REGIONALISATION OF THE ZEBRAFISH FOREBRAIN
DURING EARLY DEVELOPMENT

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ABSTRACT

I have been interested in understanding how the morphologically simple anterior neural plate develops into the complex structures of the adult forebrain. The zebrafish was chosen for this project as it is ideal for looking at the earliest stages of development and a number of genes had already been isolated which were useful for this study. In addition, a large scale mutagenesis using zebrafish has recently been conducted and several of the mutants from these screens were relevant to my project.

Through in situ hybridisation studies, I have shown that genes predicted to be involved in early forebrain development are expressed in restricted patterns in the anterior neural plate from the onset of gastrulation. In particular, I have studied the anterior-posterior boundaries present in the prospective neural plate from early gastrulation and the differences between rostral and caudal gene expression patterns.

Whilst characterising mutants with disturbed neural plate patterning, I isolated a line of fish which lacks the expression of the anterior neural plate marker anf. anf encodes a homeodomain containing transcription factor and is one of the earliest restricted anterior neural plate markers so far identified. The surprising result was that the absence of anf expression did not lead to any obvious developmental defects.

To address how patterning genes control later development, I studied their role in the regulation of cell proliferation. To address this, I developed a new technique using an antibody to a phosphorylated histone. I studied the correlation between patterning gene expression and patterns of cell proliferation within the neural plate.

During the laboratory's mutagenesis screen looking for early lethal mutants, a novel pigmentation mutant was isolated. I have characterised its pigmentation and other phenotypes.
ACKNOWLEDGEMENTS

A big thank you to Steve Wilson for guidance and help during my PhD and being so good when my outside life got so so busy (see below). Also, thanks to Nigel Holder for advice and guidance on science, Enfield and fatherhood!

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To my family for keeping me in the real world and supporting me during my seemingly endless years of education, thanks to Dad, Audrey, Andrew, Steve, Caitriona, Kirsty, Alex, Martin and Jan.

This thesis is dedicated to my lovely daughter rebecca, for being herself and providing such a positive contribution to my life, and to Rachel for being the mother of my child with all that entails and for helping me throughout the past few years. This thesis is in memory of my mum for providing me with the ambition and confidence to go as far as I can during my life.
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<tbody>
<tr>
<td>ace</td>
<td>acerebellar</td>
</tr>
<tr>
<td>anf</td>
<td>anterior neural fold</td>
</tr>
<tr>
<td>ANR</td>
<td>anterior neural ridge</td>
</tr>
<tr>
<td>AP</td>
<td>antereoposterior</td>
</tr>
<tr>
<td>AVE</td>
<td>anterior visceral endoderm</td>
</tr>
<tr>
<td>bhh</td>
<td>banded hedgehog</td>
</tr>
<tr>
<td>BMP-4</td>
<td>bone morphogenetic protein-4</td>
</tr>
<tr>
<td>BMP-7</td>
<td>bone morphogenetic protein-7</td>
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<td>boz</td>
<td>bozock</td>
</tr>
<tr>
<td>cls</td>
<td>colourless</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>carboxy-terminal Shh</td>
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<td>cyclops</td>
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<td>dino</td>
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<td>decapentaplegic</td>
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<td>dorsalin-1</td>
</tr>
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<td>DV</td>
<td>dorsoventral</td>
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<tr>
<td>en</td>
<td>engrafted</td>
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<td>ENU</td>
<td>ethyl-N-nitrosourea</td>
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<tr>
<td>eph</td>
<td>ephrin</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>flh</td>
<td>floating head</td>
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<td>fkd3</td>
<td>forkhead 3</td>
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<td>HGF</td>
<td>hepatocyte growth factor</td>
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<td>Hh</td>
<td>Hedgehog</td>
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<td>paired box</td>
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<td>Ptc</td>
<td>Patched</td>
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<tr>
<td>PCP</td>
<td>prechordal plate</td>
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<tr>
<td>PH3</td>
<td>phosphorylated histone H3</td>
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<td>PKA</td>
<td>protein kinase A</td>
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<td>puz</td>
<td>puzzle</td>
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<td>RA</td>
<td>retinoic acid</td>
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<td>sonic hedgehog</td>
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<td>silberblick</td>
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<tr>
<td>Smo</td>
<td>Smoothened</td>
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<td>spa</td>
<td>sparse</td>
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<td>sqt</td>
<td>squint</td>
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<tr>
<td>tdo</td>
<td>touch down</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
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<tr>
<td>tri</td>
<td>trilobite</td>
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<tr>
<td>val</td>
<td>valentino</td>
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<tr>
<td>VMZ</td>
<td>ventriular marginal zone</td>
</tr>
<tr>
<td>wnt</td>
<td>wingless</td>
</tr>
<tr>
<td>YSL</td>
<td>yolk syncitial layer</td>
</tr>
<tr>
<td>ZLI</td>
<td>zona limitans intrathalamica</td>
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and hindbrain. These regions are further subdivided and may give rise to segments or neuromeres (reviewed by Guthrie 1995). At the same time, some cells migrate away from the point of neural tube closure to form a transient mass of cells called the neural crest. These cells disperse and migrate away ventrally and laterally to form numerous cell types including sensory cells and ganglia, pigment cells and the bones, muscles and connective tissues of the head.

This thesis will mainly be concerned with neural plate and early neural tube stages of development.

1.2 The regional organisation of the neural plate

1.2.1 Neural plate regional organisation can be studied in several ways

The neural plate is a dynamic structure, especially during neural tube closure (for example Keller 1992a and 1992b). An enormous amount of cell movement (Keller et al 1985, Rakic 1971, LaVail and Cowan 1971) and cell proliferation (Hartenstein 1989) makes it difficult to determine the regional organisation of the neural plate at any given moment (Eagleson and Harris 1990). Therefore, several complementary methods have been adopted to study neural plate organisation.

The regional organisation of the neural plate has been studied using spatial maps that give different types of information. Fate maps show what type of tissue the cells from different regions will form. Commitment maps show when cells in different regions decide what specific cell types to form. Gene expression maps use molecular markers to define molecularly distinct regions in the neural plate. Many fate maps have been composed in a number of species although these have been carried out at numerous developmental stages. Less attention has been paid to commitment and gene expression maps. Fate maps will be considered before outlining the results of commitment maps and gene expression maps.

1.2.2 Several methods can be used to make fate maps

Several methods have been used to construct vertebrate fate maps. Firstly, single cells or small numbers of cells are labelled with vital dyes at the stage of interest and followed to see where they end up and what cell types they produce. However, these dyes are diluted at each cell division so the label becomes harder to follow with time. Therefore, these techniques usually follow fate to a relatively early stage of development which makes final assignment of fate difficult. An alternative to dyes is to graft tissue between closely related species such as quail and chicks (Le Douarin 1973) or differently pigmented strains of frogs or axolotls (reviewed by Eagleson 1996) as the cells from the
donor and the recipient can be unambiguously distinguished later on and dilution does not happen. However, there still remain frequent cell migrations and cell divisions which make fate mapping difficult (Eagleson and Harris 1990). In spite of these limitations, the fate maps of the neural plate of several vertebrate species have shown that the organisation is highly conserved (reviewed by Rubenstein et al 1998a).

1.2.3 Neural plate fate maps in different species give broadly similar results

Neural plate fate maps have been made for several vertebrate species including chick, axolotl and zebrafish and they show similar regional organisation (reviewed by Tam and Quinlan 1996, Rubenstein et al 1998a). The main similarities are in AP and dorsoventral (DV) pattern and the sizes of certain regions (for example, Figure 1.1 shows a Xenopus fate map). The maps differ with respect to the organisation of the eyefield and the fate of the anterior tip of the neural plate.

1.2.4 Fate maps show that AP pattern is present in the neural plate

Most neural plate fate maps have been made after gastrulation is complete and only the zebrafish neural plate has been fate mapped at the beginning of gastrulation (Woo and Fraser 1995). This fate map shows the telencephalon, diencephalon, midbrain and hindbrain are in the correct AP order at the start of gastrulation. Neural plate fate maps of Xenopus (Eagleson and Harris 1990), axolotl (Jacobson 1959), chick (Couly and Le Douarin 1987) and mouse (Tam 1989) show that AP pattern is established by the end of gastrulation. Therefore, fate maps suggest that AP pattern is established by early gastrulation and these fate maps have also examined when DV pattern appears.

1.2.5 Fate maps show that DV pattern appears during gastrulation

Fate maps of many species suggest that DV pattern is not present in the neural plate at the start of gastrulation but is by the end of gastrulation. Fate mapping studies of the zebrafish neural plate at the beginning of gastrulation indicates that refined DV pattern is not clearly established (Woo and Fraser 1995). However, fate maps of the neural plate at the end of gastrulation have DV pattern. For instance, prospective dorsal structures, such as the epiphysis and telencephalon, are located laterally whereas prospective ventral structures, such as the ventral thalamus and ventral hypothalamus, are situated medially in the neural plates of zebrafish, axolotl, Xenopus and chick (Woo and Fraser 1995, Jacobson 1959, Eagleson and Harris 1990, Couly and Le Douarin 1987). These data suggest that DV pattern is established in the neural plate during gastrulation.
Figure 1.1 Fate map of the *Xenopus* neural plate.

Fate map of the presumptive brain areas of the *Xenopus* neural plate (stage 15). The main subdivisions of the brain are shown on the left side and the regions fate mapped are shown on the left. Neural plate fate maps of other vertebrates are similar to *Xenopus* except for a few regions (see text). Key: 1, olfactory bulb; 2, lateral and dorsal cortex; 3, medial cortex; 4, lamina terminalis; 5, preoptic area; 6, magnocellular preoptic nucleus; 7, suprachiasmatic nucleus; 8, chiasma; 9, ventral hypothalamus/infundibulum; 10, ventromedial thalamic nucleus; 11, posterior tuberculum; 12 habenular commissure; 13, anterior thalamic nucleus; 14, central thalamic nucleus; 15, posterior thalamic nucleus; 16, praetectum; 17, optic tectum; 18, hypophysis (anterior pituitary); 19, cerebellum; 20, epiphysis; 21, tegmentum dorsale; 22, tegmentum ventrale; 23, hindbrain choroid plexus; 24, medulla oblongata. Adapted from Eagleson and Harris (1990).
1.2.6 The relative size of regions in the neural plate is similar in different vertebrate fate maps

The relative size of regions, such as the prospective telencephalon and retina, is similar in the neural plates of several vertebrate species. It has been found that the prospective telencephalon is small and the prospective retina (eyefield) is large relative to their final size in all of the available vertebrate neural plate fate maps including chick, axolotl, zebrafish and Xenopus (Jacobson 1959, Eagleson and Harris 1990, Woo and Fraser 1995, Couly and Le Douarin 1985). The small size of the prospective telencephalon suggests this region of the neural plate undergoes a lot of cell proliferation during later development. Thus, fate maps agree on the relative sizes of different regions but not necessarily on how these regions are spatially arranged.

1.2.7 The eyefield is initially single but separates into left and right primordia in neural plate fate maps

Depending upon the stage, fate mapping studies of the neural plate have shown the eyefield is either in two separate halves or a single domain spanning the midline. For instance, studies of axolotl, Xenopus and chick (Jacobson 1959, Brun 1981, Eagleson and Harris 1990, Couly and Le Douarin 1987) found two separate eyefields. However, studies of newts and zebrafish (Manchot 1929, Mangold 1931, Woo and Fraser 1995) found a single eyefield joined across the midline. The reason for the differences in the organisation of the eyefield in different fate maps is because the eyefield is initially a single domain which splits into left and right primordia (Li et al 1997).

1.2.8 Fate maps do not agree over the fate of the most rostral neural plate

Fate maps disagree over the fate of the most rostral neural plate, the anterior neural ridge (ANR). In some vertebrate fate maps, the medial ANR is fated to form the upper border of the anterior commissure in the telencephalon (Burr 1922, Jacobson 1959, Puelles 1987, Woo and Fraser 1995). Other studies suggest the medial ANR forms the anterior pituitary (Couly and Le Douarin 1985, Osumi-Yamashita 1994). Further studies suggest the medial ANR forms both the telencephalon and the anterior pituitary (Eagleson et al 1986, Eagleson and Harris 1990, Eagleson et al 1995, Kawamura and Kikuyama 1992, Houart et al 1998). Thus, it is uncertain if the medial ANR forms the telencephalon, anterior pituitary or both.
Fate maps of the lateral regions of the ANR agree that it forms telencephalon (Woo and Fraser 1995, Jacobson 1959, Eagleson and Harris 1990, Puelles 1987). Fate maps of several species agree that the medial region of neural plate caudal to the ANR forms the ventral diencephalon, although the precise region formed is uncertain (Jacobson 1959, Kawamura and Kikuyama 1992, Eagleson and Harris 1990, Couly and Le Douarin 1987). The available zebrafish neural plate fate map assigns the neural plate caudal to the ANR as prospective retina but the diencephalon is thought to be immediately caudal to this (Woo and Fraser 1995). Thus, fate maps agree that the rostral neural plate forms the forebrain but do not fully agree over which precise regions.

1.2.9 Conclusion to fate maps

Fate maps of the vertebrate neural plate agree on the general layout, the timing of AP and DV patterning, but not on the detailed organisation of particular regions relative to each other. These differences are probably caused by differences in the stage when maps are made and by the limited resolution of fate mapping techniques.

Fate maps show where cells fated for a tissue arise from not when cells make fate decisions. As there is not much cell mixing in the neural plate (Wetts and Fraser 1989, Warga and Kimmel 1990) fate maps may give an impression of order earlier then it is specified as cells may acquire their fate by default (Wilson et al 1993). For instance, cells in the anterior tip of the neural plate will not be fated to form spinal cord as they are unlikely to move to the posterior neural plate. Therefore, commitment maps of the neural plate have been made to show when cells make fate decisions (Woo et al 1995).

1.3 Commitment studies of the neural plate

Fate maps show where the cells to form a given tissue will come from but do not say when these cells are irreversibly committed to their particular fate. The degree of overlap in fate maps (for example Woo and Fraser 1995) suggests that fate is not fully determined in the neural plate. Commitment studies have been used to determine when cells make fate decisions.

The question of when commitment is established has been approached in several ways. Regions of the neural plate have been isolated and cultured in vitro to see at which stage they are able to adopt the same identity as their donor region. Other studies have transplanted pieces of neural plate to non-neural regions to see at which stage a neural plate regional identity has been irreversibly established. An alternative approach is to transplant pieces of the neural plate to ectopic locations in the neural plate to see at which stage the transplanted tissue is not able to be respecified. The results from studies of
several vertebrate species give reasonably consistent results as to the timing of commitment.

In zebrafish, transplants of neural plate cells outside the neural plate or to ectopic positions within the neural plate show that cells are not committed until the end of gastrulation (Ho and Kimmel 1993, Woo and Fraser 1997). This suggests that the AP order in the early gastrulation zebrafish fate map is not due to commitment but that the fate map at the end of gastrulation probably contains committed regions (Woo and Fraser 1995). Explants of zebrafish animal caps indicate neural commitment is gradually established from early gastrulation but final fate decisions are not made until the end of gastrulation (Sagerstrom et al 1996, Grinblat et al 1998). Studies of other vertebrates have found that AP and DV fates are committed at different times.

Studies of amphibia and chicks suggest AP fates are committed before DV fates. For instance, in axolotl and *Xenopus* it has been shown that AP is set before DV (Roach 1945, Sladecek 1952, 1960, Jacobson 1964) and that fate is established gradually (Jacobson 1964, Model 1982). Studies of the chick neural plate also show that AP is established before DV (Simon et al 1995, Garcia-Martinez et al 1997). Thus, commitment studies suggest that AP fates are established before DV fates and that fate is gradually established.

