Early posterior neural tissue is induced by FGF in the chick embryo

Kate G. Storey1,†, Anne Goriely1, Catherine M. Sargent1, Jennifer M. Brown1, Helen D. Burns2, Helen M. Abud2,*, and John K. Heath2

1Department of Human Anatomy, University of Oxford, South Parks Rd, Oxford, OX1 3QX, UK
2Department of Biochemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

*Present address: Peter MacCallum Cancer Institute, Smorgon Family Building, Andrews Place, East Melbourne, Australia
†Author for correspondence (e-mail: kstorey@ermine.ox.ac.uk)

INTRODUCTION

In higher vertebrates, the formation of the central nervous system (CNS) is initiated by signals that emanate from the anterior end of the primitive streak. Cells in this region express a number of fibroblast growth factors (FGFs), a group of secreted proteins implicated in the induction and patterning of neural tissue in the amphibian embryo. Here we exploit the large size and accessibility of the early chick embryo to analyse the function of FGF signalling specifically during neural induction. Our results demonstrate that extraembryonic epiblast cells previously shown to be responsive to endogenous neural-inducing signals express early posterior neural genes in response to local, physiological levels of FGF signal. This neural tissue does not express anterior neural markers or undergo neuronal differentiation and forms in the absence of axial mesoderm. Prospective mesodermal tissue is, however, induced and we present evidence for both the direct and indirect action of FGFs on prospective posterior neural tissue. These findings suggest that FGF signalling underlies a specific aspect of neural induction, the initiation of the programme that leads to the generation of the posterior central nervous system.

SUMMARY

Signals that induce neural cell fate in amniote embryos emanate from a unique cell population found at the anterior end of the primitive streak. Cells in this region express a number of fibroblast growth factors (FGFs), a group of secreted proteins implicated in the induction and patterning of neural tissue in the amphibian embryo. Here we exploit the large size and accessibility of the early chick embryo to analyse the function of FGF signalling specifically during neural induction. Our results demonstrate that extraembryonic epiblast cells previously shown to be responsive to endogenous neural-inducing signals express early posterior neural genes in response to local, physiological levels of FGF signal. This neural tissue does not express anterior neural markers or undergo neuronal differentiation and forms in the absence of axial mesoderm. Prospective mesodermal tissue is, however, induced and we present evidence for both the direct and indirect action of FGFs on prospective posterior neural tissue. These findings suggest that FGF signalling underlies a specific aspect of neural induction, the initiation of the programme that leads to the generation of the posterior central nervous system.

Key words: Fibroblast growth factor, Neural induction, Hensen’s node, Chick

INTRODUCTION

In higher vertebrates, the formation of the central nervous system (CNS) is initiated by signals that emanate from the anterior end of the primitive streak (Waddington, 1932; Gallera, 1971; Storey et al., 1992, 1995; Beddington, 1994). Grafts of this region (known as Hensen’s node in the chick embryo) induce extraembryonic epiblast cells to form an organised neural axis that expresses a range of anteroposterior marker genes and which contains differentiating neurons (Dias and Schoenwolf, 1990; Storey et al., 1992). However, the neural-inducing signals produced by Hensen’s node have yet to be identified.

In the chick, cells competent to respond to such inducing signals express L5220 (a cell-surface glycoprotein, Roberts et al., 1991; Streit et al., 1997) whose domain of expression at early primitive streak stages includes the prospective neural plate and the region of extraembryonic epiblast cells responsive to Hensen’s node (Roberts et al., 1991). Expression of the L5 antigen can be maintained by Hepatocyte growth factor/scatter factor, a secreted factor that is present in Hensen’s node, but which does not induce neural tissue (Streit et al., 1995, 1997). A number of genes expressed in the early neural plate have also been identified and shown to be induced by Hensen’s node, these include the transcription factor Sox2 (Streit et al., 1997) and a follistatin-like (FLIK) molecule (Patel et al., 1996). However, the functions of these genes and the identity of the signals that regulate them have yet to be established.

Among the first nerve cells to differentiate in the chick are hindbrain reticular neurons and spinal interneurons, the precursors of which arise adjacent to the anterior primitive streak during the early phase of neural induction (Sechrist and Bronner-Fraser, 1991; also see Lawson et al., 1991). The precursors of the posterior CNS (posterior hindbrain and spinal cord), therefore, appear to be among the first to respond to neural-inducing signals from Hensen’s node. We have recently shown that signals from the node induce expression of the basic helix-loop-helix (bHLH) transcription factor cash4 and the homeodomain-containing gene Sax-1 (Henrique et al., 1997). These genes are expressed in the precursors of the posterior CNS at HH5-6 (Hamburger and Hamilton, 1951 stages) and therefore after L5, Sox 2 and FLIK. cash4 is a homologue of the Drosophila achaete-scute genes which are required for the formation of neural precursors in the fly (for review, see Campuzano and Modolell, 1992; Campos-Ortega, 1993). Overexpression of cash4 in the Xenopus embryo results in the expansion of the neural plate and an increase in the number of neurons produced. Further, expression of this chick gene in Drosophila reveals that it is a functional as well as structural homologue of the fly achaete-scute genes; it is able to rescue sensory bristle formation in flies lacking both achaete and scute (Henrique et al., 1997). Our functional studies therefore implicate cash4 in a similar early neural specification process in the posterior regions of the chick embryo (Henrique et al., 1997).

We have also demonstrated that cash4 expression can be induced in anterior neural tissue following grafting of fibroblast growth factor (FGF)-soaked beads at primitive streak stages...
of FGF-signalling pathways.

Embryo by making use of local application of FGFs bound to induction in higher vertebrates. We have accordingly addressed midbrain development (Crossley et al., 1996a; Lee et al., 1995a,b; own observations) and might therefore play a role in the initiation of the neural programme. FGFs are characterised by shared features of sequence conservation and gene structure as well as the ability to bind to a conserved family of transmembrane signalling receptors of the tyrosine kinase class and heparan sulphate proteoglycans present in the extracellular matrix (reviewed Ornitz et al., 1996; Wilkie et al., 1995). FGF signals are therefore unlikely to diffuse far from their source and probably act locally on cells in their immediate vicinity. It is thus striking that FGFs are expressed during neural induction in and adjacent to the cells that will give rise to the posterior CNS.

