm results in a failure to properly establish the ANR signalling centre. In addition, results presented here suggest that endoderm directly induces stomodaeal ectoderm in the region anterior to the neural plate.

Nevertheless, definitive endoderm may also play a role in patterning the ectoderm indirectly, by modulating or stabilising the signalling properties of head process mesoderm. An active patterning influence of endoderm upon axial mesoderm has recently been reported elsewhere. Vesque and colleagues propose that signals from anterior endoderm are necessary to specify prechordal mesoderm character in the axial mesoderm, as it extends away from the node (Vesque *et al.*, 2000). Furthermore, given the important role that more lateral endoderm plays in induction of precardiac

mesoderm (Nascone and Mercola, 1995; Schultheiss *et al.*, 1995), the endoderm may operate generally to govern the character of anteriorly migrating mesoderm. Martinez-Barbera and colleagues suggest that *hex* function in the definitive endoderm may regulate expression of genes such as *cerrl* in the mesoderm; such genes are proposed to be involved in head formation (Martinez-Barbera *et al.*, 2000a). Transfer of information between successive layers may therefore be a relatively common phenomenon, co-ordinating midline identity in the different germ layers.

My preliminary results suggest that as-yet-unidentified signalling properties of the axial mesoderm, may be affected by endoderm removals. By stage 5+ and 6, removal of endoderm alone does not appear to affect later forebrain patterning. However, combined endoderm and mesoderm removal at this stage, produces forebrain morphology similar to that resulting from earlier removal of endoderm alone (stage 4+ to 5). This suggests that forebrain patterning may be stable in the presence of mesoderm alone during stage 6, but is not yet stable if prechordal mesoderm is also removed. Perhaps, then, information is transmitted to the ectoderm in a sequential layer-to-layer manner, with mesodermal properties completing their own influence on ectoderm later in development than those of the definitive endoderm. In addition, the observation that expression of several patterning genes remains intact in axial mesoderm for many hours following endoderm removal, suggests that anterior endoderm provides distinctive signals or conditions, and not just a more general protective or maintenance environment for head process mesoderm. Unfortunately, it was not possible to study the extent of endodermal signalling in the absence of axial

mesoderm; temporarily peeling back endoderm and replacing it after removing the underlying mesoderm, proved too difficult to reliably achieve.

In the mouse, axial mesoderm and definitive endoderm are thought to emerge from the node as a common population of head process cells, only later separating into their respective germ layers (Beddington, 1994; Tam and Behringer, 1997). Transfer of information from the endoderm may therefore occur through cell mixing as well as by influence of secreted signals. In chick, axial mesoderm and endoderm layers of the head process are clearly distinct as tissue leaves the node. However, I have some evidence that cells from the definitive endoderm may subsequently be incorporated into the prechordal mesoderm (see Chapter V), suggesting that information transfer by cell intercalation may also occur in the chick.

My results suggest that there is positional specificity within the definitive endoderm. Endoderm around and just anterior to the prechordal mesoderm can induce ectopic *GANF* expression in posterior neural plate, while more peripheral and lateral endoderm cannot. Restricted patterns of gene expression (such as *crescent* and *hex*), and the differing ability of endoderm regions to induce heart development, further suggest that all areas of this thin tissue layer are not functionally equivalent. This variation in signalling properties of the endoderm has made it difficult to find suitable 'neutral' regions to use as replacements, for the anterior endoderm removed in my standard operations. However, anterior lower layer from stage 4 embryos, or more posterior endoderm from stage 6 embryos, seem unable to substitute for anterior foregut endoderm and cannot fully rescue either forebrain pattern or heart migration. It

therefore appears that the endoderm is patterned at an early stage, and signals can be transmitted in a region-specific manner to the overlying layers. The close proximity of tissue layers of course makes it likely that information is transferred in all directions, with the pattern in each layer becoming progressively refined through time.