1.4 Gene expression maps of the neural plate

Gene expression studies are useful for understanding the regional specification of the CNS (Rubenstein et al 1998a). As some gene expression patterns may be stable indicators of regional identity they are helpful when comparing gene expression and fate mapping studies (Rubenstein et al 1998a). Many potential regulatory genes, such as *pax6* (Krauss et al 1991b, Puschel et al 1992), *engrailed* (Hatta et al 1991b), and *krox20* (Oxtoby & Jowett 1993), are expressed in restricted domains in the neural plate. Therefore, the expression of such genes during neural plate development probably underlies later regionalisation of the neural tube and brain (Woo and Fraser 1995). The expression patterns of potential regulatory genes has been studied in the neural tube (see for example Macdonald et al 1994, Rubenstein et al 1994) but not systematically in the neural plate. In this thesis I present detailed analysis of gene expression patterns in the neural plate of the zebrafish.

It has been suggested that the anterior neural plate has a grid-like organisation generated by a combination of longitudinal and transverse domains of gene expression (Shimamura et al 1995, reviewed by Rubenstein et al 1998). Such a grid may be used to generate regional identity in the neural plate, with different combinations of genes determining specific identities, as suggested for Hox genes in the branchial region of the vertebrate head (Hunt et al 1991).
Studies of gene expression in a number of species, including zebrafish, have identified longitudinal and transverse domains of gene expression in the neural plate. Longitudinal domains of gene expression occur most frequently in the midline of the neural plate. For instance, *nk2.2* and *sonic hedgehog (shh)* are expressed along the midline of the zebrafish neural plate (Barth & Wilson 1995, Krauss et al 1993). Transverse patterns are observed for a number of genes (reviewed by Rubenstein et al 1998) including *pax2.1* which is expressed in a transverse domain at the midbrain-hindbrain boundary (for example, see Reifers et al 1998). Studies of the zebrafish midbrain and forebrain at later stages of development have also found longitudinal and transverse domains of gene expression (Hauptmann and Gerster 1999). Thus, the neural plate may be organised into regions indicated by spatially restricted gene expression patterns.

**1.4.1 Fate maps, commitment maps and gene expression need to be compared in a single species**

For a complete understanding of the regional organisation of the neural plate it is crucial to compare gene expression maps, fate maps and commitment maps (Woo et al 1995). For instance, fate maps and commitment maps of the zebrafish neural plate draw different conclusions over the timing of regional organisation. Fate mapping suggests the neural plate is regionally organised at the start of gastrulation (Woo and Fraser 1995) whereas commitment maps suggest the neural plate is not organised until the end of gastrulation (Ho and Kimmel 1993, Woo and Fraser 1997). Thus, it will be useful to compare these maps with the gene expression study of the zebrafish neural plate described in this thesis to elucidate when regional organisation commences and how it progresses.

**1.5 Induction of the neural plate**

In 1924, Spemann and Mangold showed that transplantation of the dorsal blastopore lip of an early amphibian gastrula to the ventral side of a host of the same stage could induce a secondary axis (Spemann and Mangold 1924). As the donor dorsal blastopore lip organised the host tissue it was called the organiser. The organiser is fated to form the axial mesoderm and ectoderm (Vogt 1929). Spemann and Mangold's experiment showed that the organiser was able to convert ventral ectoderm which usually forms epidermis into neural tissue which usually only arises from the dorsal ectoderm. Many studies were undertaken to identify the neural inducing factors but these were unsuccessful until recently (reviewed by Hemmati-Brivanlou and Melton 1997). It is likely that previous studies failed to identify these molecules because it was assumed that
epidermis rather than neural was default and it is hard to separate mesoderm induction from neural induction (reviewed by Kelly and Melton 1995). Neural inducers were successfully identified when it was realised that neural may be the default state for ectoderm.

Recent studies suggest that the neural plate arises from antagonism between non-neural and neural inducers. The first evidence that epidermal, rather than neural, fate requires inducers was from the finding that dissociated *Xenopus* animal caps develop directly into neural tissue without first inducing mesoderm (Grunz and Tacke 1989, Green and Smith 1990). Thus, a signal which normally induces epidermal fate is lost during the dissociation process and neural identity results by default. Secondly, it was found that inhibition of the *transforming growth factor-β (TGF-β)* related signalling molecule *activin* also leads to direct neural induction (Hemmati-Brivanlou and Melton 1992, 1994) which was unexpected as *activin* normally induces mesoderm (Asashima et al 1990). The dominant negative *activin* receptor used in these experiments may have interfered with other *TGF-β* signalling to cause neural induction. These data led to the theory that *activin* or a related molecule normally suppresses neural fate by inducing epidermal fate (reviewed by Tanabe and Jessell 1996).

The epidermal inducing signal is now thought to be *bone morphogenetic protein-4 (BMP4)* rather than *activin* (reviewed by Tanabe and Jessell 1996). *BMP4* is expressed in the early ectoderm and is excluded from the neural plate during neural induction (Hemmati-Brivanlou and Thomsen 1995, Schmidt et al 1995, Fainsod et al 1994). *BMP4* can suppress neural markers and promote epidermal differentiation in dissociated cells (Wilson and Hemmati-Brivanlou 1995). Therefore the organiser induces neural fate by antagonising BMP signalling.


It is not clear why there are several molecules which mediate neural induction in the same way and which are produced in the same cells at the same time (Tanabe and Jessell 1996). Knock-out mice provide some evidence of redundancy as neither *activin* (Matzuk et al 1995a, reviewed by Smith 1995), *BMP4* (Winnier et al 1995) or *follistatin*
(Matzuk et al 1995b) mutant mice have defective neural induction. Studies of zebrafish mutants also suggest there is redundancy in the neural induction pathway. For instance, the dino phenotype is caused by a null mutation to chordin yet these embryos have relatively normal neural induction (Hammerschmidt et al 1996, Schulte-Merker et al 1997). Therefore, redundancy amongst neural inducers seems likely.

Studies of chicks have identified two further neural inducing factors, hepatocyte growth factor (HGF) and fibroblast growth factor (FGF). HGF is expressed in the organiser and may mediate neural induction either by promoting the competence of the ectoderm to respond to neural inducing signals or by causing neural induction directly (Streit et al 1995). FGF is able to induce posterior neural tissue in non-neural ectoderm although it is unclear if FGF acts directly or indirectly (Rodriguez-Gallardo et al 1997, Storey et al 1998). Studies of Xenopus have previously implicated FGF in neural induction (Lamb and Harland 1995, reviewed by Mason 1996) but when FGF signalling is blocked, neural induction is not prevented (Kroll and Amaya 1996). The role of endoderm in neural induction has not been fully explored but the finding that the neural inducer cerberus is expressed in the anterior endoderm (Bouwmeester et al 1996) and also that noggin and chordin affect endoderm differentiation in addition to neural induction suggests the endoderm may play a role (reviewed by Streit and Stern 1999).

Thus, the identification of several neural inducing factors suggests that the process of neural induction is complex and may involve some redundancy (Streit and Stern 1999).

1.6 AP patterning of the neural plate

Until recently, the accepted model of AP patterning of the neural plate was that the organiser induced a neural plate with an anterior character and subsequently caudal regions were respecified with posterior identity. The signals mediating the "posteriorisation" of the caudal neural plate were thought to either emanate vertically from the underlying axial mesoderm or pass through the plane of the neural plate. However, in both cases these signals were produced by the posteriorly positioned organiser (Doniach 1993, Ruiz i Altaba 1994). Studies of amphibia have shown that the dorsal mesoderm blastopore lip is the organiser (Spemann and Mangold 1924). Studies of other species have found equivalent tissues, including the embryonic shield of fish (Oppenheimer 1936), the node of mice (Beddington 1994) and Hensen's node of chick (Waddington 1934). AP patterning was believed to be induced by the organiser in all of these species.

Recent studies of anterior development of Xenopus, mouse and zebrafish have challenged this view and shown that anterior patterning information may emanate from the rostral end of the neural plate. In this section, I will outline what has been learnt from
studies within the traditional framework and then consider recent studies of anterior development.

1.6.1 Evidence for vertical signals

The first evidence that vertical signals induced AP patterning in the neural plate came from experiments which showed that mesoderm from different AP levels could induce different AP identities in the overlying neural plate (Mangold 1933). For instance, the first mesoderm to involute was able to induce head and trunk identities but later mesoderm could only induce trunk identity. Furthermore, it was also shown that neural induction could not happen without mesoderm and that isolated mesoderm developed AP pattern (Holtfreter 1933). More recent experiments (Eyal-Giladi 1954) found that the posterior neural plate is initially specified as anterior and as the axial mesoderm migrates rostrally, the overlying caudal neural plate is respecified as posterior. Thus, these experiments showed that AP pattern develops in mesoderm independently of the ectoderm and also that the ectoderm requires the mesoderm for neural induction and patterning.

A number of studies have found that the mesoderm becomes gradually regionalised in its inductive abilities during gastrulation. For instance, it has been found at mid-gastrulation, mesoderm from all AP levels induces anterior markers in the neural plate (Saha and Grainger 1992). However, by late gastrulation, although both anterior and posterior mesoderm can induce anterior markers, only posterior mesoderm is able to induce posterior markers in the neural plate (Hemmati-Brivanlou et al 1990, Sharpe and Gurdon 1990). Other studies have found that otx2 is restricted to the anterior neural plate by positive signals from the anterior mesoderm and repressed from the posterior neural plate by signals from the posterior mesoderm (Ang and Rossant 1994). Recent studies of hox gene expression in the zebrafish notochord have shown the vertebrate notochord is patterned along the AP axis (Prince et al 1998c) so the notochord may provide different AP signals along its AP length. Thus, the inductive abilities of the mesoderm become spatially restricted during gastrulation and this may be mediated by hox genes.

It is unclear how the imprecise information in the dorsal mesoderm becomes converted into precise AP information in the neural plate. The assumption is that interactions within the neural plate would have to occur to achieve final pattern (Ang 1996). However, other studies have shown that AP pattern can develop without vertical signalling from the mesoderm.

1.6.2 Evidence for planar signals
As vertical signals from the mesoderm are only able to induce an approximate AP pattern in the neural plate, it has been proposed that signals within the plane of the neural plate are also needed for precise AP patterning (Ruiz i Altaba 1994, Doniach 1993). Studies using Keller explants, which allow planar but not vertical signalling between the mesoderm and ectoderm (Keller and Danilchik 1988), have shown that planar signals are sufficient to induce most features of the neural plate AP pattern. These studies have also found that the mesoderm is needed for neural induction as the absence of mesoderm blocks neural plate formation (Dixon and Kintner 1989). It has also been found that planar signals are able to induce neural plate markers in the correct AP order to a rostral limit of at least the diencephalon (Doniach 1992, Ruiz i Altaba 1992, Papalopolu and Kintner 1993). Thus, planar signalling from the mesoderm is sufficient to induce much of the AP pattern in the neural plate.

Planar signals do not seem to be sufficient to induce all features of nervous system development. For instance, floor plate cells, motor neurons and eyes are absent in Keller sandwiches (Keller and Danilchik 1988, Dixon and Kintner 1989). In addition, gene expression along the AP axis of the neural plate is abnormal without vertical signals as genes including Xash3 and wntl are not expressed correctly (Zimmerman et al 1993, Ruiz i Altaba 1994). Thus, both planar and vertical signalling from the mesoderm are required for complete AP patterning of the neural plate (Ruiz i Altaba 1994).

1.6.3 Models of AP neural plate patterning

Several models have been proposed to account for AP patterning of the neural plate (Figure 1.2; reviewed by Doniach 1992, 1993).
Figure 1.2  Models of AP patterning of the neural plate.
Models to account for induction of AP patterning information in the vertebrate neural plate. Spatial patterns of gene expression could be determined by (A) different signals arising from different regions of the mesoderm; (B) concentration gradient; (C) time of exposure to inducer; (D) ectodermal competence; (E) prepattern with certain latitudes preassigned to express certain genes such as otx2 (black), fgf8 (green) or hoxA1 (red); (F) self-organisation: in which inducers enter the ectoderm through either the planar (green arrows) or vertical (black arrows) route, and the pattern is sorted out in the ectoderm (red arrows); (G) two-signal model, the first signal (red line) is induces forebrain development and the second signal (green line) in combination with the first signal induces posterior identity. Adapted from Doniach (1993).
A Qualitative model

Ectoderm

A

1' 2' 3' 4'

P

E Prepattern

Mesoderm

A

1 2 3 4

P

B Concentration gradient

[inducer]

P A

F Self-organisation

vertical signals

C Time of exposure to signal

hoxA1 fgfB otx2

P A

G Two signal model

hoxA1 fgfB otx2

P A
**Qualitative Model**

In the Qualitative Model, AP pattern at different AP levels in the neural plate is induced by the mesoderm using different vertical signals at each AP level (Figure 1.2A; Mangold 1933). In favour of this model is the finding that there are differences in the inductive abilities of different AP levels of the dorsal mesoderm. For instance, the anterior mesoderm induces *otx2* but the posterior mesoderm suppresses it (Ang and Rossant 1994). For this to happen, the mesoderm and the neural plate must be adjacent but as it has been shown that planar signalling is sufficient to induce some AP pattern the Qualitative Model is unlikely.

**Single Gradient Model**

The single gradient model proposes that a single inducer from the organiser induces the neural plate and provides it with AP pattern (Figure 1.2B; Doniach 1993). A high concentration of the inducer would generate posterior identity and a low concentration would induce anterior identity. A prediction of the model is that a reduction in the concentration of the inducer by loss of posterior mesoderm would be expected to cause a reduction of posterior neural identity as this needs high concentrations of the inducer. Conversely, an increase to posterior mesoderm would be expected to cause a reduction to anterior neural identity as this needs a low concentration of the inducer. However, *floating head* (*flh*) mutant zebrafish embryos have less posterior mesoderm, but do not have defects in the AP patterning of their neural plate (Talbot et al 1995) which suggests the neural plate is not patterned by a single gradient of an inducer originating in the mesoderm.

**Timing Model**

Differences in timing of exposure or of competence to respond to AP signals from the organiser could generate the AP pattern in the neural plate (Figure 1.2C,D). Evidence in favour of timing models includes the finding that mesoderm from early and late stages of development induces different AP values in the neural plate (Mangold 1933). However, studies have shown that when mesoderm involution is delayed, AP pattern develops normally (Eyal-Giladi 1954) and that the ectoderm is not differently competent at different times (Sharpe and Gurdon 1990). Thus, timing is probably not used to generate AP pattern.

**Prepattern Model**

Prepattern models suggest that the ectoderm is prepatterned along the AP axis and this information is interpreted when the neural plate is induced (Figure 1.2E). Studies which have shown that AP restricted markers, such as *En2*, can be expressed at the wrong AP level in Keller explants suggests there is not a prepattern in the ectoderm or if present...
it can be over-ridden (Doniach et al 1992). However, recent in vivo studies have provided evidence to the contrary. These studies transplanted the zebrafish organiser, the embryonic shield, to ectopic locations and found it induced secondary axes with an AP pattern independent of the level of the ectopic shield but the same as the primary axis (Koshida et al 1998). This strongly suggests there is an AP prepattern in the ectoderm of zebrafish embryos independent of the organiser and further studies have sought to identify the tissue which generates the prepattern.