In the early amphibian embryo, FGFs have been shown to have general posteriorising effects ( Slack and Tannahill, 1992) which act on both mesodermal and neural tissues (e.g. Isaacs et al., 1994; Cox and Hemmati-Brivanlou, 1995; Pownall et al., 1996). However, experiments assessing the function of FGFs in the induction of the amphibian nervous system are at present inconclusive. While FGFR1-mediated signalling appears not to be required for the induction and anteroposterior patterning of the nervous system in the intact embryo (Kroll and Amaya, 1996) and some explants of prospective epidermis (the animal cap) do not form neural tissue in response to FGF (Cox and Hemmati-Brivanlou, 1995), other reports suggest that this tissue does express neural genes in response to FGF and that FGF signalling in the ectoderm is required for its later response to neural-inducing signals (Kengaku and Okamoto 1993, 1995; Lamb and Harland, 1995; reviewed by Doniach, 1996; Launay et al., 1996). Further, mice mutant for FGFR1 do not survive long enough for the role of FGF signalling in anteroposterior patterning to be assessed (Deng et al., 1994; Yamaguchi et al., 1994). Indeed, while some neural tissue appears to be induced in these embryos, they fail to undergo regression movements and may therefore fail to form more posterior regions of the CNS; a phenotype similar to that observed in chick embryos treated globally with molecules that bind FGF, heparin or suramin (Riese et al., 1995). Finally, while mouse embryos chimeric for FGFR4 exhibit apparent outgrowth of the CNS (Abud, 1995), mice homozygous for the null allele of Fgfr4 fail to develop far enough to assess the role of FGF signalling in early neural development (Feldman et al., 1995).

In all these studies, cells are subjected to activation or inhibition of FGF signalling over prolonged periods of time in a spatially unrestricted manner. It is therefore difficult, using these experimental approaches, to define the action of FGF signalling at specific times during neural development. While the role of Fgfr8 has recently been specifically assessed during midbrain development (Crossley et al., 1996a; Lee et al., 1997), there has been no study of FGF function during neural induction in higher vertebrates. We have accordingly addressed the role of FGF in the induction of neural tissue in the chick embryo by making use of local application of FGFs bound to beads, which permits tightly defined temporal and spatial activation of FGF-signalling pathways.

Using this approach, we have assessed the neural-inducing activity of FGFs presented to extraembryonic epiblast cells that we have shown to be responsive to neural-inducing signals from Hensen’s node, but which normally give rise to extraembryonic membranes (Storey et al., 1992; 1995). We show that FGF-soaked heparin beads can maintain and/or induce the L5 epitope in extraembryonic epiblast cells and induce neural tissue that specifically expresses markers of the early posterior nervous system, including Sax-1 and cash4. Strikingly, this FGF-induced tissue does not express anterior neural markers and does not undergo neuronal differentiation. Induction of early posterior neural genes takes place in the absence of axial mesoderm. However, FGF signalling does induce prospective mesoderm (indicated by the presence of cells co-expressing Brachury (Bra) and Delta-1). This raises the possibility that posterior neural genes are induced indirectly, by signals provided by prospective mesoderm. Significantly, this ‘indirect’ effect may also be FGF mediated as Fgfr8 is induced shortly after Bra in response to FGF beads and is expressed in prospective mesoderm cells and the adjacent posterior neuroepithelium itself. The co-localisation of Bra and cash4 in epiblast cells around FGF beads also supports the possibility that FGF acts directly within the prospective neuroepithelium, downstream of bra expression in this tissue.

This study is the first to assess the role of FGF signalling in the induction of neural tissue in an amniote embryo. It shows that FGF signals can mimic a very specific aspect of the neural-inducing capacity of the organiser region; the initiation of the programme that leads to the generation of the posterior nervous system.

MATERIALS AND METHODS

Preparation of FGF proteins

Human FGF-2 protein was expressed under the control of the trp promoter of plasmid pFC80 in E. coli strain FICE127 (both a gift of Dr Antonella Isacchi, Pharmacia Biopharmaceuticals) and purified by heparin affinity chromatography. Murine FGF-8A, FGF-8B and FGF-9 were expressed in E. coli as glutathione-s transferase fusion proteins, purified by glutathione affinity chromatography, proteolytic cleavage and heparin affinity chromatography. Recombinant human FGF-4 protein was a gift of Dr David Rogers (Genetics Institute). Recombinant FGF-7 was purchased from R and D Systems (Europe) Ltd. The identity of all recombinant proteins was verified by amino acid sequencing.

Preparation and detection of DIG-FGF-2

Digoxigenin-labelled FGF-2 (DIG-FGF-2) was prepared as described by Gleizes et al. (1994). Digoxigenin malemide was purchased from Dr Antonella Isacchi, Pharmacia Biopharmaceuticals) and purified by heparin affinity chromatography. Murine FGF-8A, FGF-8B and FGF-9 were expressed in E. coli as glutathione-s transferase fusion proteins, purified by glutathione affinity chromatography, proteolytic cleavage and heparin affinity chromatography. Recombinant human FGF-4 protein was a gift of Dr David Rogers (Genetics Institute). Recombinant FGF-7 was purchased from R and D Systems (Europe) Ltd. The identity of all recombinant proteins was verified by amino acid sequencing.

Implanting FGF-soaked beads

Heparin-coated acrylic beads (selected to be approx. 75-100 μm in diameter; Sigma H5263) were soaked in a FGF of choice for 30 minutes at room temperature and washed three times in phosphate-buffered saline (PBS) prior to implantation. Heparin-coated beads simply rinsed in PBS served as controls. Embryos at stages HH3-4+ were set up in New culture (New, 1955) ventral side up and individual beads were positioned
in contact with extraembryonic epiblast cells. Embryos with implanted beads were incubated at 37°C for desired periods of time up to 36 hours.

**Dil-labelling**

Epiblast cells were labelled with the lipophilic dye DiI (1,1’-dioctadecyl-3,3,3¢,3¢-tetrachloroindocarbocyanineperchlorate, Molecular Probes) using standard procedures (e.g. Stern, 1990; Izpisúa-Belmonte et al., 1993). The spatial relationship between the bead and labelled cells was confirmed using a cooled CCD camera to detect low level fluorescence through a fluorescence filter set (to reduce photobleaching and the production of toxic free-radicals) prior to incubation of embryos for the desired period. Embryos were then fixed in formal saline/EGTA and the pattern of fluorescently labelled cells observed using a rhodamine filter set to reveal cell movement. Fluorescent and bright-field images were superimposed using Adobe Photoshop.

**In situ hybridisation and immunocytochemistry**

Following New culture, embryos were fixed in 4% formaldehyde/PBS for 1 hour to overnight. Whole-mount in situ hybridisation protocol was adapted after Izpisúa-Belmonte et al. (1993). In some cases, embryos were further processed for immunocytochemistry with the Brachyury antibody (TN-1, kindly provided by Prof. Bernhard Herrmann). Briefly, embryos were fixed for 30 minutes in formal saline at 4°C, washed in PBS and placed in a blocking solution (PBS containing 3% bovine serum albumin (BSA), 1% Triton X-100, 0.01% thimerosal and 5% heat-inactivated normal goat serum) for 1 hour to overnight. Whole-mount immunocytochemistry with Not-1, Inv4D9 and 3A10 antibodies were added 1:500 and embryos incubated for 6 hours at room temperature. After extensive washing in PBS, embryos were incubated in blocking solution with peroxidase-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch) (1:50) overnight at 4°C. Embryos were then washed, underwent the usual dianinobenzidine tetrahydrochloride reaction and were postfixed in 4% formal saline. Whole-mount immunocytochemistry with Not-1, Inv4D9 and 3A10 antibodies were described as described by Storey et al. (1992, 1995) and the L5 antibody was used as described by Streit et al. (1995).