Expression of several patterning genes in the axial mesoderm remain unaffected by endoderm removal. There is also morphological evidence to suggest that ventral midline signalling is still intact at the 12 somite stage, even though these brains exhibit forebrain pattern deficits. In the operated embryos presented here, the simple prosencephalic vesicle is moderately expanded and early ventral midline morphology is normal. In addition, it is likely that rostral diencephalic ventral midline cells (RDVM) are still specified, since *BMP7* expression in the prechordal mesoderm appears unaffected by endoderm removal (Dale *et al.*, 1997; Dale *et al.*, 1999). In contrast, disruption of *Shh* gene function produces a distinctive narrow, proboscis-like forebrain that lacks ventral midline morphology (Chiang *et al.*, 1996; Towers *et al.*, 1999). Furthermore, removal of prechordal mesoderm, by either surgical or genetic means, causes defects in DV forebrain patterning but does not appear to affect segregation of anterior brain territories along the AP axis (Pera and Kessel, 1997; Schier *et al.*, 1997). Taken together, these phenotypes demonstrate that DV and AP patterning of the brain can be separated. The experimental embryos presented here do not show any obvious loss in ventral midline patterning. Instead, it appears that the forebrain territories which would normally respond to midline-derived signals have not been specified, so that distinctive growth programmes of structures such as the optic vesicles are not initiated. These embryos therefore presumably lack one or more

signals that operate in combination with midline patterning to achieve normal regionalisation of more dorsal forebrain territories.

Development of the floorplate at the ventral midline of the neural tube has long been thought to depend on inductive signals provided by the underlying notochord (reviewed in Placzek, 1995). Notochord-derived signals can induce floorplate differentiation both *in vitro* and *in vivo*, and selective removal of the notochord *in vivo* results in failure of floorplate differentiation. However, Le Douarin and colleagues have recently challenged this view (see Placzek *et al.*, 2000; Le Douarin and Halpern, 2000 for latest discussion). They propose that floorplate cells originate from the node, unlike the rest of the neural plate, and that specification of floorplate identity begins in the node, before these cells are underlain by any mesoderm. This debate concerns the relationship between neural and mesodermal midline cells at posterior brain and spinal cord levels, and relatively little is known about interactions at more anterior brain levels. Preliminary observations presented here suggest that removal of endoderm and mesoderm produces negligible additional effects on early CNS ventral midline specialisation and growth, in comparison to removal of endoderm alone. This suggests that at forebrain level, midline CNS specification may therefore not be entirely dependent upon signals from axial mesoderm. Unfortunately, this issue could not be examined in detail, due to time constraints. However, it is potentially very interesting and merits further investigation.

CHAPTER VIII

GENERAL CONCLUSIONS AND FUTURE WORK

The main focus of the work presented in this thesis has been to investigate the strategies of anterior neural patterning in amniote embryos. It is necessary therefore, finally, to consider the data as a whole and examine whether differences between head induction in mouse and chick really do exist, or whether the experimental approaches used for each organism have led to the establishment of apparently different models. There is now considerable genetic and embryological evidence to suggest that the anterior visceral endoderm plays an essential role in head induction in the mouse. The AVE is thought to act in synergy with the early gastrula organiser to initiate induction and patterning of the forebrain. In chick, however, results so far suggest that the equivalent tissue, the hypoblast, is not essential for production of complete neural pattern. My experiments described here directly compare the inducing ability of mouse and chick nodes. A graft of a chick node is sufficient to induce a second axis with complete neural pattern; however, a mouse node graft in the same system cannot induce forebrain markers and produces a truncated axis. This does therefore suggest a fundamental difference in the location and/or timing of initial forebrain inductive events between mouse and chick.

Later patterning and maintenance events, performed by node-derived tissues, appear much more conserved. Prechordal mesendoderm has been shown to have an important positive influence on development of forebrain character in overlying neural tissue, in both species. In addition, recent analysis of the *hex* mutant mouse has provided evidence for an important role of the definitive foregut endoderm in maintenance of forebrain character. I propose, on the basis of the work presented here, that the definitive endoderm plays a similar role in the chick. In addition, the effects observed

following interference with this signalling ability in both species, are compatible with a mechanism whereby the definitive endoderm normally acts to establish a signalling centre in the overlying anterior neural ridge. This ANR centre is then thought to be necessary during early somite stages to continue the growth and development of the forebrain anlage. The maintenance of an anterior neural identity at head process stages, and further embellishment of the AP pattern, therefore appear to involve remarkably similar mechanisms in both mouse and chick.