There is evidence that the germ ring is the source of the signals which establish the ectodermal pre-pattern in zebrafish (Koshida et al 1998, Woo and Fraser 1997). The signal itself is unknown although it is not thought to be FGF8 (Koshida et al 1998, Woo and Fraser 1997). Further evidence that the shield is not the source of AP information comes from studies of the zebrafish mutant bozozk (boz) which lacks the organiser and yet develops AP pattern in the nervous system (Solnica-Kretzel 1996, Fekany et al 1999) and of zebrafish embryos with ablated organisers which still develop AP pattern (Shih and Fraser 1995). Thus, there is evidence of a prepattern in the ectoderm which is involved in AP patterning the neural plate.

Self-organisation Model

In the self-organisation model, AP information is derived from interactions within the neural plate (Figure 1.2F). It is possible that either all AP patterning information arises from within the neural plate or the mesoderm induces an approximate AP pattern which is refined by interactions within the neural plate. As it has been shown that the mesoderm induces at least some AP pattern in the neural plate (Nieuwkoop 1952), it is doubtful that all of the AP information arises from within the ectoderm. However, there is evidence that the neural plate can self-organise after induction without further contact with the mesoderm (Saha and Grainger 1992) and that there are organising centres acting within the plane of the neural plate.

Neural plate organising centres have been identified in the prospective midbrain and telencephalon and may be responsible for AP patterning of the neural plate. The midbrain is patterned by planar signals from the isthmus which can induce midbrain in ectopic locations through much of the neural plate (Crossley et al 1996). The isthmus organising centre is mimicked by fgf8 (Crossley et al 1996) and acts by repressing nearby genes including otx2 (Martinez et al 1999). There is also evidence that the telencephalon is patterned by a signalling centre. Ablation of the anterior row of cells in the zebrafish neural plate causes a loss of telencephalic gene induction but, unlike the isthmus, the signal is not fgf8 (Houart et al 1998). It is not yet known if such an organiser occurs in the rostral neural plate of other vertebrates. However, ablation of the ANR of the Xenopus neural plate causes a reduction to the size of the telencephalon (Corner 1963). As the inductive properties of the ablated tissue were not tested it is not certain if the loss
of an anterior organiser causes the defect. Studies of mouse anterior neural development suggest that there is an FGF8 dependant signalling centre at the rostral edge of the neural plate which is capable of inducing anterior genes such as *BF1* (Shimamura and Rubenstein 1997). Therefore, it is likely that signalling centres occur at least in the midbrain and telencephalon to self-organise the neural plate.

**Two Signal Model**

The two signal model was proposed independently by two groups (Figure 1.2G; Nieuwkoop 1952, Saxen and Toivonen 1961). Saxen and Toivonen proposed that a first signal, the neuralising inducer, induces anterior neural tissue (forebrain). A second signal, the mesodermal inducer, induces mesoderm on its own but when combined with the neuralising inducer induces posterior neural tissue (hindbrain and spinal cord). It was proposed that the neuralising inducer is distributed evenly along the AP axis and the mesodermal inducer in an AP gradient with the highest concentration at the posterior. The combination of these inducers leads to the full AP neural pattern.

Similarly, Nieuwkoop proposed that the first signal, the activator, induces anterior neural (forebrain) and the second signal, the transformer, induces posterior neural tissue. The activator is either at a high concentration in the anterior neural plate or evenly distributed along the AP axis of the embryo and induces forebrain throughout the neural plate. The transformer respecifies caudal neural plate as posterior in a dose dependant manner with a high concentration at the posterior end. The two signal model can be adapted to support both vertical and planar signalling (Doniach 1993).

**Evidence for the Two Signal Model**

A number of studies suggest that the caudal neural plate is initially specified as anterior before being respecified as posterior. The first study to show this found that the posterior neural plate was first able to differentiate into anterior cell types before it was able to differentiate into posterior cell types (Eyal-Giladi 1954). More recent studies have found that anterior neural plate markers are initially expressed posteriorly and gradually shifted rostrally (Sive et al 1989). Other markers, such as *otx2*, have been found to be initially expressed across the entire neural plate and become gradually restricted to anterior regions by signals from the mesoderm (Blitz et al 1995, Ang and Rossant 1994). Thus, in support of the two signal model there is evidence that the neural plate is initially specified as anterior along its entire length and is subsequently respecified as posterior in caudal regions.

There are several candidates for the first signal including *noggin*, *follistatin* and *chordin*, which are expressed in the axial mesoderm and can induce anterior neural plate identities directly, that is, without first inducing mesoderm (Lamb et al 1993, Hemmati-Brivanlou and Melton 1994, Sasai et al 1994, Smith and Harland 1992). There is
probably redundancy between these molecules as the neural plates of null mouse and zebrafish mutants do not have AP patterning defects (for example, see Matzuk et al 1995b, Schulte-Merker et al 1997). Retinoic acid (RA) and FGF have been proposed as candidates for the second signal.

RA is able to induce anterior defects in neural tissue (Durston et al 1989, Sive et al 1990), has a graded distribution along the AP axis (Chen et al 1992, 1994, Hogan et al 1992, Wagner et al 1992), can suppress anterior genes, such as \textit{otx2} and \textit{Xrx1} (Ang and Rossant 1994, Pannese et al 1995, Cararosa et al 1997), and studies of zebrafish support an AP patterning role for RA (Costardis et al 1996). However, RA cannot suppress posterior genes and its effects may be restricted to the hindbrain (for example see Holder and Hill 1991, Marshall et al 1992). Attempts to resolve the conflicting results have included analysing the expression of RA receptors but they have a complex distribution that does not indicate how RA acts in vivo (Ellinger-Ziegelbauer and Dreyer 1991). Studies have also analysed the distribution of RA in chick but do not find RA rostral to the spinal cord (Maden et al 1998). At present, studies indicate that if RA is a transforming signal other factors are needed for a complete AP pattern (Blumberg et al 1997).

Studies of FGF as a candidate for the second signal have also produced conflicting results (reviewed by Doniach 1995). A particular difficulty with studying the role of FGF in neural patterning is that it also induces mesoderm which complicates interpretation of results (Doniach 1995). In support of FGF as the second signal, it has been shown that FGF can induce posterior neural identities (Lamb and Harland 1995, Cox and Hemmati-Brivanlou 1995, Kengaku and Okamoto 1995, Storey et al 1998). However, other studies have found that posterior neural patterning is established without FGF signalling (for example see Kroll and Amaya 1996, Woo and Fraser 1997). Thus, at present there is not strong evidence to support either FGF or RA as the second signal of the two signal model.

1.6.4 Evidence for an anterior organiser

A number of recent studies have produced evidence which suggests AP patterning and head induction involves an anterior organiser alongside the traditional role of the Spemann organiser (reviewed by Bouwmeester and Leyns 1997, Ruiz i Altaba 1998, Beddington and Robertson 1999). These studies indicate that anterior patterning information derives from the endoderm and the anterior neural plate.

\textit{Anterior patterning information may derive from the endoderm}

Studies of \textit{Xenopus} suggest that anterior patterning information derives from the endoderm. \textit{cerberus} is expressed in the non-involuting dorso-anterior yolky endoderm which forms the foregut and liver and is not expressed in the PCP (Bouwmeester et al
However, ectopic expression of *cerberus* induces partial head structures which lack axial mesoderm and are cyclopic suggesting the endoderm plays a role in anterior patterning (Bouwmeester et al 1996). As ectopic expression of *cerberus* induces *Xotx2* expression in the anterior endoderm, *cerberus* may be upstream of *Xotx2* which induces anterior identity in the overlying rostral neural plate (Bouwmeester et al 1996). At present the signalling pathways are not certain, but may involve suppression of Wnt signalling.

There is evidence that Wnt signalling is also involved in anterior patterning. For instance, blocking *wnt8* signalling affects head development (Hoppler et al 1996) and the simultaneous suppression of BMP and Wnt signalling is sufficient for head induction (Glinka et al 1997). Furthermore, *frzb* is a Wnt inhibitor expressed in the endoderm and PCP that increases head size at the expense of the trunk when ectopically expressed (Leyns et al 1997). *dickkopf* is also a Wnt inhibitor which can induce complete heads but is expressed in the PCP not the endoderm (Glinka et al 1998). Therefore, suppression of Wnt signalling and BMP signalling is necessary for head induction in *Xenopus* but it is not certain if head induction is due to signals from the endoderm or PCP. Studies of anterior patterning in mammals also suggest there is an anterior organiser in the endoderm.

**Mammalian anterior organiser**

Studies of mammals suggest that anterior pattern develops independently of the organiser, the node. For instance, the node is unable to induce forebrain (Beddington 1994, Popperl et al 1997, Tam et al 1997) and there are morphological signs of AP pattern before the node develops (Viebahn et al 1995, Downs and Davies 1993). Recent data suggests the extra-embryonic anterior visceral endoderm (AVE) provides AP patterning information during early mouse development (reviewed by Beddington and Robertson 1999).

Several lines of evidence support a role for the AVE in anterior patterning in mammals. Independently of the node, the AVE expresses anterior markers early in development, such as *hex* and *cerberus-related-1* (Thomas et al 1998, Belo et al 1997). Furthermore, mutations in genes expressed in the AVE, such as *lim1*, *otx2* and *hess1*, affect anterior development (Shawlot and Berringer 1995, Matsuo et al 1995, Ang et al 1996, Dattani et al 1998). The AVE can ectopically induce anterior neural markers in mouse and chicks (Ding et al 1998, Knoetgen et al 1999) and ablation of the AVE affects forebrain patterning (Thomas and Beddington 1996). More direct evidence of the role played by genes expressed in the AVE in anterior patterning comes from studies of chimeric mutant mice.

Chimeric mouse embryos, composed of wild type and mutant cells, have been useful in determining if the AVE is needed for anterior patterning. Embryonic stem cells introduced to a blastocyst do not populate the visceral endoderm or extraembryonic
ectoderm but participate in the embryo proper so this method can be used to study the need for a gene in these tissues separately (Varlet et al 1997a, Beddington and Robertson 1989). For instance, single mutants of \textit{HNF3}\(\beta\), \textit{gsc} or \textit{nodal} did not suggest a role in anterior patterning, but chimeras of \textit{HNF3}\(\beta\) heterozygotes and \textit{gsc} (Filosa et al 1997) or \textit{nodal} (Varlet et al 1997b) provide evidence that these genes are needed in the AVE for anterior patterning. Chimeric mice have been used to show \textit{otx2} is required in the AVE to induce anterior neural plate pattern but that \textit{otx2} is also needed in the neural plate or PCP to maintain anterior identity (Rhinn et al 1998, Acampora et al 1998). Thus, numerous studies of mice and \textit{Xenopus} indicate that there is an anterior organiser in the endoderm required for induction of anterior neural identity. Anterior patterning information also appears to originate from the rostral neural plate.

\textit{Anterior information from the rostral neural plate}

Recently, it has been shown that zebrafish embryos have an anterior organiser in the rostral neural plate (Houart et al 1998). The most rostral row of cells at the border of neural and non-neural ectoderm, Row 1, are needed for normal forebrain development as ablation causes the loss of anterior genes, such as \textit{emx1} (Houart et al 1998). This region has similar abilities as demonstrated for the anterior neural fold of mouse embryos (Shimamura and Rubenstein 1997) but acts earlier and is \textit{fgf8} independent (Houart et al 1998). The yolk syncitial layer (YSL) may induce Row1 in zebrafish (Houart et al 1998) as it has been shown that the YSL has inductive abilities as it can induce mesoderm and expression of \textit{boz} mRNA in the YSL is sufficient to rescue the \textit{boz} phenotype (Mizuno et al 1996, Fekany et al 1999).

At present it is unclear how the endodermal and neural plate anterior organisers are related. As the AVE is beneath the border of the neural and non-neural ectoderm where Row 1 is found, the AVE may induce a Row 1 type organiser in mice (Ruiz i Altaba 1998) and the cerberus expressing endoderm may induce a Row 1 organiser in \textit{Xenopus} (Ruiz i Altaba 1998). Therefore, anterior neural pattern is induced by one or more anterior organisers which may interact with the mesodermal organiser to induce AP pattern in the neural plate.

1.7 \textbf{DV patterning of the neural plate}

1.7.1 \textit{Ventral patterning in the caudal neural plate}

DV patterning is best understood in the caudal neural tube due to extensive studies of DV patterning in the spinal cord (reviewed Tanabe and Jessell 1996). These studies have focused on the induction of the floor plate, motor neurons and interneurons. The main source of ventral patterning information in the caudal neural plate is the notochord.
The notochord is a rod of mesodermal cells beneath the neural plate which extends from the caudal spinal cord to the ZLI (Placzek et al 1990).

The notochord induces the adjacent neural plate to form floor plate and may pattern the ventral neural plate more extensively (Placzek et al 1990, 1991). Grafts of notochord adjacent to the dorsal spinal cord can induce ectopic floor plate and motor neurons (Yamada et al 1991). Conversely, when the notochord is removed, floor plate and motor neurons do not form (Yamada et al 1991). Floor plate induction by the notochord is contact dependant (Placzek et al 1990, 1993) but motor neuron induction is by long range signalling initially from the notochord and subsequently from the floor plate (Yamada et al 1991, 1993). Grafts of floor plate cells are able to mimic the notochord's inductive properties suggesting there is a common signal (Yamada et al 1991).

Although, the floor plate has been shown to induce motor neurons in the ventral spinal cord, interneuron induction appears to be dependant on at least two parallel pathways. *islet 1 (isll)* null mutant mice lose both *isll* expressing motor neurons and interneurons which do not express *isll* (Pfaff et al 1996). In the *isll* mutants, interneurons can be induced by motor neuron containing regions of the neural tube (Pfaff et al 1996). Thus, motor neurons may be required to induce some types of interneurons. However, evidence of a motor neuron independent pathway is provided by studies of *HNF3β* null mutant mice which lose notochord, floor plate and motor neurons but still develop interneurons (Ang and Rossant 1994). Recent studies have shown that although *shh* is sufficient to induce some types of interneuron, *shh* is not needed in vivo or in vitro for interneuron induction (Pierani et al 1999). Instead, retinoids provide a *shh* independant interneuron induction signal (Pierani et al 1999). Therefore, there appear to be motor neuron dependant and retinoid dependant pathways which induce interneurons.

Thus, studies of the caudal neural plate suggest that the notochord induces floor plate, which in turn induces motor neurons and that these inductions may use a common signal. The best candidate for this signal is *shh*.

### 1.7.2 *shh* is a ventral inducing signal

*shh* is a vertebrate homologue of *Drosophila Hedgehog* (*Hh*). Homologues of *Hh* have been identified in chick (Riddle et al 1993), mouse (Echelard et al 1993), zebrafish (Krauss et al 1993, Ekker et al 1995b), rat (Roelink et al 1994) and *Xenopus* (Ekker et al 1995a). The expression pattern of *shh* implicates it as the inductive signal in ventral patterning of the caudal neural plate as it is expressed in the notochord from early gastrulation and later in the floor plate.

*Floor plate induction by shh*
A number of studies suggest that shh induces floor plate cells in the ventral neural plate. Ectopic expression of shh in zebrafish (Krauss et al 1993), mouse (Echelard et al 1993) and Xenopus (Roelink et al 1994) induces floor plate markers, including HNF3β and F-spondin. COS cells expressing shh induce floor plate differentiation in neural plate explants (Roelink et al 1994). Furthermore, inhibiting shh prevents floor plate induction.

Removal of shh signalling blocks the induction of the floor plate by the notochord. Blocking antibodies to Shh prevent floor plate induction by the notochord (Ericson et al 1996). shh null mutant mice develop a notochord in caudal regions but do not induce floor plate cells (Chiang et al 1996). Thus, shh is needed from the notochord to induce floor plate cells. Studies of flh (Talbot et al 1995) and no tail (ntl, Schutle-Merker et al 1994) zebrafish mutant embryos found they have floor plate cells in rostral regions without a differentiated notochord because shh is expressed in the notochord precursors and is sufficient to induce floor plate even in the absence of notochord. Thus, there is strong evidence that short range shh signalling induces floor plate. It seems likely that shh also acts as a long range signal.