**Marker gene expression patterns**

**Mesoderm markers**

All the following genes or proteins are expressed in mesodermal tissues, however, all but the first are also expressed in the other tissues including the nervous system:

- **The Not-1 antibody recognises a notochord-specific antigen** (Yamada et al., 1991) and is expressed shortly after this axial mesoderm emerges from Hensen’s node at HH7.
- **goosecoid** is expressed in the node and later in the emerging prechordal plate (mesoderm and endoderm) and in the anterior neural folds from HH8 (Izpisúa-Belmonte et al., 1993).
- **Brachyury** (Brachyury) is expressed in posterior regions of the chick embryo from primitive streak stages; it is found in prospective mesodermal cells in the primitive streak and in surrounding epiblast cells (Kispert et al., 1995). These domains of expression retain their spatial relationship with the regressing node as the posterior CNS is laid down. Bra is later also expressed in the notochord from HH5, in prospective posterior endoderm, and in the neural tube anterior to the first formed somite (Kispert et al., 1995; Knezevic et al., 1996). Bra is therefore a marker of all three germ layers in posterior regions of the embryo.
- **fgf8** is also expressed in multiple germ layers; it is found in the anterior primitive streak (excluding the node) and flanking epiblast and in a number of discrete sites in the developing CNS, including the forebrain, the isthmus of the midbrain and the newly formed posterior neural plate (Heikinheimo et al., 1994; Crossley and Martin 1995; Mahnood et al., 1995; own observations).
- **Deltal-1** is expressed in presomitic mesoderm and nascent neurons (Henrique et al., 1995).

**Neural markers**

- **tailless** is a steroid-hormone receptor expressed in the diencephalon from HH10 (Yu et al., 1994).
- **Engrailed-2** (En-2) is expressed in the posterior midbrain and rhombomere 1 of the hindbrain (Patel et al., 1989).
- **Krox-20** is expressed in the hindbrain in rhombomeres 3 and 5 from HH9 (Wilkinson et al., 1989).
- **cash4** is expressed at HH5 in the epiblast flanking Hensen’s node which will give rise to the posterior CNS and maintains its spatial relationship with the regressing node during the laying down of this region of the nervous system (Henrique et al., 1997). cash4 expression extends laterally into prospective epidermal tissue and also has an extraembryonic domain in the forming blood islands. Thus while it is the first gene to distinguish prospective posterior neural tissue from the anterior neural plate, it is not a neural-specific gene.
- **Sax1** is expressed after cash4 in the prospective CNS either side of the regressing Hensen’s node at HH6-7 (Spann et al., 1994; own observations). In contrast with cash4, Sax-1 expression is confined to the nervous system and is therefore the earliest neural-specific marker expressed in the posterior regions of the embryo.
- **Hox b9** is expressed (at HH8-9) in prospective spinal cord and posterior mesoderm, its anterior boundary of expression is later established below the level of the somite 9 in the central nervous system, while its mesodermal domain is located more posteriorly at the 18/19 somite level.
- The L5 antibody recognises an antigen expressed in the epiblast cells throughout the anterior two thirds of primitive streak stage embryos but which becomes restricted to the neural plate by HH8 (Roberts et al., 1991; Streit et al., 1995, 1996).
- The 3A10 antibody (a gift of Dr Jane Dodd) recognises a neurofilament-associated antigen expressed in the chick by early differentiating neurons in the posterior hindbrain, anterior spinal cord and diencephalon at HH11-12 (Yamada et al., 1991; Storey et al., 1992).

**RESULTS**

Grafts of Hensen’s node can induce extraembryonic epiblast cells to form an ectopic neural axis consisting of both anterior and posterior CNS that contains differentiating neurons (Storey et al., 1992). We have used this in vivo approach to assess the extent to which FGF signalling can mimic these organisational properties by presenting FGF4 on heparin-coated beads to the same extraembryonic epiblast cells. FGF4 was chosen because it contains a signal sequence and because it binds to a number of FGF receptors and is therefore likely to emulate the activity of other FGFs. In all cases, beads were soaked in 50 µg/ml FGF4 (FGF beads) (see below) grafted into HH3-3+ embryos (Fig. 1) and then incubated for 20-22 hours in New culture (New, 1955) until they reached HH8-9 (unless stated otherwise).

**FGF induces early posterior neural tissue in extraembryonic epiblast**

FGF beads, but not control PBS washed beads (PBS beads), induce ectopic structures expressing the early posterior neural markers Sax-1 and cash4 (Table 1; Fig. 2A-D). Hoxb9, which is also expressed posteriorly in neural tissue and mesodermal tissue at HH8-9, is also induced in response to FGF beads (Table 1; Fig. 2E). FGF beads implanted in embryos incubated until they reached HH5 (n=4/4) as well as to HH8-9 (Table 1) maintain and/or induce expression in extraembryonic tissue of the L5 epitope (Fig.2H), an early pan-neural marker and possible indicator of neural competence (Roberts et al., 1991; Streit et al., 1995).
FGF does not induce anterior neural markers

To ascertain that FGF4 specifically initiates the generation of the posterior neural tissue, we assessed its ability to induce ectopic expression of a panel of neural genes characteristic of anterior regions: a forebrain marker, tailless, a midbrain/hindbrain boundary marker, Engrailed 2 and a marker of rhombomeres 3 and 5 of the hindbrain, Krox-20. FGF beads do not induce ectopic expression of any of these genes when placed in the extraembryonic epiblast of HH3-3+ embryos (Table 2; Fig. 2F,G and 2I-L).

Hensen’s nodes grafted into later stage hosts (HH4) induce truncated axes, which appear to constitute largely anterior CNS (Storey et al., 1992). It has also been observed in the amphibian embryo that competence to respond to FGF changes during development (Lamb and Harland, 1995) such that age ectoderm loses competence to respond to FGF by expressing posterior neural markers, but gains the ability to express progressively more anterior neural genes. We therefore assessed the ability of the extraembryonic epiblast to respond to FGF beads in later stage embryos. While the ability to induce ectopic Sax1 and cash4 is lost by HH4, this activity is not replaced by the induction of the more anteriorly expressed markers (tailless, Engrailed and Krox-20) (Table 2). An alternative hypothesis is that, while high FGF concentrations induce expression of posterior neural genes, low levels induce genes characteristic of the anterior CNS (Kengaku and Okamoto, 1995; Lamb and Harland, 1995). We therefore also assessed the ability of FGF to induce expression of tailless and Krox-20 at lower concentrations (Fig. 4). Neither Krox-20, nor tailless are induced by FGF concentrations 10-fold lower (beads soaked in 5 μg/ml FGF4) than those able to induce cash4 and Sax-1 (Fig. 4). Indeed, FGF beads placed in HH4 hosts and beads soaked in this lower FGF concentration elicited only a small ectopic accumulation of epiblast cells. In contrast with grafts of the young Hensen’s node, therefore, FGF signalling does not induce anterior neural tissue, but specifically elicits expression of genes characteristic of the inchoate posterior CNS.