On the basis of this outcome then, it could be suggested that initiation of head development begins earlier in mammalian embryos than it does in avian embryos. This shift in onset of forebrain development during mammalian evolution may reflect the relatively large size of the mammalian cerebral hemispheres, which develop from the telencephalon. Indeed, even at early somite stages, the anterior neural folds in the mouse are extremely large and well-developed, and anterior development is visibly more advanced than the posterior. This imbalance is not so apparent in the chick embryo.

We cannot be sure that timing differences exist, however, until the question of when initiation of anterior neural induction occurs has been answered. It could be that establishment of forebrain character in chick is initiated just as early as in mouse embryos, but by a planar signalling route within the epiblast and not involving the extra-embryonic hypoblast. In the mouse, it could be that AVE cells at the distal tip and EGO progenitors in the proximal epiblast regionalise the epiblast even before overt gastrulation begins. Such influences may sensitise the epiblast, making it

competent to respond to inducing signals during pre-streak and streak stages, and form anterior neural tissue. In the chick as well, a 'pre-neurulation' state is proposed to exist in the unincubated (blastula stage) embryo, and regionalised expression of the competence marker L5 is seen very early.

Formation of the endoderm and mesoderm during gastrulation involves extensive cell re-arrangement. In fact, the AP position of cells in these layers is almost reversed, since the most posterior cells ingress through the streak first and move to an anterior position, followed by originally more antero-lateral epiblast cells. The cell arrangement within the ectoderm, however, changes relatively little during gastrulation. Therefore, although it may be unrealistic to suggest that AP patterning in all tissue layers is initiated prior to gastrulation, it is still feasible that some AP polarity in the ectoderm is established extremely early, while the embryo is still single-layered. One could speculate that the ectoderm may even serve as a point of reference for the underlying tissue layers as they are formed.

Another important question that remains to be answered is when does neural induction itself begin, if indeed it is separable from initial neural patterning. The discovery of more and more potential endogenous inducing molecules suggests that neural induction involves a sequence of cell states and multiple signals. It is intriguing that the organiser expresses such a cocktail of diverse secreted proteins and transcription factors. It may be that these structurally unrelated secreted proteins recognise and antagonise partially non-overlapping subsets of targets. This could therefore enable the organiser to perform its diversity of functions, with the synergistic interactions of the

proteins it secretes, producing a greater effect than one molecule alone. However, it may be that several of the molecules are redundant during neural induction, but are evolutionarily maintained due to later distinct essential signalling functions.

The list of molecules with head inducing activity also continues to grow. A current model proposes that the formation of anterior neural structures requires the combined inhibition of TGFβ and Wnt signalling pathways. The genes encoding TGFβ and Wnt inhibitors are expressed in the relevant tissues for head formation in several organisms. However, gene inactivation studies for some of these genes in mouse have shown no effect on the initiation of anterior neural patterning. Once again, therefore, either the level of genetic redundancy is very high, or other signal(s) still to be identified play a crucial role in the initial steps of anterior neural induction.

Finally, an important concept that has emerged from the recent influx of evidence in the mouse, is that anterior brain development is not a default fate of the neural ectoderm. The discovery of the mouse AVE demonstrates that anterior identity is not passively established as the opposite side of the embryo to the streak. Indeed, the anterior pole looks to be formed by active signalling and cell movement, and may actually be established prior to the posterior end, at the site of streak formation. Based on Nieuwkoop's activation-transformation model, it has long been thought that all neural tissue is initially broadly anterior in character, and that all neural patterning signals are posteriorising and dominate over this initial character. However, recent evidence argues for the presence of anteriorising patterning signals. Default neural character, if it exists, is therefore more likely to be in an intermediate state, requiring

both anterior and posterior patterning signals (in all species) for full elaboration of the AP axis.

Future directions

There are a number of approaches that could be taken to extend the studies presented in this thesis, and to further examine development of the anterior brain in amniote embryos. Firstly, the grafting studies involving young (6.5dpc) mouse AVE could be continued; whether the AVE can synergise with a young node (or EGO) to induce a complete axis, when grafted together into chick, is an important assay and needs further investigation for a decisive answer.