Motor neuron induction by shh

A number of studies suggest that shh acts as a long range signal to induce motor neurons. COS cells expressing shh induce floor plate and motor neuron differentiation in neural plate explants (Roelink et al 1994). Blocking antibodies to shh prevent motor neuron induction by the notochord (Ericson et al 1996) and shh null mutant mice do not develop motor neurons (Chiang et al 1996). It has been suggested that shh initiates a common pathway for all motor neurons and local factors assign subtype identity (reviewed by Pfaff and Kintner 1998). Retinoid signal signalling between early and late born motor neurons may form part of this mechanism (Sockanathan and Jessell 1998). The finding that shh acts as a long range signal to induce motor neurons has led to the suggestion that it is a morphogen (Roelink et al 1995).

shh may be a morphogen

shh is needed as a short range contact dependant signal to induce floor plate and as a long range contact independent signal to induce motor neurons (Roelink et al 1994, Tanabe et al 1995, Ericson et al 1995). Short range floor plate induction is direct as it is without protein synthesis (Roelink et al 1995) but the mechanism of motor neuron induction is unclear as several cell divisions occur before motor neuron markers, such as is11, are expressed (reviewed by Johnson and Tabin 1995). Recent studies of pax6 mutant mice and nklx2.2 mutant mice have shown that pax6 and nklx2.2 expression is induced at different DV positions by different concentrations of shh (Ericson et al 1997a, Osumi et al 1997, Briscoe et al 1999). Thus, shh induction of a number of different cell types along the DV axis may be due to shh acting as a morphogen (reviewed by Ericson et al 1997b).
Shh protein is autocatalytically cleaved into two peptides (Porter et al 1995). The 19K amino-terminal (N-Shh) is the signal for both short and long range actions of Shh and is conserved in evolution (Bumcrot et al 1995, Fan et al 1995, Fietz et al 1995, Hynes et al 1995, Lai et al 1995, Lee et al 1994, Marti et al 1995, Porter et al 1995, Roelink et al 1995). The carboxy-terminal (C-Shh) is less conserved and has not been shown to be active as a signal. Instead, C-Shh is required for auto-proteolysis of the Shh peptide which causes the attachment of cholesterol to the carboxy-terminus of N-Shh. This modification may be required to associate N-Shh to the surface of expressing cells to create a high concentration of Shh protein at the site of production (reviewed by Rubenstein and Beachy 1998). Without cholesterol biosynthesis, developmental defects arise that are similar to the phenotype of shh null mutants (Chiang et al 1996). Therefore, the addition of cholesterol to Shh protein is important for its signalling activities (reviewed by Rubenstein and Beachy 1998). Thus, Shh protein may act as a morphogen or initiate a relay signalling cascade to generate short and long range signalling activities (reviewed by Johnson and Tabin 1995, Ericson et al 1997b).

**Downstream targets of Shh**

A number of downstream targets of Shh have been identified. For instance, cAMP dependant protein kinase A (PKA) activators antagonise N-SHH (Fan et al 1995, Epstein et al 1996) so Shh may act by inhibiting PKA. In support of this, it has been shown that PKA inhibition phenocopies the zebrafish cyclops (cyc) mutation (Ungar and Moon 1996).

Shh signalling has also been shown to be activated by binding to the multipass membrane proteins Patched (Ptc; Chen and Struhl 1996, Stone et al 1996, Maringo et al 1996, Goodrich et al 1996) and Smoothened (Smo; van den Heuvel and Ingham 1996, Xie et al 1998, Alcedo et al 1996) which are involved in ligand reception and signal transduction. Binding of Shh to Ptc relieves Ptc-mediated repression of Smo, thereby activating the signalling pathway. The targets activated include Ptc itself, which creates a negative loop that may restrict further diffusion of Shh protein (Porter et al 1996, Chen and Struhl 1996). *hip* encodes a membrane glycoprotein Hh binding protein (Chuang and McMahon 1999). Hip binds all three mammalian Hh related proteins, Shh, Indian hedgehog and Desert hedgehog, with similar affinities to Ptc-1 (Chuang and McMahon 1999). *hip* is expressed in cells adjacent to shh and is induced by ectopic shh expression and lost in shh mutants (Chuang and McMahon 1999). Therefore, *hip* may be part of a negative regulatory feedback loop which could modulate responses to Shh signalling by binding to Shh protein.

A number of transcription factors have also been shown to be regulated by Shh signalling, including Gli family genes (Lee et al 1997, Hynes et al 1997, Dahmane et al 1997, Marine et al 1997). *Gli1* and *Gli2* are induced by Shh and are able to induce motor

The zebrafish cyc mutation causes severe ventral defects including the loss of rostral shh expression, the loss of floor plate cells and cyclopia (Hatta et al 1991a, 1994, Macdonald et al 1995). The recent discovery that the cyc phenotype is caused by a mutation to a nodal related gene, nr1, raises the possibility that shh interacts with TGFB family members to confer ventral identity (reviewed by Blader and Strahle 1998). nr1 may act by mediating target cell responses to shh (Blader and Strahle 1998). Although, cyc mutants lack rostral shh expression and floor plate cells, they have motor neurons so cyc mutants still have a functional shh pathway as well as some remaining nodal signalling (Feldman et al 1998). Therefore, both shh and nr1 are necessary for floor plate induction but nr1 is not needed for motor neuron induction (reviewed by Blader and Strahle 1998).

Further components of the Shh signalling pathway undoubtedly remain to be identified and will be necessary for a full understanding of how shh mediates so many aspects of ventral neural development.

1.7.3 HNF3β may regulate notochord development


There is evidence that HNF3β plays a role in floor plate induction. For instance, ectopic HNF3β expression in the dorsal midbrain induces ectopic floor plate and ventral gene expression whilst suppressing dorsal genes (Sasaki and Hogan 1994). HNF3β null mutant mice lose ventral tissues, such as notochord, node, floor plate and motor neurons and dorsal genes, such as pax3, are expanded across the midline (Ang and Rossant 1994, Weinstein et al 1994). In shh null mutant mice, HNF3β is expressed in the notochord but it is not maintained suggesting shh is required to maintain but not induce HNF3β (Chiang et al 1996). Studies of gsc:HNF3β double-mutants suggest that they interact to regulate
shh expression in the notochord (Filosa et al 1997). Recent studies of regulatory regions of shh have found HNF3-binding sites in some regions suggesting that HNF3β regulates some aspects of shh expression (Epstein et al 1999, Muller et al 1999). Thus, HNF3β is probably required for notochord induction and some aspects of shh expression, whereas shh induces ventral cell types including floor plate and motor neurons.

1.7.4 Dorsal neural tube patterning

Dorsal neural plate identity may either be induced as ventral appears to be, or be a default state that is suppressed at the ventral midline (Dickinson et al 1995). It is unlikely that signals from the ventral midline pattern the dorsal neural plate directly (reviewed by Ruiz i Altaba 1994). For instance, shh null mutant mice have normal dorsal gene expression (Chiang et al 1996) and dorsal cell types, such as neural crest, can differentiate without a notochord (Liem et al 1995). Several studies have shown that signals from the non-neural ectoderm, which is adjacent to the neural plate, may provide dorsal neural plate identity.

The non-neural ectoderm and dorsal neural plate have been shown to interact to induce dorsal cell types, such as the neural crest (Moury and Jacobson 1989, 1990, Selleck and Bronner-Fraser 1995). Furthermore, when ventral neural plate explants are cultured in contact with non-neural ectoderm dorsal genes and cell types are induced in the ventral neural plate, such as pax3, slug and wnt1 (Liem et al 1995, Dickinson et al 1995). The non-neural ectoderm is able to induce dorsal cell types in the presence of notochord but is unable to fully suppress the notochord's ventral inducing ability as cells immediately adjacent to the notochord are not respecified as dorsal (Dickinson et al 1995). Thus, the non-neural ectoderm produces signals, which may be contact-dependant, that can induce dorsal cell identity.

The best candidates for the contact-dependant signal from the non-neural ectoderm are the polypeptide growth factors belonging to the TGF-β superfamily, BMP4, BMP7 and dorsalin-1 (dsl1; Liem et al 1995, Dickinson et al 1995, Basler et al 1993). BMP4 (Fainsod et al 1994, Hemmati-Brivanlou and Thomsen 1995, Nikaido et al 1997) and dsl1 (Basler et al 1993), are initially expressed in the non-neural ectoderm and subsequently expressed in the dorsal neural tube so are in the appropriate tissues to provide a dorsalising signal. dsl1 has been shown to inhibit ventral cell types, such as motor neurons, and promote dorsal cell types, like neural crest (Basler et al 1993). The culture media from COS cells expressing BMP4 and BMP7 mimics the contact-dependant dorsalising effect of the non-neural ectoderm (Liem et al 1995). Overexpression of BMP4 in zebrafish embryos suppresses the expression of ventral midline genes, such as shh, and reduces the number of ventral motor neurons (Neave et al 1997). Analysis of the zebrafish mutant, swirl (Mullins et al 1996), shows that it is caused by a mutation to
bmp2b (Nguyen et al 1998, Kishimoto et al 1997) and that the absence of bmp2b signalling causes a loss of neural crest cells (Nguyen et al 1998). Thus, there is strong evidence that BMPs act to pattern the dorsal neural plate.

The mechanism of action by BMPs is uncertain as there is evidence to support both a long-range and short-range mechanism (reviewed by Smith 1996b). In the role of mesoderm induction in *Xenopus*, BMP4 has been shown to act at a short distance (Jones et al 1996) and as a long range morphogen (Dosch et al 1997). The *Drosophila* homolog of BMP4, decapentaplegic (*dpp*; Padgett et al 1987), is involved in wing development and studies have presented evidence showing that *dpp* acts as both a long range (Nellen et al 1996) and short range (Lecuit et al 1996) signal. Recent studies of BMP signalling in zebrafish have also provided evidence for both a short range (Nikaido et al 1999) and long range (Nguyen et al 1998) mechanism. Thus, it is unclear how BMPs mediate dorsal signalling by the non-neural ectoderm.

The non-neural ectoderm may induce a dorsalising centre in the neural tube (in a similar way that the notochord induces the floor plate ventralising centre) which maintains dorsalising signals from the non-neural ectoderm (Basler et al 1993, Fan and Tessier-Lavigne 1994). The roof plate is analogous to the floor plate in that neither forms neurons, both are transient and both express common genes, such as Wnt family genes (Wilkinson et al 1987, Roelink and Nusse 1991, McGrew et al 1992). Thus, dorsoventral patterning of the caudal neural plate is likely to involve opposing *shh* ventralising signals and BMP dorsalising signals (Liem et al 1995).

### 1.7.5 Ventral patterning of the forebrain

As the notochord and floor plate do not extend into the forebrain beyond the ZLI (Placzek et al 1990, Hatta et al 1991a, Placzek et al 1993) different DV patterning mechanisms may be used rostral to the ZLI. The prechordal plate (PCP) is in the rostral midline beneath the neural plate, in an equivalent position to the notochord more caudally. Therefore, the PCP may ventralise the rostral neural plate in the same way that the notochord ventralises the caudal neural plate (Dale et al 1997). Furthermore, differences between the forebrain and more caudal regions of the CNS may be caused by differences in the signals produced by the notochord and PCP or by differences between the rostral and caudal neural plate (Dale et al 1997).

Like the notochord, the PCP is a source of ventralising signals. The zebrafish mutation *cyc* has a reduced PCP, severe defects to the ventral forebrain and alterations to gene expression (Hatta et al 1991a, 1994, Barth and Wilson 1995, Macdonald et al 1995, Ekker et al 1995b, Thisse et al 1994). *one-eyed pinhead (oep)* mutants do not develop the PCP and have severe defects to their ventral forebrains (Schier et al 1996a). The PCP can induce ventral markers in the dorsal part of the rostral neural plate (Shimamura and
Rubenstein 1997). Therefore, the PCP may play an equivalent role to the notochord as it is able to induce ventral identities in the rostral neural plate.

The PCP has other similarities with the notochord. Some of the same genes are expressed in both the PCP and notochord, including *shh, chordin, HNF3β* and *oep* (Echelard et al 1993, Krauss et al 1993, Sasaki and Hogan 1993, Sasai et al 1994, Roelink et al 1994, Zhang et al 1998). The notochord and PCP have similar developmental origins (Shih and Fraser 1996, Psychosos and Stern 1996) and morphologies (Sulik et al 1994).

There are also some differences between the PCP and notochord. For instance, the PCP expresses some genes not expressed in the notochord, including *gsc, otx2, ntl* and *flh* (Pannese et al 1995, Blitz et al 1995, Thisse et al 1994, Talbot et al 1995, Schulte-Merker et al 1994). In addition, the PCP cannot induce floor plate in the caudal neural plate (Placzek et al 1993) and the notochord cannot induce ventral identity in the rostral neural plate (Placzek et al 1993) so the PCP and notochord have different inductive abilities. However, both the notochord and PCP mediate their ventral patterning activities with *shh*.

The PCP needs *shh* signalling to induce ventral identity in the rostral neural plate. Overexpressing *shh* in the forebrain affects ventral specification, including the expansion of *nk2.2* and the reduction of *pax6* (Barth and Wilson 1995, Krauss et al 1993, Ekker et al 1995b, Macdonald et al 1995). *shh* can induce ventral identity in the prospective forebrain of chicks, including the induction of *nk2.1* expression (Ericson et al 1995) which is needed for forebrain development (Kimura et al 1996). *shh* null mutant mice have ventral defects along their whole rostrocaudal length, including the forebrain (Chiang et al 1996). Experiments which block *shh* signalling with an anti-SHH antibody prevent the PCP inducing ventral identity (Dale et al 1997). It has recently been shown that *shh* may regulate ventral identity indirectly in the forebrain through *pax6* and *nk2.2* (Briscoe et al 1999). Therefore, the PCP uses *shh* signalling to induce ventral identity.

The finding that the PCP induces rostral rather than caudal ventral identity in the posterior neural plate even though the PCP produces *shh* which normally induces floor plate in the posterior neural plate (Dale et al 1997) suggests the PCP produces signals in addition to *shh* which induce ventral identity in the rostral neural plate. A candidate for this signal is *BMP7* as it has been shown that *BMP7* and *shh* are able interact to induce ventral identity in the rostral neural plate (Dale et al 1997). However, as *BMP7* is also co-expressed with *shh* in regions of the notochord, it is likely that other factors are involved (Dale et al 1997). Recent studies have found that ventral midline cells of the rostral diencephalon are initially specified with caudal ventral identity and subsequently respecified with rostral ventral identity and this may be mediated by the downregulation of *chordin* (Dale et al 1999). Therefore, the PCP uses *shh, BMP7* and the suppression of *chordin* to induce ventral identity in the rostral neural plate.
1.7.6 Dorsal patterning of the forebrain

Very little is known about the dorsal patterning of the rostral neural plate. However, it appears likely that members of both $Hh$ and BMP families are involved (reviewed by Rubenstein and Beachy 1998).

$Hh$-related genes have been shown to be involved in dorsal forebrain patterning. Studies of *Xenopus* have shown that *banded hedgehog* (*bhh*) is expressed in the dorsal rostral neural plate and may be involved in specifying dorsal structures, such as the cement gland (Ekker et al 1995a). In the mammalian telencephalon it has recently been shown that $shh$ is required for induction of two regions of the basal ganglia, the globus pallidus and striatum (Kohtz et al 1998). BMP related genes also mediate dorsal neural plate patterning.