FGF signalling does not elicit neuronal differentiation

To determine whether FGF expression in the extraembryonic epiblast is sufficient to elicit a neural programme that proceeds to the onset of neuronal differentiation, embryos were grafted with FGF beads or PBS beads and allowed to develop to a later stage, HH12-13. The resulting ectopic structures were then assessed for the expression of a neurofilament-associated protein recognised by the antibody 3A10. Hensen’s nodes derived from HH3-4 embryos and implanted in extraembryonic epiblast of HH3-3+ embryos induce ectopic neural axes expressing this neurofilament-associated antigen within this period of time (Storey et al., 1992). FGF beads however, do not induce 3A10-positive cells within the same period (n=12; Fig. 3A) nor is this antigen detected around FGF beads implanted within the extraembryonic epiblast in ovo and cultured for a much longer period to HH20-24 (n=6; Fig. 3B,C). These data suggest that, while FGF can initiate the neural programme in extraembryonic epiblast cells, it is not sufficient to elicit neuronal differentiation, which therefore requires the action of additional signals.

FGF DOES NOT INDUCE AXIAL MESODERM IN EXTRAEMBRYONIC EPIBLAST

It is important to establish in this assay whether FGF beads induce posterior neural tissue directly or as a consequence of the prior induction of axial mesoderm. To test this, we therefore assayed for the induction of the Not-1 antigen, which specifically identifies the notochord from HH7 and for the expression of goosecoid, which at the time assessed is expressed in prechordal mesendoderm anterior to the emerging notochord as well as in the ventral diencephalon. Neither the Not-1 antigen (0/6 FGF beads, 0/4 PBS beads) nor goosecoid mRNA (0/7 FGF beads, 0/6 PBS) were induced in response to FGF or PBS beads placed in contact with the extraembryonic epiblast at HH3+ (Fig. 5A-C). Together these findings suggest that axial mesoderm is not formed in response to FGF.

However, brachyury (bra) (Herrman et al., 1990; see Beddington et al., 1992 for review) is induced in response to FGF beads at HH3-3+ and in older HH4-4+ hosts (Table 2; Figs 4, 5C). Expression of this T-box-containing transcription factor is an immediate early response to FGF signalling (Smith et al., 1997). These findings show that FGF signalling can induce neural tissue, which expresses a combination of transcription factors characteristic of early posterior neural tissue.

Table 1. Induction of neural genes by FGF in HH3-3+ extraembryonic epiblast

<table>
<thead>
<tr>
<th>Gene</th>
<th>HH3-4 FGF</th>
<th>HH3-3+ PBS</th>
<th>HH3-4 PBS</th>
<th>HH4-4+ FGF</th>
<th>HH4-4+ PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>saxl</td>
<td>0/2</td>
<td>0/4</td>
<td>0/4</td>
<td>0/2</td>
<td>0/4</td>
</tr>
<tr>
<td>L5</td>
<td>0/2</td>
<td>0/6</td>
<td>0/6</td>
<td>0/2</td>
<td>0/6</td>
</tr>
<tr>
<td>hostb</td>
<td>0/2</td>
<td>0/4</td>
<td>0/4</td>
<td>0/2</td>
<td>0/4</td>
</tr>
</tbody>
</table>

Table 2. FGF induces posterior, but not anterior neural genes in HH3-3+ hosts and does not elicit neural genes in older HH4-4+ host embryos

<table>
<thead>
<tr>
<th>Gene</th>
<th>HH3-3+ FGF</th>
<th>HH4-4+ FGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>tlx</td>
<td>0/2</td>
<td>0/5</td>
</tr>
<tr>
<td>en2</td>
<td>0/11</td>
<td>0/6</td>
</tr>
<tr>
<td>krox20</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>saxl</td>
<td>9/16</td>
<td>0/4</td>
</tr>
<tr>
<td>cash4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>bra</td>
<td>7/10</td>
<td>4/4</td>
</tr>
</tbody>
</table>

Table 3. FGF induction of neural genes in HH3-3+ host embryos

<table>
<thead>
<tr>
<th>Gene</th>
<th>HH3-3+ FGF</th>
<th>HH3-3+ PBS</th>
<th>HH4-4+ FGF</th>
<th>HH4-4+ PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>saxl</td>
<td>0/2</td>
<td>0/4</td>
<td>0/2</td>
<td>0/4</td>
</tr>
<tr>
<td>L5</td>
<td>0/2</td>
<td>0/6</td>
<td>0/2</td>
<td>0/6</td>
</tr>
<tr>
<td>hostb</td>
<td>0/2</td>
<td>0/4</td>
<td>0/2</td>
<td>0/4</td>
</tr>
</tbody>
</table>

Table 4. FGF induction of neural genes in HH4-4+ host embryos

<table>
<thead>
<tr>
<th>Gene</th>
<th>HH3-3+ FGF</th>
<th>HH3-3+ PBS</th>
<th>HH4-4+ FGF</th>
<th>HH4-4+ PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>saxl</td>
<td>0/2</td>
<td>0/4</td>
<td>0/2</td>
<td>0/4</td>
</tr>
<tr>
<td>L5</td>
<td>0/2</td>
<td>0/6</td>
<td>0/2</td>
<td>0/6</td>
</tr>
<tr>
<td>hostb</td>
<td>0/2</td>
<td>0/4</td>
<td>0/2</td>
<td>0/4</td>
</tr>
</tbody>
</table>
Early posterior neural genes induced by FGF

477

Early posterior neural genes induced by FGF (1991; Isaacs et al., 1994) and in the chick Bra is expressed in presumptive mesodermal tissues of the primitive streak and subsequently in notochord and endodermal tissues as well as in the posterior CNS (Kispert et al., 1995; Knezevic et al., 1996). As Bra is only co-expressed with Delta-1 in the primitive streak, we combined in situ hybridization for Delta-1 with immunocytochemistry for the Bra antibody in order to identify cells that have characteristics of the prospective mesoderm. In most cases (16/17), Bra and Delta-1 are co-expressed in response to FGF. In many cases (8/16), they are expressed together in a block of cells at a distance from the bead which resembles the regressed primitive streak (Fig. 5D-F). However, not all Bra-expressing cells co-express Delta-1 (Fig. 5F) and some Bra-positive cells are also found in the immediate vicinity of the bead (Fig. 5E). Together, these findings indicate that, while ectopic FGF does not induce axial mesoderm, it can induce expression of genes characteristic of prospective mesoderm.