Given that the definitive endoderm is proposed to play an equivalent role in maintaining and embellishing anterior neural identity in both mouse and chick, it would be interesting to directly compare these activities, in a manner similar to the present study of the node. A chick node could be grafted to the periphery of the *area pellucida* for 6 hours, by which time general/broad neural markers such as *sox 3* and *otx 2* should have been induced. The graft could then be removed and replaced with a piece of either chick or mouse definitive endoderm. After 18 hours further culture, the presence of anterior neural markers in the host tissue overlying the endoderm graft could be assayed. If this procedure provided interesting initial results, it could be extended to include grafting definitive endoderm from mouse mutants such as *hex* -/- embryos, thus continuing analysis of the signalling defects in these embryos.

The syndrome produced following removal of the definitive foregut endoderm has produced some interesting insights into the normal functions of this tissue during early development. In order to make the study more complete, it would be good to confirm with molecular markers that other regions of endoderm are unable to perform the same role, when grafted as a replacement. It would also be good to try and develop ways of culturing the embryos for longer following operations, since one of the limitations of this study is that effects of brain pattern are assayed at a stage when the telencephalon is still extremely small.

The present study also proposes two novel functions for the definitive endoderm in the chick, which could be investigated further; establishment of a forebrain signalling centre in the ANR, and induction of stomodaeal ectoderm. The role of the ANR could be examined using a similar method to the mouse; explants of anterior neural plate could be cultured with or without the ANR, and assayed for expression of *BF-1*. The ability of FGF 8 protein to induce *BF-1* expression in isolated anterior neural plate explants could also be examined. A possible strategy for exploring the induction of stomodaeal ectoderm also involves explant culture. Pieces of young (stage 4) anterior ectoderm could be cultured with or without anterior definitive endoderm (stage 4+ to 5), and then inserted beneath the ventral diencephalon of a 10 somite embryo, from which the ventral head ectoderm had been removed. Following further incubation, it could be assayed whether the period of culture with the definitive endoderm increased the incidence of expression of Rathke's pouch markers in the grafted ectoderm.

Finally, the role of axial mesoderm in ventral forebrain patterning merits additional study. A more comprehensive examination of DV forebrain patterning in embryos following endoderm only and endoderm + mesoderm removals should be conducted, using ventral markers such as *Nkx2.1*. In addition, the expression of *chordin* and *shh* in the ventral neural tube at 12 somite stages could be assayed in embryos after axial mesoderm removal at stage 5. Suitable controls would involve mesoderm removal immediately prior to fixation, so that relative intensity of *in situ* hybridisation signal could be properly compared in the two situations. A node grafting strategy may be usefully employed again here, to address the issue of floorplate formation in the ventral neural tube. Previous studies using quail tissue have shown that a grafted node does not contribute any cells to the neural plate induced in the chick host. Therefore, floorplate character in a second axis must have been induced vertically by the underlying node derivatives, and not by insertion of floorplate progenitors into the midline ectoderm. The onset of *chordin* and *shh* expression in neural tissue of second axes could therefore be examined in detail in this novel situation, and compared with normal expression patterns of these genes. In addition, it has been reported that following implants of FGF4-soaked beads in the chick, neural tissue is induced but no axial mesoderm at all is formed. This scenario therefore provides a converse situation; any floorplate character that is present in these ectopic neural plates must have been produced independently of underlying mesoderm. Combined analysis of floorplate gene expression in these different situations may therefore shed light on a currently controversial issue.

In summary, information regarding the early inductive and patterning events in the embryo is expanding at an ever-increasing rate. General principles have begun to be elucidated from the use of several 'model organisms', and insight into the evolution of vertebrate development is being gained. Moreover, the remarkable conservation of many molecular mechanisms adds strength to a comparative approach, and allows some extrapolation of results from different species. Tissue grafting and removal operations in the chick embryo exploit the specific advantages of this system, and can continue to make valuable contributions to our understanding of vertebrate development; in this case, the induction and patterning of the embryonic brain.

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