$BMP4$ and $BMP7$ are expressed in the non-neural ectoderm adjacent to the neural plate (Shimamura and Rubenstein 1997, Furuta et al 1997). It has been shown that dorsal cell types are induced along the entire length of the neural tube by $BMP4$ (Muhr et al 1997). BMPs induce neural crest markers at the hindbrain level but at telencephalon level, $msx1/2$ is induced in vitro (Muhr et al 1997, Furuta et al 1997). Conversely, non-neural ectoderm from the telencephalon level cannot induce neural crest at the hindbrain level, suggesting that different signals may be used at different AP levels (Muhr et al 1997). As BMP antagonists, such as *noggin* are also expressed in the forebrain (Shimamura et al 1995, Knecht and Harland 1997) it seems likely that these modulate BMP signalling in the forebrain as elsewhere (Rubenstein and Beachy 1998). Mutants have not shown a role for BMPs in dorsal neural plate patterning but a number of other mutations have been identified which cause dorsal forebrain phenotypes.

Mutations to a number of transcription factors cause dorsal forebrain defects and are likely to be downstream of the BMP and $Hh$ related signalling molecules that mediate dorsal identity. For instance, *small-eye* mice with a naturally occurring *pax6* mutation, have defective cerebral cortex lamination and patterning of the dorsal diencephalon (Schmahl et al 1993, Stoykova et al 1996, Warren and Price 1997). $Emx1$ mutant mice have defects to the dorsal midline causing the loss of the corpus callosum (Qiu et al 1996, Yoshida et al 1997) and $Emx2$ mutant mice have severe abnormalities including the loss of the hippocampal dentate gyrus (Yoshida et al 1997, Pelligrini et al 1996). Zebrafish *flh* mutants have defective differentiation of the dorsal diencephalic epiphysis (Masai et al 1997). Zebrafish *masterblind* (*mbl*) mutants lack the telencephalon and eyes and have an rostral expansion of the dorsal diencephalon (Heisenberg et al 1996, Masai et al 1997). Thus, it appears that dorsoventral patterning in the both the rostral and caudal neural plate involves members of the BMP and $Hh$ gene families acting synergistically or antagonistically to regulate transcription factors and other downstream targets.
1.8 Models of the segmental organisation of the forebrain

A series of lateral bulges and invaginations that form in the neural tube have led to the suggestion that the early neural tube is segmented (Kimmel 1993, Puelles and Rubenstein 1993). The criteria used to characterise the segments have included morphological features, reiterative patterns of neurogenesis, serial homology of neuronal cell types, cell lineage restrictions and gene expression (reviewed by Guthrie 1995).

There are two models of forebrain segmentation, the His-Herrick Model which divides the forebrain into longitudinal columns and the neuromeric model which divides the forebrain into transverse segments (reviewed by Northcutt 1995).

1.8.1 His-Herrick Model

In the His-Herrick Model, the CNS is divided into longitudinal columns which are most obvious in caudal regions. In the hindbrain and spinal cord these are several longitudinal columns, the roof plate, paired lateral plates and a floor plate. The roof and floor plates remain thin and form non-neural ependymal elements lining the central lumen. The lateral plates thicken and are divided into alar and basal plates by the sulcus limitans (His 1893). The alar plate is the sensory (afferent) zone and the basal plate the motor (efferent) zone of the CNS (Strong 1895, Herrick 1899, Heijdra and Niewenhuys 1994). Thus, there is evidence supporting division into longitudinal columns in the caudal CNS but it is less certain if these columns occur in rostral regions.

Longitudinal columns are not as obvious in rostral as caudal regions of the CNS. For instance, the rostral extent of the alar and basal plates is not certain and may extend to the anterior end of the neural tube (His 1893, Rendahl 1924, Keyser 1972, Puelles 1987) or only as far as the midbrain (Kingsbury 1922, Herrick 1948, Heijdra and Niewenhuys 1994). The His-Herrick model suggests that the diencephalon is divided by three sulci into four longitudinal zones (Herrick 1899, 1948) and the telecephalon is thought to be subdivided by two sulci into three longitudinal zones (reviewed by Kuhlenbeck 1973). The uncertainty over the arrangement of longitudinal divisions has led to criticism of the His-Herrick model.

A major criticism of the His-Herrick model is that because the longitudinal columns in the forebrain do not correspond to the columns in the caudal CNS, it is unclear how such columns would independently arise at different rostrocaudal levels (Northcutt 1995). The alternative to the His-Herrick model is the neuromeric model.

1.8.2 Neuromeric Model
In the neuromeric model the brain is subdivided by transverse furrows along the rostrocaudal axis (von Baer 1828) into segments called neuromeres (Orr 1887). The neuromeric segmentation of the hindbrain is most clearly understood. It has been suggested that there are between six and eight neuromeres (rhombomeres) in the hindbrain (Kuhlenbeck 1973, Lumsden & Keynes 1989, Gilland & Baker 1993). These rhombomeres are transient developmental structures (Vaage 1969) which were previously thought to be artefacts or of no functional importance (Kuhlenbeck 1973). However, the identification of serially repeated brainstem neurons (Hanneman et al 1988, Trevarrow et al 1990), of rhombomere specific gene expression (Wilkinson et al 1989) and of the rhombomeric origin of branchiomeric cranial nerves (Gilland & Baker 1993) led to the recognition that the rhombomeres are important developmental structures (Lumsden and Krumlauf 1996). Rhombomeres affect several aspects of cell behaviour.

Rhombomeres affect cell proliferation, cell migration and cell mixing. Rhombomeres have been shown to be sites of organised cell proliferation (Guthrie et al 1991, Wullimann and Puelles 1999), to be sites of specific cell migration (Puelles 1987, Layer & Alber 1990, Figdor & Stern 1993) and have boundaries that restrict cell mixing (Fraser et al 1990). Rhombomeres may acquire their distinct characteristics through rhombomere specific cell adhesion which may be mediated by Eph signalling (Xu et al 1999). Similar characteristics are likely to apply to neuromeres of the forebrain.
Figure 1.3  Prosomeric model of forebrain segmentation.
Drawing of a schematic lateral view of the developing brain of a chick embryo, with the segmental neuromeric divisions projected onto it. The prosomeres are identified as p1-p6 and the two mesomeres as m1 and m2. Extract from Butler and Hodos (1996).
It is unclear how the forebrain is divided into neuromeres (prosomeres) although several models have been proposed (reviewed by Northcutt 1995, Fishell 1997). Initially it was suggested that there were three prosomeres in the forebrain corresponding to the telencephalon, diencephalon and pretectum (Von Kupffer 1906). Subsequent models have sub-divided the telencephalon (secondary prosencephalon) into three prosomeres (Berquist and Kallen 1954, Vaage 1969). Recent neuromeric models have subdivided the forebrain into further divisions.

In the most recent neuromeric model, the Prosomere Model (Figure 1.3; Puelles and Rubenstein 1993), the forebrain is divided into six prosomeres. There are three prosomeres each in the diencephalon (p1-p3) and telencephalon (p4-p6). The divisions of the Prosomere Model are based upon the morphological features and patterns of gene expression in the neural tube (Rubenstein et al 1994). The prosomeres are thought to roughly subdivide the neural tube and signalling centres, such as the ANR, are thought to generate the detailed regionalisation of the forebrain (Shimamura and Rubenstein 1997).

Though widely accepted, the prosomere model has been criticised for two main reasons (Northcutt 1995). Firstly, the model divides the embryonic brain on the basis of both adult and transient embryonic structures which do not occur at the same time. Secondly, it does not include several important adult telencephalic structures, such as the olfactory bulbs. These shortcomings are caused by limitations to the resolution of fate maps and the extensive cell migration in the telencephalon so it is difficult to define prosomeres during early development (Rubenstein et al 1998). In spite of these criticisms, the Prosomere Model is currently the most widely accepted theory of forebrain segmentation.

1.9 Evolution of the vertebrate forebrain

1.9.1 Origin of the neural plate

The auricularia hypothesis (Garstang 1894) offers an explanation for the origin of the neural ridge and lateral eyes. This hypothesis proposes that the dorsal nerve cord of chordates originates from dorsally converging ciliary bands in an ancestral larva as are found in larvae of modern echinoderms such as auricularia or tornaria (Figure 1.4). This hypothesis is unproven but is widely accepted and there is some evidence to support it (reviewed by Lacalli et al 1994, Young 1995). In the auricularia larva the ciliary band divides into two in the anterior region of the larva, with an apical band at the front of the neural tube and more anterior transverse band remaining outside the neural tube. The apical band of an auricularia-like ancestor may have evolved into the chordate retinal photoreceptors.
Figure 1.4  Auricularia hypothesis of neural fold evolution.

Garstang (1894) proposed the vertebrate neural ridges arose as ciliated bands on an auricularia like ancestor. A) an auricularia like larva, showing ciliary bands and apical organ (ap.). The preoral transverse band (t.b.) lies forward of the apical organ and defines a ventral preoral field. B) ancestral chordate in which the ciliary bands are converted into neural ridges. C) a model based on studies of amphioxus, which suggests the anterior-most cells of the neural tube are derived from the apical plate and the preoral band remains outside of the CNS.

Adapted from Lacalli et al 1994.
and the transverse band may have evolved to form either the hypophysis or olfactory placodes. Thus, regional organisation of the vertebrate neural plate originates in a very primitive ancestor. The major regions of the vertebrate brain are also very ancient.

1.9.2 Origin of the chordate nervous system

The major regions of the vertebrate brain, the forebrain, midbrain and hindbrain arose early during vertebrate evolution as they are present in all vertebrates including the most primitive groups, the agnathan fishes (Forey and Janvier 1993). Thus, in order to understand the evolutionary origin of the vertebrate brain it is necessary to examine the nervous systems of more primitive chordates (Holland and Holland 1998). In addition to the vertebrates, the phylum Chordata consists of the invertebrate urochordates (ascidians) and cephalochordates (amphioxus). Ascidian larvae and amphioxus possess many of the features expected of ancestral chordates (reviewed by Butler and Hodos 1996). Therefore, the nervous systems of ascidian larvae and amphioxus have been intensively studied in an effort to elucidate the steps that may have led to the evolution of the vertebrate brain.

1.9.3 Ascidian larvae have a primitive nerve cord

The typical ascidians are the sea squirts, the tunicates, which are bottom living filter feeders with no obvious trace of a chordate-like form at all. However, the ascidian tadpole larval stage has a notochord and other chordate features (reviewed by Young 1995). Furthermore, the Larvacea Class of ascidians have undergone neotony as the tadpole stage has become sexually mature and this may be how vertebrates arose. The neural tube of ascidian larva forms in the same way as the neural tube of vertebrates.

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The nervous system of ascidian larvae is formed by folds making a hollow dorsal nerve cord extending into the tail. The anterior nerve cord is enlarged into a cerebral vesicle, within which are a simple eye, the ocellus, and a single statocyst cell carrying an otolith which is sensitive to gravity. The visual cells project through a pigmented cup and have packed microvilli which provide the directional tropisms of the animal. The statocyst and ocellus are used to direct the larva to a suitable place for attachment and metamorphosis.

Although simple, the ascidian neural tube may have the beginnings of the DV pattern typical of the vertebrate neural tube (reviewed by DiGregorio and Levine 1998, Graham 1997). The notochord, but not the paraxial mesoderm, expresses brachyury suggesting brachyury has a more ancient role in notochord development than in mesoderm patterning (Holland et al 1995). An ascidian homolog of HNF3β is expressed in the notochord and the ventral ependymal cells adjacent to the notochord which has led
to the suggestion that these cells are fore-runners of the ventral floor plate cells (Corbo et al 1997). Studies of the morphology of several species of ascidian larvae suggest there may be a rudimentary floor plate (Corbo et al 1997, Shimauchi et al 1997, Olsen and Jeffrey 1997). A dorsal marker, HrPax37, which is a homologue of the vertebrate genes, Pax3 and Pax7, is expressed in the dorsal nerve cord of ascidian larva and when overexpressed dorsalisises ventral cell types (Wada et al 1997). Gene expression in ascidian larva has also been studied along the AP axis.

The larval ascidian nerve cord also appears to be patterned along the AP axis. A pax6 homolog is expressed in the anterior lateral ependymal cells of the neural tube of an ascidian and it has been suggested that this region is the ancestor of the secondary prosencephalon (Glardon et al 1997). Other studies suggest there are at least three AP subdivisions in the ascidian nerve cord. A homolog of the vertebrate forebrain and midbrain marker otx2, Hrot, is expressed anteriorly (Wada and Saiga 1999), a homolog of the vertebrate hindbrain marker Pax2, pax2/5/8, labels a caudal region and a homolog of the vertebrate spinal cord marker HoxA1, HrHox1, labels a caudal region (Wada et al 1998). Thus, the nerve cord of ascidian larva is regionalised along both AP and DV axes (DiGregorio and Levine 1998). The development of the nervous system of amphioxus has also been studied as amphioxus is thought to be a close relative of the ancestor of vertebrates.

1.9.4 Amphioxus has the features of the vertebrate ancestor

Amphioxus (Branchiostoma), the lancelet, may be the closest living relative of the vertebrates and the nearest to their immediate ancestor (reviewed by Lacalli et al 1994, Young 1995, Holland and Holland 1998). Amphioxus is a free-swimming animal which mainly burrows and has no obvious head. There are no separate eyes, nose or ears and no jaws. Gill slits are not external but covered by lateral folds of the body and a notochord is present along the entire length of the body. Amphioxus has a hollow dorsal nerve cord as in vertebrates which is modified anteriorly into a short swollen region, the cerebral vesicle.

Studies of the structure of the amphioxus cerebral vesicle suggests homologies with regions of the vertebrate nervous system (Lacalli et al 1994). The cerebral vesicle is divided into anterior and posterior parts which contain different cell types. The infundibular cells in the ventral cerebral vesicle mark the border between these regions and has similarities to the vertebrate infundibulum. The lamellar body contains cells with cilia which produce lamellae and may be photoreceptive and is probably homologous to the vertebrate epiphysis. The paired rostral nerves arising from within the cerebral vesicle may be homologous to the olfactory nerves. It is uncertain if amphioxus has a homologous structure to the midbrain but the large ventral motor neurons caudal to the
cerebral vesicle may be homologous to the vertebrate tegmentum. Amphioxus possesses a single anterior pigment spot which may be homologous to the paired lateral eyes of vertebrates. Further information about the regionalisation of the amphioxus nerve cord has been obtained from studies of gene expression.

Analysis of regionally restricted molecular markers has been useful in determining the extent of AP patterning of the amphioxus neural tube (Holland and Holland 1998). The expression of AmphiHox3 and AmphiHox1 suggests amphioxus has an extensive hindbrain (Holland and Holland 1998). Structural analysis indicates the posterior dorsal region of the cerebral vesicle is homologous to the vertebrate midbrain (Lacalli et al 1994). However, although engrailed is expressed at the midbrain-hindbrain boundary of vertebrates, the amphioxus homolog AmphiEn is not expressed in the proposed midbrain region suggesting either AmphiEn does not label the midbrain or amphioxus lacks a midbrain (Holland and Holland 1998). The forebrain has been analysed with a homolog of otx which labels the vertebrate forebrain and midbrain, AmphiOtx, and a homolog of dlx which labels the vertebrate forebrain and in the ventral and lateral walls more posteriorly (Williams and Holland 1996). AmphiDII is expressed in the extreme rostral cerebral vesicle and in the dorsal walls more posteriorly (Holland et al 1996). The expression of the homolog of the vertebrate telencephalic marker BF1 in a small number of cells in the cerebral vesicle suggests that telencephalic precursor cells may be present in amphioxus (Torresson et al 1998). Thus, analysis of molecular markers suggests the cerebral vesicle is similar to the forebrain but it is uncertain if a midbrain is present (Holland and Holland 1998, Lacalli et al 1994). Molecular markers have also been used to study the regionalisation of the DV axis in the amphioxus neural tube.