Induction of early posterior neural genes is preceded by expression of brachyury and Delta-1

The presence of Bra/Delta-expressing cells in this assay indicates that prospective mesodermal cells might be induced by FGF and it is possible that, in turn, these cells produce signals that elicit expression of early posterior neural genes. We investigated this possibility further by assessing the temporal sequence in which bra, Delta-1 and cash4 are expressed. FGF induces bra and Delta-1 in extraembryonic epiblast cells within 6 hours, while cash4 is only expressed 10-12 hours after FGF.
is presented to extraembryonic epiblast cells (Table 3; Fig. 6A-D). The expression of \(b\)ra and Delta-1 is therefore a prelude to cash4 induction. It is thus possible that FGF signalling acts ‘indirectly’ via non-FGF signals provided by prospective mesodermal tissue, to induce early posterior neural genes.

FGF4 induces Fgf8

In view of the different kinetics of bra/Delta-1 and cash4 induction by FGF signalling, we investigated the possibility that FGF4 might also induce activation of other FGF genes (as reported by Crossley et al., 1996b). We find that Fgf8 is induced by FGF4 beads in the extraembryonic epiblast within 8 hours and therefore prior to cash4 (Fig. 6E; Table 4). Fgf8 is normally expressed in both prospective mesoderm (the primitive streak) and also in the posterior neural plate where its domain of expression overlaps with Bra and cash4 as well as Sax1 (see below Fig. 7). This finding therefore identifies Fgf8 as a signal downstream of FGF4 signalling which may act ‘directly’ on or within prospective neural tissue to induce early posterior neural genes (see below).

Bra and cash4 are co-expressed in a subset of epiblast cells

After 6 hours incubation, Bra is expressed in cells surrounding FGF beads, however, by 12 hours cells closest to the bead now express cash4. In embryos in which cash4 in situ hybridisation is combined with the Bra antibody, we find that some cells express both cash4 and Bra (Fig. 6F). This suggests that exposure to FGF elicits a succession of gene expression from Bra to cash4, in the same cell population. Strikingly, this sequence of events mirrors the temporal pattern in which these two genes are normally expressed in prospective posterior neural tissue. Bra is expressed prior to the onset of cash4 at HH5-6 in the epiblast adjacent to the primitive streak, (Fig. 3 in Kispert et al., 1995; Henrique et al., 1997) and, by HH8-9, the overlap between these two genes (and Sax-1 and Fgf8) is confined to the most recently formed posterior neural plate (Fig. 7A-J; and see Fig. 4 in Kispert et al., 1995). These findings further support the direct action of FGF signals on the prospective neuroepithelium, which could act via brachyury expression in this tissue.

A specific subset of FGFs induce cash4 and Bra

We assessed the ability of a variety of FGF family members with differing receptor specificities to induce expression of cash4 and Bra in order to establish the ligand-specificity of this effect and to deduce the possible receptor-mediated signalling pathways involved (Table 5). While FGF7 does not induce Bra or cash4, FGF2, FGF4-FGF9 and FGF8b all induce expression of both these genes. Given the binding specificities of these FGFs for specific receptors (reviewed by Ornitz et al., 1996), we can rule out transduction through FGFR2 (IIib) which specifically mediates the activity of FGF7. Chick FGFR1 and FGFR2 are both expressed in the extraembryonic epiblast at HH3-3+, while FGFR3 is expressed most highly in the primitive streak and only weakly in surrounding epiblast cells (data not shown). The most likely receptors transducing FGF presented to extraembryonic epiblast are therefore the IIIC splice variants of FGFR1 and/or FGFR2. It is significant that FGF8b is able to induce cash4 as well as Bra, as this supports an endogenous role for this gene in the induction of early posterior neural genes.

Concentration effects and cell movements in response to FGF

Previous studies of the activity of FGF bound to heparin-coated beads (e.g., Cohn et al., 1995; Crossley et al., 1996) have not defined the concentration of FGF required for signalling. It is, however, important to establish that FGF-induced neural induction occurs at physiologically relevant concentrations of ligand (Slack, 1994). We soaked heparin-coated beads in a range of FGF4 concentrations (0.5-50 µg/ml) and assessed their ability to induce expression of Bra (Fig. 4). We find that beads soaked in a concentration between 0.5 and 5 µg/ml elicit Bra expression in half the cases examined, while a maximal response is achieved when beads are soaked in 50 µg/ml FGF4. By comparison, the data of Ornitz et al. (1996) indicate that

![Fig. 4. Heparin-coated beads were soaked in a range of FGF4 concentrations (µg/ml) and juxtaposed with extraembryonic epiblast cells in HH3-3 embryos, which were then cultured until they reached HH8-9. The activity of different concentrations of FGF presented by the beads was assessed in terms of the ability to induce expression of Bra, cash4, Sax-1, Krox20 and Tlx. While Bra is induced by FGF presented by beads soaked in 0.5 µg/ml cash4 and Sax-1 are only expressed in response to higher FGF concentrations. Krox20 and tailless expression are not elicited by FGF signalling. (*a* values on columns tops).](image-url)

| Table 3. Response times of Bra, Fgf8 and cash4 to FGF signalling |
|------------------|------------------|------------------|
|                  | 4 hours | 6 hours | 8 hours | 10 hours |
| bra              | 0/4 (0%) | 8/8 (100%) | 6/10 (100%) | ND         |
| Delta-1          | ND | 6/12 (50%) | 10/10 (100%) | ND         |
| Fgf8             | 0/4 (0%) | 0/4 (0%) | 6/10 (60%) | ND         |
| cash4            | 0/4 (0%) | ND | 0/8 (0%) | 5/12 (71%) |

| Table 4. A subset of FGFs induce expression of Bra and cash4 |
|------------------|------------------|------------------|
|                  | conc. | Bra | cash4 |
| FGF2             | 50 µg/ml | 4/4 | 4/4   |
| FGF4             | 50 | 6/6 | 4/6   |
| FGF7             | 300 | 0/12 | 0/12 |
| FGF8a            | 60 | 0/10 | 0/10 |
| FGF8b            | 6 | 2/2 | 2/7 |
| FGF9             | 50 | 4/4 | 4/4   |
the Ed50 for FGF4 activating FGF-R2c and R1c in transfected cell lines is about 0.5 nM. Combined with a clear dose-response curve (Fig. 4), this suggests that FGF beads present physiological levels of ligand, although we cannot calculate the exact amount of FGF available to surrounding cells. FGFs bind locally to cell surface receptors and heparan sulphate proteoglycans present in the extracellular matrix (Wilkie et al., 1995). FGFs should therefore only be presented to cells in the immediate vicinity of a bead. In order to define the cell population directly exposed to FGFs in this assay, the distance of diffusion from heparin beads was assessed by immunocytochemical detection of digoxigenin (DIG)-labelled FGF2. While this growth factor is detected only on the surface of beads soaked in 0.5 μg/ml DIG-FGF (Fig. 8A), DIG-FGF is restricted to cells immediately adjacent to beads soaked in concentrations of 5 μg/ml (Fig. 8B). At 50 μg/ml DIG-labelled FGF2 is detected 4-6 cell diameters from the beads (Fig. 8C; also assessed in sections, not shown). The lower level of our immunocytochemical detection of DIG-FGF therefore correlates with the lowest concentrations at which we can detect Bra expression around a bead. Together, these data indicate that beads soaked in 50 μg/ml FGF present local physiological levels of growth factor.