The amphioxus neural tube is patterned along the DV axis (reviewed by Graham 1997). A homolog of the vertebrate ventral marker HNF3β is expressed in the notochord and ventral nerve cord of amphioxus (Schimeld 1997). Furthermore, homologs of the vertebrate dorsal markers, snail and Dlx, mnx, snail and AmphiDll, are expressed dorsally in the amphioxus neural tube (Holland et al 1998, Langeland et al 1998). Thus, studies of ascidian and amphioxus neural development reveal the likely features of the early vertebrate brain.

1.9.5 The organisation of the early vertebrate brain

The organisation of the earliest vertebrate brain can be inferred from studies of amphioxus and ascidian (reviewed by Butler and Hodos 1996). The olfactory, retinal photoreceptive and pineal photoreceptive senses were probably present in the common ancestor of cephalochordates and vertebrates. The major subdivisions of the brain, the forebrain, midbrain, hindbrain and spinal cord were probably present. Sensory inputs
were established in early vertebrates, such as taste and auditory, but not necessarily at the same time. Some of the major ascending sensory pathways and descending motor pathways were also established in early vertebrates. A major step in the evolution of vertebrates that influenced forebrain evolution was the innovation of the neural crest.

1.9.6 Neural crest precursors

Vertebrates differ from their ancestors in having an enlarged and highly specialised head (Gans and Northcutt 1983). The innovation of neural crest led to the evolution of jaws, allowing new modes of predation, and to sensory development which led to selection of a new integrative forebrain.

The neural crest is a transient population of migratory cells which originate in the dorsal neural tube and give rise to a number of different tissues throughout the body (reviewed by Anderson 1997). These include pigment cells, peripheral glia and autonomous and sensory ganglia. In the head, the neural crest form the muscles, bone and connective tissues rostral to the end of the notochord, the cartilage of the branchial arches, the anterior part of the neurocranium, and a number of the cranial ganglia and their sensory ganglia (Butler and Hodos 1996). Neural crest are segmentally specified at their point of origin by hox genes and carry segmental identity as they migrate between the paraxial mesoderm and surface ectoderm (Hunt et al 1991). Neural crest cells are thought to be a novel vertebrate innovation (Gans and Northcutt 1983).

Recent studies have identified cells which may be precursors of the vertebrate neural crest in ascidian larva and amphioxus. In ascidian larva, homologs of the vertebrate neural crest markers snail and Pax3, snail and Hrpax37, are expressed in cells which by position and morphology may be neural crest precursors (Wada et al 1996, Corbo et al 1997).

Although there are no neural crest cells or neural crest derivatives, such as jaws, in amphioxus, the finding that AmphiDLL and snail are expressed in cells flanking the neural plate suggests there may be precursors of the neural crest in amphioxus (Holland et al 1996, Langeland et al 1998). Amphioxus does not undergo neurulation but instead a sheet of epidermis grows over the neural plate. These epidermal cells have features of the neural crest, such as expressing AmphiDLL, being adjacent to the neural plate and extending filopodia. However, unlike neural crest, these cells do not differentiate into other cell types and they express AmphiDLL before growing over the neural plate, and move as a sheet rather than individually. Therefore, although neural crest cells have not been found in invertebrate chordates it seems likely that there were precursors of neural crest cells in the ancestors of vertebrates.

1.9.7 Jaws led to major developments of the forebrain
The appearance of neural crest may have led to the evolution of jaws which was a significant step in vertebrate evolution as it broadened potential diet and lifestyles (reviewed by Gans and Northcutt 1983, Hanken 1993). The development of the postotic skull and true bony vertebrae also happened in early vertebrates. The entire brain of gnastosomes has been influenced by developments related to the new opportunities presented by an active predatory lifestyle (reviewed Butler and Hodos 1996).

Increased mobility needed to find prey or evade predation led to selection of improved motor control through the development of regions such as the cerebellum. The midbrain of early vertebrates would have been relaying sensory information to the forebrain, had a topographic map and been involved in motor responses to sensory stimuli. The advent of jaws probably led to an expansion in size and sophistication of the midbrain for improved spatial analysis and orientation to stimuli. The increased size of the forebrain in amniotes and fish may have happened independently but was probably associated with an actively predacious lifestyle made possible by the evolution of jaws (Butler and Hodos 1996).

1.10 Zebrafish as a developmental model

1.10.1 Features of the zebrafish

The zebrafish is a good model organism for identifying genes essential for vertebrate development (Streisinger 1981, Kimmel 1989, Rossant and Hopkins 1992, Nusslein-Volhard 1994). Zebrafish are small, hardy, freshwater fish which can live in high density in small tanks without affecting their development or reproduction. The female lays 200-300 eggs per week which undergo external fertilisation and development so all stages of development can be studied and manipulated. Furthermore, the embryos are transparent during early stages of development so internal structures can be visualised without disturbing the embryo. Mutant screens of a number of organisms including zebrafish have been used to identify genes affecting early developmental events.

Mutant screens have been carried out in a number of organisms, including Drosophila (Nusslein-Volhard and Weischaus 1980) and Caenorhabditis elegans (Brenner 1974). These mutagenesis screens led to the identification of a large number of mutations affecting many important developmental processes. The main advantage of mutagenesis is that it selects for the small proportion of genes with unique and at least partially non-redundant functions as genes with redundant functions are not detected (Nusslein-Volhard 1994). Recently mutagenesis screens have been carried out using zebrafish.
Zebrafish has been used for mutagenesis screens because of its advantages as an embryological and genetic system (Streisinger et al 1981). The availability of large numbers of transparent, synchronous stage embryos allows screening for morphological traits with a dissecting microscope. In addition, mutations can be induced chemically with high efficiency as demonstrated during recent screens (Driever et al 1996, Haffter et al 1996, reviewed by Eisen 1996). Presently, the identification of mutant genes in zebrafish is by mapping or positional cloning.

Mapping mutations and candidate genes is an efficient way of identifying genes causing mutant phenotypes. Mapping has been used for a number of mutants, such as no isthmus (noi) which is caused by a mutation to pax2.1 (Brand et al 1996). Positional cloning has also been used to identify genes causing mutant phenotypes, such as the oep gene which is a novel EGF-related gene (Zhang et al 1998). New methods are being developed to improve the efficiency of identification of developmental genes.

An alternative method to chemical mutagenesis is insertional mutagenesis using either a retroviral vector (Schier et al 1996) or transposon (Weinberg 1998). This allows the genes to be identified much more quickly and easily than through positional cloning or mapping. Insertional mutagenesis of zebrafish has been tried on a small scale and has identified several novel developmental genes, such as dead eye and pescadillo (Allende et al 1996, reviewed by Schier et al 1996b). Therefore, several strategies have been employed to identify developmental genes in zebrafish.

A potential drawback of the zebrafish as a genetic system has come from the finding that teleost fish, including zebrafish, underwent an additional genome duplication after diverging from the last common ancestor of teleost fish and tetrapods (Postlethwait et al 1998, reviewed by Wittbrodt et al 1998). Although this is likely to lead to increased genetic redundancy, zebrafish will be useful for separating the multiple functions of genes as the functions will often be carried out by separate duplicate genes which have diverged rather than by a single gene as in mammals (Blader and Strahle 1998). Furthermore, the imminent development of reverse genetics in zebrafish, perhaps using ribozyme (Xie et al 1997) or double-stranded RNA injection (Fire et al 1998, Kennerdell and Carthow 1998), will allow knock-outs to be generated of known genes.

1.10.2 Stages of development of the zebrafish

The stages of development of the zebrafish are well characterised (Kimmel et al 1995). Zebrafish development is divided into seven periods - zygote, cleavage, blastula, segmentation, pharyngula, and hatching periods. These main developmental processes occur over the first three days after fertilisation. This thesis is mainly concerned with stages from beginning of gastrulation (6 hours post fertilisation) to the segmentation period (24 hours post fertilisation; Figure 1.5).
Early development is fundamentally similar to other vertebrates

Although, during early development, zebrafish exhibit morphologically different modes of development to other vertebrates, fate maps at the late blastula and of the neural plate are remarkably similar to other vertebrates (Kimmel et al 1990, Woo and Fraser 1995). During gastrulation, involution occurs, with cells at the blastoderm margin on the presumptive dorsal side (termed the shield), moving from superficial locations into the interior of the embryo. Thus the inner mesoderm and endoderm forms the hypoblast with an overlying ectoderm forming the epidermis and neuroectoderm (Warga and Kimmel 1990). The embryonic shield is thought to be equivalent to the organiser of other vertebrates on the basis of conserved gene expression, and the ability to induce secondary axes (Shih and Fraser 1995).

Development of the zebrafish brain

The dorsal epiblast thickens abruptly near the end of gastrulation at around bud stage (10h) as the neural plate becomes visible morphologically (Kimmel et al 1995). The neural plate is thicker in the head region and underlain by the prechordal plate hypoblast, the polster. The neural plate is formed from a monolayer of pseudostratified neuroepithelium which is comprised of columnar cells and so is raised above the adjacent non-neural ectoderm (Papan and Campos-Ortega 1994). Many important events in brain development happen during somitogenesis.
Figure 1.5  
Stages of zebrafish development.
Sketches of embryos at selected stages, animal pole to top at early stages and rostral to left at later stages. Scale bar: 250µm. Adapted from Kimmel et al (1995).
The neural keel forms during early somitogenesis. Before cavitation the anterior part of the neural keel undergoes regional morphogenesis. The eye rudiments begin to develop from the lateral walls of the diencephalon. By mid-somitogenesis ten neuromeres have formed, the telencephalon, diencephalon, midbrain and seven rhombomeres in the hindbrain. During later somitogenesis, the ventral diencephalon expands as the primordium of the hypothalamus, and the primordium of the epiphysis appears as a small well-delineated swelling in the midline of the diencephalic roof. The midbrain primordium subdivides horizontally to form the dorsal midbrain, tectum, and the ventral midbrain, tegmentum. The cerebellum forms near the end of somitogenesis as a prominent dorsal domain in the region of the midbrain-hindbrain boundary. The neural crest also appears during somitogenesis. The neural crest delaminates and migrates from the dorsolateral wall of both the brain up to the diencephalon and spinal cord primordia beginning at neural keel stage, and continuing until after cavitation of the neural keel.

1.11 Research strategies

The first aim of this study was to analyse the regionalisation of the zebrafish neural plate by patterns of gene expression in wild type and mutant lines. I also investigated the regulation of early neural plate markers using tissue transplantation and RNA overexpression. Next, I characterised a mutant line which I identified that did not express the early neural plate marker, anf. Unexpectedly this mutant line did not affect early neural development. Thirdly, I characterised an immunohistochemical method for labelling dividing cells and used it to carry out a preliminary analysis of cell proliferation in the zebrafish neural plate. Finally, I was involved with a mutagenesis screen carried out to isolate mutations affecting early development. As part of this screen, I characterised a mutation affecting neural crest development which led to pigmentation and other defects.
CHAPTER 2

MATERIALS AND METHODS

2.1 Fish maintenance and embryo preparation

Breeding fish were maintained at 28.5°C on a 14 hour light/10 hour dark cycle. Fertilised eggs were collected from natural spawnsings and raised in fish tank water with 0.1% methylene blue at 28.5°C. Embryos were staged according to Kimmel et al (1995). one-eyed pinhead (oep$^{m134}$, oep$^{tr257}$), masterblind (mb$^{m13}$), squint (sqt$^{c1}$), knollase (kas$^{t^{y}122}$), and silberblick (slb$^{tz216}$) carrier fish were obtained from Carl-Philipp Heisenberg. cyclops (cyc$^{b16}$) carrier fish were obtained from Charles Kimmel and Stefan Schulte-Merker. acerebellar (ace$^{ii282a}$) and no isthmus (noi$^{lb21}$) carrier fish were obtained from Michael Brand. Fixed knypek (kny$^{m119}$) and trilobite (tri$^{m209}$) embryos were obtained from Lilianna Solnica-Krezel. Fixed dino (din$^{tm84}$) embryos were obtained from Matthias Hammerschmidt.

2.1.1 Obtaining non-pigmented embryos

When required, the formation of pigment was prevented by incubating embryos from fertilisation in 0.2mM phenylthiocarbamide (PTU, Sigma) in tank water and staged as described above. Previous research has shown that PTU inhibits pigment formation in teleosts but has little affect on growth rate during the first two weeks of development (Vischer 1989). Prior to fixation, embryos were washed in tank water without PTU.

2.1.2 Observation of living embryos

Living embryos were viewed after manual removal of their chorions in 3% methyl cellulose using Nikon dissecting and compound microscopes.

2.2 Immunohistochemistry

2.2.1 Fixation of tissue

Embryos and larvae older than 24h were anaesthetised in 0.03% MS222 (3-amino benzoic acid ethyl ester, Sigma) prior to experimental procedures. Whole embryos in their chorions if younger than 18 somites, otherwise dechorionated, and larvae up to 48h were fixed in PEM fix (3.7% paraformaldehyde in PIPES buffer; 0.1M PIPES, 2mM EGTA, 1mM MgSO$_4$, pH 6.95) for 3 hours at room temperature or overnight at 4°C. Larvae over 48h were washed out of MS222 anaesthetic into phosphate-buffered saline (PBS, Oxoid), then fixed in 2% trichloroacetic acid (TCA; BDH) for 3 hours at room
2.2.2 Labelling

Immunohistochemistry methods were based on that of Wilson et al (1990) and Macdonald et al (1997). Embryos older than 48h were permeabilised with chilled 0.25% trypsin solution for 4 minutes on ice.

Embryos were rinsed with PBT at room temperature before incubation in 10% normal goat serum (Sigma) in PBS with 0.8% Triton X-100 (PBST) for 1 hour at room temperature to block non-specific binding. After rinsing in PBST, embryos were incubated in primary antibody diluted in 1% goat serum in PBST overnight at 4°C, although embryos older than 48h were left for 2-3 days. Embryos were washed in PBST for 2 hours at room temperature and then incubated in secondary antibodies (Sigma) diluted in PBST plus 1% goat serum at room temperature for 2-3 hours or overnight at 4°C, although embryos older than 48h were left for 2-3 days. In general, horse raddish peroxidase (HRP)-conjugated secondary antibodies were diluted 1:200. For embryos older than 48h, endogenous peroxidases were inactivated before the secondary antibody incubation by treating the embryos with 0.3% H₂O₂ in absolute methanol for 10 minutes at room temperature. After secondary antibody incubation, the embryos were washed 6 times in PBST over 2 hours at room temperature. For HRP-conjugated secondary antibodies, localisation of the antibodies was visualised by incubating the embryos in PBS in 2mM diaminobenzidine (DAB; Sigma) for 20 minutes at room temperature then activating the colour reaction by addition of 0.002% H₂O₂. The reaction was stopped by washing three times in PBS and embryos were postfixed in 4% paraformaldehyde overnight at 4°C. Embryos were dehydrated into 70% glycerol for dissection, mounting and microscopy.

The primary antibodies used included anti-PH3 polyclonal (L. Mähadevan unpublished data) diluted 1:1000; anti-Fkd2 polyclonal (Warga and Nusslein-Volhard 1999), 1:1000; anti-acetylated tubulin monoclonal (Sigma), 1:1000; HNK-1 monoclonal (Kruse et al 1984), 1:20; anti-Fret43 (Masai et al 1997) 1:500.