FGFs are known to stimulate both cell movement and proliferation (Wilkie et al., 1995). In order to define further the cell population exposed to FGF, we compared the movement of Dil-labelled extraembryonic epiblast cells in response to beads washed in PBS or soaked FGF. We observe no dramatic difference between the movement of cells in contact with PBS (n=14) or FGF beads (n=23). In both cases, Dil-labelling is found in the vicinity of the bead and in trails of cells that have moved away from the bead. Thus, although cells move away from FGF beads placed in the extraembryonic epiblast, this behaviour appears to reflect normal morphogenetic movements in this region, rather than a response evoked by FGF signalling (Fig. 8D,E). We also assessed the possibility that FGF-stimulated extraembryonic epiblast cells recruit cells from the embryonic epiblast. Epiblast cells at the lateral edge of the prospective neural plate were labelled with Dil at HH3-3+ and an FGF bead placed in the adjacent extraembryonic epiblast (Fig. 8F). In all cases (14/14), no Dil-labelled cells were found in the vicinity of the bead, following overnight culture (to HH8-9) (Fig. 8G). Thus, while cell proliferation may expand the population of cells responding to this local source of growth factor, we show that embryonic epiblast cells are not recruited in response to local application of FGF to the extraembryonic epiblast.

**DISCUSSION**

This is the first study to examine the role of FGF signalling in the induction of neural tissue in an amniote embryo. FGFs are expressed in the organiser region Hensen’s node/anterior primitive streak; a source of signals that induces both anterior and posterior neural tissue which subsequently undergoes neuronal differentiation. We demonstrate that, in comparison with Hensen’s node, FGF signalling at primitive streak stages leads to the induction of a very specific subset of genes associated with the laying down of the posterior nervous system. We show that ectopic FGF maintains and/or induces expression of the early neural marker L5 in extraembryonic epiblast cells and elicits the expression of the transcription factors *cash4* and *Sax-1*, genes expressed transiently during the formation of the posterior nervous system. Further, this early posterior neural tissue forms without the induction of anterior neural markers and does not undergo the complete neural programme culminating in neuronal differentiation. We present evidence for both direct and indirect actions of FGF signalling on the prospective neuroepithelium. While axial mesoderm does not form in response to FGF signalling, the co-expression of Bra and Delta-1 in some cells suggests that presumptive mesodermal tissue is formed. Neural-inducing signals could therefore be provided indirectly by presumptive mesoderm. However, these ‘indirect’ signals may also be FGF-mediated as FGF4 beads induce *Fgf8*, which is expressed in both prospective mesoderm and the forming posterior neural plate. The co-localisation of Bra and *cash4* in cells around FGF beads further suggests that the induction of posterior neural genes could be mediated directly via Bra expression in the prospective neuroepithelium. These findings therefore show that FGF signalling in an early amniote embryo can mimic a specific subset of node activities, which include the maintenance of neural competence as well as the induction of early posterior neural genes.

**The assay**

We have used the large and accessible chick embryo to analyse the effects of FGF activity in a spatiotemporally defined manner. By placing FGF-soaked heparin beads in contact with extraembryonic epiblast cells, previously shown to be competent to respond to endogenous neural-inducing signals, we have tested the neural-inducing activity of FGF. While the extraembryonic epiblast may not be a completely naïve tissue, it contrasts favourably with *Xenopus* animal cap ectoderm which contributes to embryonic structures and appears to be prepatterned (Sokol and Melton, 1991). In comparison, chick extraembryonic epiblast is fated to give rise to extraembryonic membranes and is physically remote from the embryonic axis. Our Dil-labelling studies also demonstrate that FGF beads placed in the extraembryonic epiblast do not recruit embryonic epiblast cells that might have been exposed to neural-inducing signals from Hensen’s node. Further, although only the medial half of the extraembryonic epiblast is competent to respond to neural-inducing signals (Storey et al., 1992), it is striking that ectopic gene expression is often found on the side of FGF beads furthest from the host embryo (see for example Figs 2A, 6B). This pattern is inconsistent with the influence of other neural-inducing signals emanating from the embryonic axis and may reflect the radial expansion of the embryo at these stages.

By assessing the effects of FGF signalling with respect to the FGF concentration in which beads were soaked, we have also identified a ‘soaking’ concentration that delivers physiologically relevant levels of FGF. Further, using DIG-labelled FGF2, we show that at these concentrations FGF diffuses from the heparin bead and becomes associated with cells in its immediate vicinity. The induction of *Fgf8* in response to FGF4, however, may relay FGF signals beyond this region. Indeed, *Fgf8* expression, along with the morphogenetic movements of extraembryonic epiblast cells may account for patterns of gene expression that extend outside the domain defined by the spread of DIG-labelled FGFs. Comparison of the effects of six different FGF members has also allowed us to establish the ligand-specificity of observed effects and to deduce the possible receptor-mediated signalling pathways, which include IIIC splice variants of FGFR1 and/or...
FGFR2. Our study therefore further defines the extraembryonic epiblast assay and characterises this in vivo method of delivering secreted factors.

FGF can initiate the neural programme in the chick embryo

In common with grafts of Hensen’s node (Streit et al., 1997), FGF signalling is able to maintain and/or induce expression of the L5 antigen in the extraembryonic epiblast. The L5 antibody recognises the LeX oligosaccharide epitope present in two different glycoproteins one of which (L5(220)) is present early in extraembryonic tissue and is associated with neural competence, while the second (L5(450)) is a later pan-neural marker (Streit et al., 1996, 1997). As FGF beads implanted for only a short period (6-8 hours) are surrounded by an ectopic region of L5 expression, it is possible that FGF signalling maintains L5(220). However, FGF may also induce neural tissue that subsequently expresses L5(450). Thus, while these findings and the induction of neural-specific genes (see below) implicate FGF in neural competence, the specific maintenance of L5(220) by FGF signalling has yet to be determined. Like Hensen’s node, FGF also induces ectopic expression of the transcription factors cash4 and Sax-1, which are markers of early posterior neural tissue (Henrique et al., 1997; Spann et al., 1994). However, unlike organiser activity (Storey et al., 1992), FGF signalling in extraembryonic epiblast does not elicit the expression of the early neuronal marker recognised by the 3A10 antibody even in embryos cultured to HH24, suggesting that FGF does not trigger the complete neural programme leading to neuronal differentiation.

The failure to induce neurons is consistent with the induction of only early posterior neural genes (cash4 and Sax-1). cash4 is expressed transiently in precursors of the posterior CNS in a rostrocaudal wave of expression that moves in concert with the regressing node/primitive streak and is induced by signals emanating from the node (Henrique et al., 1997). It can substitute for the neural specification activity of homologous genes (achaete and scute) in Drosophila and expands the neural plate
Fig. 7. During normal development Bra, cash4, Sax-1 and Fgf8 are expressed in overlapping domains in the posterior neural plate at HH9-10 (A-J). (A) The posterior neural plate (white arrowhead) expresses bra as well as the cells in the regressing primitive streak (PS) and the notochord (black arrowhead); (B) section through A (white bar) showing expression of bra in both open neural plate and underlying mesoderm. (C) cash4 is expressed in the neural plate at this time, the caudal-most region of this domain overlaps with bra expression; (D) Section through B (white bar) showing cash4 expression in the open neural plate but not in underlying mesodermal tissues; (E) Sax-1 is expressed in a similar domain to cash4 in the posterior neural plate; (F) Section through E (white bar) showing Sax-1 expression confined to the neural plate; (G) Fgf8 is expressed in the regressing primitive streak and in the caudal end of the forming posterior neural plate in a domain that overlaps with cash4, Sax-1 and bra at HH9; (H) Section through (white bar) G showing Fgf8 expression in the mesodermal and prospective neural tissue; (I) By HH10 Fgf8 is clearly expressed in the elevating posterior neural folds; (J) Section through I (white bar) showing localisation of Fgf8 throughout neural folds as well as underlying mesodermal tissues.

when overexpressed in the frog embryo (Henrique et al., 1997). The expression of cash4 in neural precursors in the chick, together with these functional studies therefore suggests that cash4 acts early to assign neural cell fate in posterior regions of the chick embryo. Our current findings suggest that expression of cash4 is however, insufficient to trigger the cascade of gene expression that leads to neuronal differentiation.

During neural development cash4 is expressed prior to Delta-1, which in the CNS identifies the first post-mitotic neurons (Henrique et al., 1995). In this assay, Delta-1 is always co-expressed with Bra (a combination indicative of prospective mesodermal tissue in the primitive streak; Fig. 5F) suggesting that post-mitotic neurons (which do not express Bra) do not form in response to FGF. Consistent with this interpretation, overexpression of Fgf8 in the developing midbrain leads to a dramatic expansion of the neural precursor population in the ventricular zone (Lee et al., 1997). It is therefore tempting to speculate that, in line with in vitro data (Gensburger et al., 1987; Murphy et al., 1994; Temple and Qian, 1995) a common function of FGF signalling in the developing nervous system is the induction and/or maintenance of precursor cells. Indeed, the generation of post-mitotic cells and their subsequent differentiation as neurons or glia may require the down regulation of cash4 and Sax-1 and the activity of other signals, such as retinoic acid, bone morphogenetic proteins, sonic hedgehog and neurotrophins (Papalopulu and Kintner, 1996; Sharpe and Cross, 1997; Liem et al., 1995; Shah et al., 1996; Averbuch-Heller et al., 1994; Roelink et al., 1995; Jungbluth et al., 1997). FGF signalling in this assay, therefore, appears to elicit only the initial steps in the neural programme, which includes maintenance of neural competence and the induction of a combination of transcription factors characteristic of posterior neural precursors.

FGF does not induce anterior neural tissue

FGF does not induce any of the anterior neural markers tested in this assay; these include genes expressed in the diencephalon (tailless), at the midbrain/hindbrain boundary (Engrailed 2) and in the hindbrain (Krox20). This suggests that neural tissue induced in response to FGF has a more posterior regional character, which may include posterior hindbrain (at least

Fig. 8. Digoxigenin-labelled FGF2 was used to detect the spread of FGF from beads implanted in the extraembryonic epiblast (A-C), and Dil was used to follow the movement of cells exposed to FGF or PBS beads (D,E) as well as to test the possibility that FGF beads recruit embryonic epiblast cells from the host embryo (FG). The spread of DIG-FGF2 from beads soaked in concentrations of (A) 0.5 μg/ml, (B) 5 μg/ml and (C) 50 μg/ml, was detected with an anti-DIG-alkaline-phosphatase conjugated antibody following implantation in HH3-3+ embryos and overnight incubation in New culture. The dorsal surface of the extraembryonic epiblast cells was labelled with Dil and (D) PBS beads or (E) FGF beads were placed over the labelled cells and in HH3-3+ embryos, which were cultured overnight. Cells exposed to FGF did not appear to move more than those exposed to PBS beads; (F) Dil labelling of embryonic epiblast cells at the lateral edge of the prospective neural plate at HH3-3+ adjacent to a FGF bead (arrowhead); (G) Following overnight culture no Dil-labelled cells are recruited by FGF beads (arrowhead).
below the level of *Krox20* expression in rhombomere 5) and spinal cord, as judged by the induction of *Hoxb9*. The absence of more anterior neural markers might also reflect the undifferentiated state of this FGF-induced neural tissue, which is characterised by the expression of only early markers of the posterior neural precursor population.

In this assay, anterior neural markers are also not induced by low FGF concentrations. This contrasts with reports in amphibian embryos in which high FGF concentrations elicit expression of posterior neural genes, while low levels of FGF induce anterior neural markers (Kengaku and Okamoto, 1995). However, these experiments were carried out with ectoderm cells which had been previously dissociated, a treatment that can in itself lead to the formation of anterior neural tissue (Grünz and Täcke, 1989; Godsave and Slack 1991; Wilson and Hemmati-Brivanlou, 1995). Indeed, Lamb and Harland (1995) report only weak concentration effects, but they do find anterior neural markers, such as Engrailed, induced when old (late gastrula) animal caps are exposed to FGF. Ectoderm in these experiments, however, also underwent treatment that may have led to the partial dissociation of cells. A further problem with these *Xenopus* experiments is that it is not possible to distinguish between multiple actions of FGF signalling during development.

Using more precise methods afforded by the use of FGF-coated beads, our results show that in the chick there is a defined window of competence during which epiblast cells respond to FGF by expressing early posterior neural genes and that anterior neural genes are not induced in their stead in older epiblast cells. Further, using the same technique Crossley et al. (1996a) have demonstrated that much later during the regionalisation of the nervous system and formation of the midbrain, FGF signals do now elicit Engrailed expression. This emphasises, along with other studies (e.g. Shimamura and Rubenstein, 1997) that FGF signalling acts at specific times and in distinct regions of the developing nervous system. By showing that FGF signalling underlies the initial steps in the generation of posterior neural tissue, our study identifies a new role for such signals at primitive streak stages.