2.2.3 Bromo-deoxyuridine Labelling

Prior to treatment, embryos were dechorinated by hand. Embryos were incubated for 30mins to 1 hour in 10mM BrdU (Sigma B5002) in Ringers with 15% DMSO at 6°C water bath. Embryos were washed three times in Ringers and then incubated for 15-30 minutes at 28.5°C. After this, embryos were fixed in 4% paraformaldehyde at 4°C overnight.

Before processing for BrdU detection, embryos were rehydrated in PBST, incubated in 10μg/ml Proteinase K for 20 minutes and then refixed in 4%
paraformaldehyde for 20 minutes at room temperature. After fixation, embryos were rinsed in dH$_2$O and incubated in 2N HCL for 1 hour at room temperature. To stop the acid treatment, embryos were rinsed in PBST. Blocking and processing for BrdU antibody labelling and detection was as per the normal protocol. Anti-BrdU antibody (Sigma) was used at 1:1000 and anti-mouse IgG HRP conjugated secondary antibody at 1:200. Results were analysed with a Nikon compound microscope.

2.3 Standard molecular biology techniques

Standard molecular biology procedures were performed as detailed in Sambrook et al (1989) and outlined below.

2.3.1 Restriction enzyme digestion of DNA

DNA was digested with restriction enzymes according to the manufacturers instructions (Promega) in the buffer provided. Digestion was monitored by agarose gel electrophoresis.

2.3.2 Removal of protein from DNA or RNA by phenol/chloroform extraction

DNA or RNA was diluted to a volume of 200ml with dH$_2$O and extracted by addition of an equal volume of phenol/chloroform (1:1:24 tris-buffered phenol pH7.0:chloroform:isoamyl alcohol), vigorous mixing and brief centrifugation in a microfuge. The top phase containing the DNA or RNA was recovered and re-extracted by addition of an equal volume of chloroform (1:24 chloroform:isoamyl alcohol) alone.

2.3.3 Concentration of DNA or RNA by ethanol precipitation

DNA or RNA was precipitated by addition of sodium acetate (pH5.5) to a final concentration of 0.3M and 2.5 volumes of absolute ethanol. Samples were mixed and chilled at -20°C overnight or -70°C for 30 minutes then centrifuged for 20 minutes in a microfuge. The pellet was carefully washed with 70% ethanol and either air-dried or vacuum dried for 5 minutes.

2.3.4 Agarose gel electrophoresis

DNA and RNA were analysed by agarose gel electrophoresis. Generally, a 1% agarose gel in TBE buffer (0.045M Tris borate, 0.001M EDTA pH 8.0) with ethidium bromide to a final concentration of 1µg/ml was prepared.
2.4 In situ hybridisation

2.4.1 Preparation of embryos

Embryos at the appropriate stages were fixed for 3h at room temperature or overnight at 4°C in 4% paraformaldehyde in PBS, washed 3 times in PBS, rinsed in 100% methanol and stored in methanol at -20°C until required.

2.4.2 Preparation of Digoxigenin (DIG)-labelled and Flourescein-labelled riboprobe

10mg of cDNA for each probe was linearised with the appropriate restriction enzyme, purified by phenol extraction and precipitated as described in section 2.4. The DNA was resuspended to 0.5mg/ml in low TE (10mM Tris pH 8.0, 0.1 mM EDTA pH 8.0). The DIG or Fluorescein riboprobe was synthesised as described by Xu et al. (1994). 0.5mg of linearised DNA was incubated in a volume of 20ml with 10 units of RNA polymerase in transcription buffer (Promega) with 10mM DTT, nucleotide mix, 50U RNasin (Promega) for 2h at 37°C. Synthesis was analysed by agarose gel electrophoresis (1% agarose in TBE buffer, see section 2.4.4).

2.4.3 In situ hybridisation

In situ hybridisation was carried out as described in Xu et al (1994), with all steps carried out at room temperature unless stated otherwise. Embryos stored in 100% methanol at -20°C were rehydrated through a methanol series in PBT (PBS, 0.1% Tween-20, Sigma) and then rinsed in PBT. Embryos older than 24h were treated with proteinase K (10mg/ml) for 20 minutes at room temperature, washed in PBT and then refixed in 4% paraformaldehyde in PBS for 20 minutes. After washing 5 times with PBT, embryos were incubated in the hybridisation mix alone (50% formamide, 5xSSC, pH 6.0 adjusted with 1M citric acid, 50mg/ml Torula yeast RNA, 50mg/ml heparin, 0.1% Tween-20) for 2 hours at 65°C. In some cases embryos were stored at -20°C at this point prior to labelling. The hybridisation mix was then replaced with fresh hybridisation mix containing 50-200ng of digoxigenin-labelled or flourescein-labelled RNA and incubated with gentle shaking overnight at 65°C.

Following hybridisation, the embryos were washed in 25% hybridisation mix/2xSSC for 10 minutes at 65°C, once in 2x SSC and twice in 0.2x SSC for 30 minutes each. The embryos were further washed for 5 minutes each at room temperature in 75%, 50%, 25% 0.2xSSC/MABT (maleic acid buffer, Sigma; 0.1% Tween-20, Sigma), once in MABT alone then preincubated in MABT containing 2% Blocking Solution (Boehringer) overnight at 4°C. This was replaced with anti-digoxigenin-alkaline phosphatase antibody (Boehringer) diluted to 1:6000 in the same solution or anti-flourescein-alkaline phosphatase antibody (Boehringer) diluted 1:2000 in the same
solution and incubated for 4-6h at room temperature or overnight at 4°C. Prior to use, the anti-fluorescein antibody was preabsorbed by incubating overnight in PBS containing 30 fixed 36h whole zebrafish embryos at 4°C and was reused up to 10 times. After incubation, embryos were washed 6-8 times for 15 minutes each in PBT.

For a purple reaction product, embryos were rinsed in 0.1M Tris-HCl pH9.5, 0.1% Tween-20; for a light blue reaction, embryos were rinsed in 0.1M Tris-HCl pH9.5, 50mM MgCl₂, 0.1M NaCl, 0.1% Tween-20; for a red reaction product, embryos were rinsed in Tris-HCl pH8.5, 0.1% Tween-20. The colour reaction was carried out by incubating embryos in alkaline phosphatase substrate until the desired labelling intensity was obtained. For a purple reaction BM Purple (Boehringer) was used, BCIP (5-bromo-4-chloro-3-indoyl-phosphate; Boehringer) for light blue or 1 Fast Red tablet (Boehringer) diluted in 2ml 0.1M Tris-HCl pH2.2, Triton-20 0.1%. The reaction was stopped by washing in PBT then single colour in situ were incubated overnight in 4% paraformaldehyde in PBS at 4°C. Additionally, two colour in-situ were washed in glycine-HCl pH2.2 four times for 5 minutes and then rinsed in MABT with 2% Blocking Reagent (Boehringer) for at least 2h. These embryos were incubated with the appropriate secondary antibody in 2% Blocking Reagent (Boehringer) overnight at 4°C. The embryos were rinsed 6-8 times for 15 minutes each in PBT. The second colour was developed as described above. The reaction was stopped by washing in PBT and incubating overnight in 4% paraformaldehyde at 4°C.

Single-colour and two-colour in situ were washed in PBS and cleared and mounted in 70% glycerol as described for antibody labelling.

2.5 Tissue sectioning

Following in situ hybridisation, embryos for plastic sections were refixed in 4% paraformaldehyde for 3-5 days at 4°C, to prevent leaching of the stain, and then washed into 70% glycerol for storage prior to processing for sectioning. Embryos were rehydrated from 70% glycerol into dH2O and then dehydrated in a graded progression of methanol concentrations to 95% methanol in dH2O. This solution was replaced with activated Solution A (Agar Scientific; JB4 water soluble plastic embedding Solution A catalysed with 0.45g benzoyl peroxide per 50ml). Embryos were left overnight at room temperature. Next the embryos were placed into coffin moulds, inside a fume hood, and embedded in polymerised resin (Agar Scientific; one part JB4 Solution B to 25 parts activated Solution A). These moulds were placed into an airtight box, overlaid with nitrogen gas to facilitate polymerisation, and left overnight at room temperature.

After fixation, the plastic block containing each embryo was trimmed under dissecting microscope with a razor blade to enable correct orientation of the embryo prior to mounting it on a plastic chuck with Historesin (Riechert-Jung). The chuck was then
clamped into a Leica microtome and 10μm sections cut using Leica tungsten carbide blades. Sections were placed flat onto a droplet of water and allowed to dry onto an uncoated slide. Slides were then coverslipped with DPX and observed under Nikon compound microscope.

2.6 Preparation of mRNA for microinjection

Xbmp4 RNA was prepared and injected according to Neave et al (1997) and carried out in collaboration with B. Neave. The RNA was derived from the pSP64TXBMP-4 plasmid kindly provided by N. Ueno (Nishimatsu et al 1992). RNA was synthesised in vitro with SP6 RNA polymerase according to the manufacturers instructions (Boehringer), phenol extracted and precipitated twice as described in section 2.3 and resuspended in a small volume of sterile water. The concentration of RNA was determined by UV spectrophotometry (A260) and adjusted to final concentration of 100ng/ml. Several picolitres of RNA was injected into blastomeres of 1- to 4- cell embryos using a pressure-pulsed Picospritzer II (General Valve Corp.). Embryos were then fixed at the appropriate stage for further analysis. Control embryos were injected with β-galactosidase mRNA at the same concentration as shown in Barth and Wilson (1995). β-galactosidase RNA was derived from the pSP64Tβ-galactosidase plasmid (a gift from D. Wilkinson).

2.7 Embryonic shield and Hensen's node transplants

For transplantation of embryonic shield and Hensen's node (HN), the method of Koshida et al (1998) was followed and carried out in collaboration with M. Shinya and H. Takeda. Fertilised eggs at the 2- to 8- cell stage were injected with a mixture of rhodamine- and biotin-dexran (Molecular Probes) into the yolk with a glass micropipette. The dye spreads through all of the cells of the blastoderm. Embryos were transferred into dishes coated with 1% agar to prevent them sticking to the plastic dish and manually dechorionated with fine forceps in 33% Ringers solution. When the embryonic shield was visible (6h) embryos were mounted in 2% methyl cellulose and shield tissues were cut out with a sharpened tungsten needle and then transplanted through a glass micropipette into the ventral region of a shield stage host taking care not to damage the yolk membrane. Embryos were allowed to develop at 28.5°C in 33% Ringers solution in 1% agar coated dishes until they reached between 90% epiboly and bud stage when they were fixed in 4% paraformaldehyde overnight at 4°C and processed for in situ hybridisation. In some grafted embryos, the graft migrated to the dorsal side so only the embryos in which the graft remained ventrally were analysed.
For HN transplantation, 50-100μm fragments of chicken HN (HH Stage 4, definitive streak stage), which included the most potent region, the medial sector of both the epiblast and mesenchyme (Storey et al 1992), were manually removed. These were inserted through a small incision made into the ventral side of the zebrafish host during shield stage using a sharpened tungsten needle and taking care not to damage the yolk membrane. Embryos were allowed to develop at 28.5°C in 33% Ringers solution in 1% agar coated dishes until they reached between 90% epiboly and bud stage when they were fixed in 4% paraformaldehyde overnight at 4°C and processed for in situ hybridisation.

2.8 Alcian blue staining

I followed the method of Sordino et al (1995) using Alcian blue (Sigma) to stain the extracellular matrix associated with chondrocytes. This provides a clear marker of the skeletal patterning beginning at approximately 72h. Anaesthetized larvae were fixed in 5% TCA (BDH) at room temperature for 3h, and then rinsed in dH2O. Embryos were transferred into 0.1% Alcian blue dissolved in 70% ethanol/1% HCl for 3h and then into 70% ethanol/1% HCl overnight at 4°C. The embryos were dehydrated in 2 washes of 1h in 100% ethanol and cleared in 70% ethanol/1% HCl overnight at 4°C. Stained preparations were mounted in 70% glycerol and photographed with a Nikon compound microscope.

2.9 ENU mutagenesis and screening

2.9.1 Fish strains

The wild type line used for mutagenesis and outcrossing was *AB from the Orgon Laboratory.

2.9.2 Fish raising, keeping and mutagenesis

Mutants were generated using ethyl-N-nitrosourea (ENU, Sigma) as it causes lesions to single genes (Singer and Grunberger 1983), is a potent mutagen of the mouse germ line (Russell et al 1979) and can induce mutations in the proliferating germ line of male zebrafish with high efficiency (Driever et al 1996). Adult male fish were mutagenised with 3mM ENU by placing them into an aqueous solution for three 1h periods within one week. Three weeks after treatment, males were crossed to wild type females at weekly intervals and progeny generated originating from mutagenised premeiotic germ cells obtained were raised. The progeny from each mutagenised male were kept separate such that the origin of individual mutations could be traced back to the founder male.
2.9.3 Screening procedure

On the Friday prior to the screen, male and female fish to be screened were separated and given extra food over the weekend. About 20-40 pairs of fish per family, and about 2 families per week set up for egg lay in one afternoon (Monday) of the week. The following morning, eggs from successful matings were collected using a tea strainer, and 40-80 fertilised embryos were sorted within 5 hours post-fertilisation into 60mm Petri dishes in fish water with 0.1% methylene blue. Parental fish were kept in z-mod tanks labelled in accordance with the clutches of eggs. Fish that had not laid on the first day were checked again on the second day after set up. As with previous screens (Haffter et al 1996, Driever et al 1996) not all matings were successful. Fish of unsuccessful matings by the second day were returned to their tank, and more crosses of their family if necessary set up the following week. In general not more than eight crosses were evaluated with care.

For any two fish randomly taken from the F2 family, there is a 25% chance that they will both be carriers of the same mutation. The probability of finding a mutation in a family is \( P = (1 - 0.75^n) \) where \( n \) is the number of successful crosses. For \( n = 8 \), \( P \) gives 0.90. Thus, by screening a minimum of eight crosses for a family, we would lose at most 10% of the mutations.

Live embryos were scored at 2s, 24h, 48h, 72h for morphological abnormalities visible under a dissecting microscope. Embryos were also fixed at 2s in 4% paraformaldehyde for in situ hybridisation, at 30h for antibody screening and cell death screening. After labelling, embryos were screened for abnormalities on dissecting microscope. A minimum of 10 embryos per clutch was analysed for each screen.

At the 2s stage, the morphological screen was for progress of epiboly, proper formation of the axis and of the first somites. At 24h, the morphological screen was mainly for CNS defects. The shape of the telencephalon, diencephalon, epiphysis, tectum, cerebellum, midbrain-hindbrain border and hindbrain, size of the brain ventricles, presence of floor plate, shape of eyes and otic vesicle was examined. In addition, the shape and differentiation of the notochord and somites, and the overall shape of the body and yolk was examined. At 24h, an in situ hybridisation screen was carried out using a mixture of \( shh \) for midline development, \( zash1b \) for forebrain development and \( myoD \) for somite development and differentiation. The in situ hybridisation protocol followed was a shortened version of the normal protocol.

2.9.4 Screening in situ hybridisation protocol

At 24h embryos were fixed in 4% paraformaldehyde for 4 hours at room temperature. Embryos were washed three times in PBT and prehybridised in hybridisation solution (50% formamide, 5xSSC, 50\( \mu \)g/ml heparin, 500\( \mu \)g/ml Torula RNA, 0.1% Tween-20, citric acid to pH6) for 1 hour at 65°C. Fresh hybridisation solution with RNA
probe mix was added overnight at 65°C. Embryos were washed in 2xSSC, then given two 30min washes with 0.2xSSC at 65°C. At room temperature, embryos were rinsed twice in PBT and non-specific binding was blocked with 2% sheep serum and 2mg/ml BSA in PBT for 1 hour. This was replaced with fresh blocking solution with 1:5000 anti-DIG antibody for 2 hours at room temperature. Embryos were rinsed with 0.1M Tris-HCl pH9.5 before adding BM Purple (Boehringer) developing at room temperature. After staining was complete, embryos were rinsed overnight in 4% paraformaldehyde. Results were analysed using a Nikon dissecting microscope after embryos had been dehydrated in 70% glycerol.