**Do FGF signals act directly or indirectly to induce early posterior neural genes?**

It is possible that non-FGF signals provided by mesodermal tissues induce the expression of early neural genes in this assay. Axial mesodermal tissue has been shown to provide neural-inducing signals in many vertebrates, including the chick embryo (Storey et al., 1995; A. Rowan, C. D. Stern and K. G. S., unpublished data). We demonstrate that FGF signalling in this assay does not induce markers of axial mesoderm, the prechordal plate marker gooseoid and the notochord-specific antigen Not-1, confirming observations made in the *Xenopus* embryo (Cho et al., 1991; Pownall et al., 1996). However, we do find that FGF beads induce the co-expression of genes characteristic of prospective para-axial mesoderm in the primitive streak (*Delta-1* and *Bra*).

**Evidence for the indirect action of FGF**

Several lines of evidence suggest that early posterior neural genes could be induced indirectly by signals emanating from these prospective mesoderm cells: (i) *bra* and *Delta-1* expression precede *cash4* induction by at least 4 hours; and (ii) the competence to respond to FGF by expressing *bra* persists beyond that for *cash4* and *Sax-1* (Table 2). As neural competence is lost shortly after HH4 (Gallera, 1971; Storey et al., 1992; Streit et al., 1995), it may be that signals produced by *bra/Delta-1*-expressing tissue are not produced soon enough to elicit *cash4* and *Sax-1* expression.

However, the proposition that these prospective mesoderm cells are a source of neural-inducing signals is not consistent with the results of grafting experiments that compare the neural-inducing abilities of prospective axial and non-axial mesoderm cells in the anterior primitive streak (Storey et al., 1995). These experiments show on the one hand that, while prospective axial mesoderm (medial mesendoderm) is a potent neural inducer, prospective non-axial mesoderm (posterolateral mesendoderm) does not induce neural tissue in extraembryonic epiblast cells (Storey et al., 1995). On the other hand, this failure to induce neural tissue was assessed only on morphological grounds and it may be, therefore, that prospective non-axial mesoderm can induce expression of early posterior neural genes.

**Evidence for direct effects**

In this context, it is significant that an early response to the FGF beads is the induction of *Fgf8*. During development *Fgf8* is expressed in both this prospective non-axial mesoderm in the primitive streak, as well as later in adjacent developing posterior neural tissue in a domain that overlaps with those of *cash4* and *Sax-1* (Fig. 7G-I). It is therefore possible that FGF signalling mediates the action of non-axial mesoderm and also that it may act directly within the posterior neural plate. We show that *Fgf8* is induced prior to *cash4* in response to FGF beads and also that FGF8 protein can induce *cash4* (as well as *bra*) identifying it as a potential endogenous regulator of this early neural gene.

The presence of cells that co-express *Bra* and *cash4* close to FGF beads (Fig. 6F) further suggests that direct action of FGFs on prospective neural tissue could be mediated via *Bra* expression in this tissue. This *Bra*-expressing cell population most likely corresponds to those *Bra*-positive cells close to FGF beads that do not co-express *Delta-1* (Fig. 5E). In fact, the spatiotemporal pattern in which *Bra* and *cash4* are expressed in the embryo during the laying down of the posterior nervous system suggests that *bra* is a prelude to the expression of *cash4* in the same cell population (Fig. 7A-D) (Kispert et al., 1995; Henrique et al., 1997). Strikingly, a requirement for *bra* for the maintenance of *Sax-1* expression has been demonstrated in the T-mutant mouse (Schubert et al., 1995), although this may reflect the failure to form mesodermal tissue as well the absence of *bra* in the posterior neural plate. A role for *bra* in the induction of early posterior neural genes might also account for the formation of an apparently normal posterior nervous system in transgenic frogs in which FGFRI-mediated-signalling is blocked at early gastrula stages, but after the induction of *bra* (Kroll and Amaya, 1996).

The concentration effects that we observe (Fig. 4) may also support the direct action of FGFs on prospective neuroepithelium. These data show that *bra* is induced by both low and high FGF doses while posterior neural genes are only induced at high FGF concentrations. This is consistent with the early expression of *bra* in the vicinity of the bead and its later expression at a distance, while *cash4* is expressed close to FGF beads. One interpretation of these results is that cells close to the FGF bead initially express *bra*, but later acquire a neural fate. Alternatively, cells may initially turn on mesodermal genes, move away and induce neural tissue in their wake.
However, as Dil-labelling of the extraembryonic epiblast exposed to FGF (or PBS) beads shows that some cells do remain in the vicinity of these beads while others move away we cannot distinguish between these two possibilities.

In short, we find evidence for both direct and indirect effects of FGF signalling on prospective neural tissue. These two routes are not mutually exclusive. Indeed, given the induction of Fgf8 in this assay and its expression in both prospective mesodermal and neural tissue, direct and indirect effects could be mediated by the same signal in this region of the embryo.

The role of FGF in neural induction

Our results implicate FGF signalling in neural competence and show that FGF signalling can elicit expression of early posterior neural genes in the absence of anterior neural tissue. This suggests that the initial steps in the generation of posterior neural tissue take place downstream of FGF signalling independently of other node-derived signals. This contrasts with many studies in the Xenopus embryo which show that initial neural-inducing signals (such as Noggin and Chordin) elicit formation of anterior neural tissue and that this step is a prerequisite for the later induction of posterior neural genes (Nieuwkoop et al., 1952; reviewed by Hemmati-Brivanlou and Melton, 1997; see Taira et al., 1997). It is also striking that in our assay FGF signalling elicits only early and transient markers of prospective posterior neural tissue and it is possible that expression of these genes may not be indicative of caudal neural character, but rather reflects the induction of a particular cell state associated with the formation of neural precursors in this region.

In conclusion, while our data strongly suggest that FGF signalling functions during neural induction and that it specifically plays a role in the generation of precursors of the posterior nervous system, spatiotemporally defined loss-of-function experiments are required to confirm these activities. The early posterior neural genes induced in this assay are present only transiently; their expression ceases when the cells of the posterior neural plate are left behind by the regressing Hensen’s node/FGF source and no longer express Fgf8. It will be intriguing to assess the regulation of this transition in the context of signals that instigate later phases of the neural programme leading to neuronal differentiation.

We thank Helen Skaer, Jonathan Slack, Claudio Stern and Andrea Streit for critical reading of the manuscript. We are grateful to Yosef Gruenbaum for the gift of Sax-1 probe; Bernard Herrman for the gift of the Bra antibody; Domingos Henrique for gifts of cashb4 and Delta-1 plasmids; Robb Krumlauf for the gift of Hoxb9 plasmid; Gail Martin and Clive Dickson for the gift of mouse Fgf8 plasmids; Jim Smith for the gift of the bra plasmid and to Andrea Streit for the L5 antibody. This work was supported by Joint MRC small project grant for the MRC to K. G. S. and J. K. H. and by grants from the Wellcome Trust to J. K. H. and K. G. S.

REFERENCES


Early posterior neural genes induced by FGF 483