2.9.5 Screening for cell death and late defects

At 24h, a cell death screen using ApopTag In Situ Apoptosis Detection Kit-Peroxidase (Oncor Inc.) was carried out for alterations to levels of cell division, particularly in the central nervous system. At 30h, anti-acetylated tubulin antibody was used to screen for the features of the axonal scaffold in the forebrain, midbrain and hindbrain (Wilson et al 1990) and anti-Fret43 antibody was used for the shape, size and differentiation of the epiphysis (Masai et al 1997). Between 48h and 72h, morphological screening was for general body shape and pigmentation, morphology of the brain, eyes and ears, formation of the jaw and branchial arches, formation of the heart, blood and circulatory system, presence of oedema, differentiation of the notochord, somites, muscle and fins.

2.9.6 Rescreen and outcrossing

Rescreening was carried out on all parents of mutants and of families on which a decision could not be made, for instance, because too few pairs bred or clutches were too small. In the rescreen, the embryos were analysed as during the main screen and additional analyses of the specific phenotype carried out. Parents generating interesting phenotypes were outcrossed against *AB wild type fish and an F3 line established which was numbered according to the allele series U1, U2, etc. After rescreening, families not generating an interesting phenotype were discarded.
CHAPTER 3

REGIONAL ORGANISATION OF THE ROSTRAL NEURAL PLATE

3.1 Introduction

The complex vertebrate forebrain develops from the morphologically simple neural plate. The transition from two to three dimensions and the vast increase in cell numbers and cell types indicates that this is a complex process. However, a knowledge of the early steps of this process is likely to help in our understanding of the later events of forebrain development. Therefore, I have studied early forebrain development by examining the regional organisation of the neural plate.

Traditionally, neural plate regional organisation has been studied by examining regional differences in cell fate and cell commitment. However, fate mapping studies are of limited value because, although they show where cells that give rise to a structure are located, they do not show how the cells acquire their fate. Commitment maps are also limited as they look at the position of cells when they make their final fate choice but do not study the steps which led to that decision. An alternative method to study when the neural plate becomes regionalised is to study gene expression. The relationship between genes with regionally restricted domains of expression are used to infer when regional identity becomes established and how regions change during development. Therefore, I analysed gene expression in the rostral neural plate of the zebrafish embryo. The gene expression study was used to address several questions regarding rostral neural plate development which have not been resolved by fate mapping and other studies.

Firstly, I addressed when the AP pattern is visible within the rostral neural plate. Zebrafish neural plate fate mapping studies suggest AP pattern is established at the start of gastrulation (Woo and Fraser 1995) but studies of commitment suggest AP pattern is not established until the end of gastrulation (Ho and Kimmel 1993, Woo and Fraser 1997). Commitment studies of several vertebrate species have also shown the AP axis is established before the DV axis (for example, see Simon et al 1995). Therefore, I studied gene expression to determine when AP pattern is first established in the rostral neural plate.

Secondly, I examined the validity of recent models of neural plate patterning. The two signal model proposes that initially the entire neural plate is anterior and a second signal is required to confer caudal characteristics (Neuiwkoop 1952, Saxen and Toivonen 1961). This model predicts that during early development, anterior genes will be expressed across the entire neural plate and later will be downregulated as posterior genes become expressed in caudal regions. I examined gene expression in the zebrafish neural plate during early development to see if I could find evidence to support this model.
Thirdly, I used the gene expression map to look for differences between the organisation of the rostral and caudal regions of the neural plate. For instance, the prosomere model (Rubenstein et al 1994) divides the entire rostral neural plate into transverse and longitudinal domains (Shimamura et al 1995). I looked at early gene expression patterns to ask if I could see any differences in rostral and caudal regions of the neural plate and if these were consistent with the prosomere model.

Finally, I also studied the expression and regulation of the homeodomain transcription factor encoding gene anf which is expressed very early in the rostral neural plate.

The aims of this study were to analyse the level of organisation in the rostral neural plate using gene expression and to characterise the expression and regulation of anf. I also sought to determine if the patterning of the zebrafish neural plate is consistent with models developed from studies of other vertebrate species.

3.2 Results

To examine neural plate patterning I used a variety of genes with regionally restricted expression domains during gastrulation. Amongst the earliest regionally restricted markers of the anterior neural plate are members of the Anf gene family (reviewed by Kazanskaya et al 1997). Anf family genes encode homeobox transcription factors and have been cloned in several vertebrate species including the zebrafish (Zaraisky et al 1995).

3.2.1 anf expression profile

anf is expressed in the anterior neural plate, telencephalon and anlage of the anterior pituitary

In zebrafish embryos, anf expression was first detected at around 50% epiboly in a large region of the anterior neural plate (Fig. 3.1A,B). anf expression begins to be lost at the midline at 75% epiboly (Fig. 3.1C,D), then anf expression disappears laterally from the midline at 80% epiboly (Fig. 3.1E,F) until the central area of expression was lost by 90% epiboly (Fig. 3.1G,H). anf was restricted to a crescent at the rostral most neural plate by bud stage (Fig. 3.1I,J). This domain fused at the dorsal midline during early somitogenesis (Fig. 3.1K,L). At 24h anf was expressed in the prospective anterior pituitary gland (Fig. 3.2A,B) and dorsal telencephalon (Fig. 3.2A,C).

anf is not expressed in the yolk syncitial layer

The mouse homolog of anf is hesxl (also called rpX; Thomas and Rathjen 1992, Thomas and Beddington 1996, Hermesz et al 1996). anf is 72% related to hesxl at the
Figure 3.1  *anf* is expressed in the rostral neural plate during gastrulation.

Embryos labelled as whole mounts by in situ hybridisation with rostral to the left. Animal pole (A,C,E,G,I,K) or lateral (B,D,F,H,J,L) views.  

A,B) at 50% epiboly *anf* is expressed broadly across rostral neural plate.  
C.D) by 75% epiboly, *anf* expression begins to be lost in the midline (arrowhead).  
E,F) at 80% epiboly, *anf* downregulation spreads laterally (arrowhead).  
G,H) by 90% epiboly, *anf* is downregulated further in an arrowhead shaped domain.  
I,J) at bud stage, *anf* is downregulated except for a rostral crescent of expression.  
K,L) by 4s, the crescent of *anf* expression meets at the dorsal midline.  

Abbreviations: nne, non-neural ectoderm; np, neural plate; t, prospective telencephalon.  
Scale bar: 125μm.
Figure 3.2 Expression of *anf* in the forebrain at 24h.

Embryos labelled as whole mounts by in situ hybridisation. Rostral to the left. Views of lateral with dorsal to top (A), ventral (B) or dorsal (C) orientations.

A,B) *anf* is expressed in the prospective anterior pituitary by 24h.

A,C) *anf* expression is restricted to the dorsal telencephalon by 24h.

Abbreviations: *ap*, anterior pituitary; *h*, hypothalamus; *t*, telencephalon.

Scale bars: 25μm.
amino acid level and hesxl has been implicated in mouse anterior development (Hermesz et al 1996, Thomas and Beddington 1996, Dattani et al 1998). hesxl is expressed in the extra-embryonic anterior visceral endoderm (AVE) before neural plate expression begins (Thomas and Beddington 1996, Hermesz et al 1996). In zebrafish embryos, the yolk syncitial layer (YSL) may perform the same function as the AVE (Beddington 1998, Houart et al 1998) but it has not been shown if the YSL and AVE express the same genes.

To determine if the YSL expresses the same genes as the AVE, the AVE marker hex (Thomas et al 1998), which is unrelated to anf and hesxl, was examined in zebrafish embryos. hex (C.Y. Ho, R. Beddington and D. Stainier unpublished) was expressed in the dorsal YSL at 50% epiboly (Fig. 3.3A,B) which showed that the YSL expresses AVE markers observed in other organisms. However, anf was not expressed in the YSL at any of the stages examined (Fig. 3.3C). Therefore, the YSL expresses the AVE marker hex but does not express anf.

**The anterior border of anf expression is at the rostral edge of the neural plate and the posterior border is in the mid-diencephalon**

To define the anterior border of anf expression, embryos were double-labelled with anf and either the placodal marker dlx3 (Akimenko et al 1994) or the non-neural ectoderm marker gta3 (Neave et al 1995). At 90% epiboly, the anterior border of anf met both dlx3 (Fig. 3.4A,B) and gta3 but did not significantly overlap (Fig. 3.4C,D). Thus, at 90% epiboly the anterior border of anf is at the rostral most edge of the neural plate.

The posterior border of the anf expression domain was defined using a marker of the prospective mid-diencephalon, flh (Talbot et al 1995), and a marker of the prospective midbrain boundary, pax2.1/noi (Krauss et al 1992a, Brand et al 1996, Lun and Brand 1998). Double-labelling with flh and pax2.1 (Fig. 3.5A) showed that they retain the same relative positions in the neural plate as during later development. Double-labelling with pax2.1 and anf (Fig. 3.5B) revealed that pax2.1 does not meet anf at 95% epiboly. Double-labelling with flh and anf (Fig. 3.5C) revealed that the posterior border of anf and the anterior border of flh meet at bud stage. Thus, during late gastrulation, the posterior border of anf is at the prospective mid-diencephalon.

**flh does not directly regulate the posterior boundary of anf**

As flh is expressed adjacent to the posterior border of anf during late gastrulation, it was possible that flh may regulate this boundary. To determine if flh regulates the posterior border of anf, anf expression was examined in flh mutant embryos. When late gastrulation flh mutant embryos were double-labelled with anf and pax2.1 it was found the posterior boundary of anf did not change (Fig. 3.5D). Therefore, flh does not appear to regulate the posterior boundary of anf.
**Figure 3.3** *hex*, but not *anf*, is expressed in the yolk syncitial layer.

Embryos labelled with *hex* (A,B) or *anf* (C) as whole mounts by in situ hybridisation (A) and sectioned (B,C). Views of animal pole with rostral to left (A), transverse view of 30-50μm midline section (B), or transverse views of 10μm plastic section with animal pole to the top (C).

A,B) at 50% epiboly, *hex* is expressed in the dorsal yolk syncitial layer.

C) *anf* is not expressed in the yolk syncitial layer at 50% epiboly.

Abbreviations: AP, animal pole; D, dorsal; epi, epiblast; evl, enveloping layer; V, ventral; Y, yolk cells; YSL, yolk syncitial layer.

Scale bars: A,B) 125μm. C) 100μm.
Figure 3.4 The rostral border of anf is at the rostral edge of the neural plate.
Embryos labelled with anf and dlx3 or anf and gata3 as whole mounts by in situ hybridisation. Animal pole (A,C) or lateral (B,D) views with rostral to the left.
A,B) at 90% epiboly, the rostral border of anf (purple) meets dlx3 (light blue), a marker of the non-neural ectoderm including the placodes, at the the boundary between neural and non neural (arrowheads).
C,D) at 90% epiboly, the rostral border of anf also meets gta3, a non-neural ectoderm marker, at the boundary between neural and non-neural (arrowheads).
Abbreviations: nne, non-neural ectoderm; np, neural plate.
Scale bar: 125μm.
Figure 3.5 The posterior border of anf is in the mid-diencephalon.
Embryos labelled as whole mounts by in situ hybridisation. Animal pole orientations with rostral to the left.
A) at bud stage, flh, a marker of the prospective mid-diencephalon, is expressed rostral to pax 2.1, a marker of the prospective midbrain.
B) at 95% epiboly, the posterior border of anf (purple) is rostral to the anterior border of pax2.1 (red).
C) at bud stage, the posterior border of anf meets the anterior border of flh.
D) at 95% epiboly, the space between the posterior border of anf and the anterior border of pax2.1 is indistinguishable in flh mutants and siblings.
Scale bar: 125μm.
A

flh  pax2.1

B

anf  pax2.1

C

anf  flh

D

flh

anf  pax2.1
3.3.2 AP patterning of the neural plate

There are at least three AP boundaries in the neural plate during early gastrulation

I used gene expression to analyse when the neural plate is first patterned along the AP axis and to follow how this pattern changes during development. To determine how many AP boundaries are present in the neural plate during early gastrulation embryos were labelled with anf, hoxA1 (Alexandre et al 1996), otx2 (Li et al 1994, Mori et al 1994) and fkd3 (Odenthal and Nusslein-Volhard 1998). The posterior borders of anf (Fig. 3.6A) and otx2 (Fig. 3.6B) were rostral to the anterior border of hoxA1. The anterior border of fkd3 met the posterior border of anf (Fig. 3.6C,D). The posterior border of otx2 was caudal to the posterior border of anf (Fig. 3.6E,F). Thus, three AP boundaries were identified in the early gastrulation neural plate at 65%-70% epiboly, firstly, the posterior border of anf and anterior border of fkd3; secondly, the posterior border of otx2 and thirdly, the anterior border of hoxA1.

At the end of gastrulation further AP boundaries are present

Further AP boundaries form during late gastrulation. Embryos double-labelled with anf and hoxA1 (Fig. 3.7A), otx2 and hoxA1 (Fig. 3.7B), anf and otx2 (Fig. 3.7C) or anf and fkd3 (Fig. 3.7D) showed that the same order in the AP boundaries identified at the start of gastrulation is present at 95% epiboly. Further AP boundaries were defined using fgf8 (Furthauer et al 1997, Reifers et al 1998), wnt1 (Krauss et al 1992b), pax2.1 and flh which are not expressed at earlier stages.

Additional AP boundaries were identified at late gastrulation. The posterior border of anf met the anterior borders of fkd3 (Fig. 3.7D) and flh (Fig. 3.7E). The posterior border of flh met the anterior border of wnt1 (Fig. 3.7F) but not the anterior border of pax2.1 (Fig. 3.7G). The anterior border of wnt1 (Fig. 3.7H) appeared to be closer than the anterior border of pax2.1 (Fig. 3.7I) to the posterior border of anf. The posterior border of wnt1 (Fig. 3.7J) and otx2 (Fig. 3.7B) were similar distances rostral to the anterior border of hoxA1. Thus, otx2 and wnt1 may share the same posterior border with wnt1 overlapping the caudal domain of otx2 expression domain (Fig. 3.7K). The posterior border of otx2 was rostral to the anterior border of fgf8 (Fig. 3.7K) and the posterior border of fgf8 met the anterior border of hoxA1 (Fig. 3.7L). Previous studies have shown that there is a small space between the posterior border of pax2.1 and the anterior border of hoxA1 (Alexandre et al 1996), thus the pax2.1 expression stripes may fit into the space between the posterior border of otx2 and the anterior border of fgf8 (Fig. 3.7K).

Thus, by combining these results, five AP boundaries could be defined in the neural plate at the end of gastrulation. The most rostral was defined by the posterior border of anf and the anterior borders of flh and fkd3. Moving caudally, the next boundary was defined by the posterior border of flh and the anterior border of wnt1. The
Figure 3.6 There are at least three AP boundaries in the rostral neural plate by early gastrulation.

Embryos labelled as whole mounts by in situ hybridisation (A-E) and sectioned (F). Animal pole orientations with rostral to the left (A-E) or, transverse 30-50μm midline section with animal pole to the top and rostral to the left (F).

A) at 65% epiboly, the posterior border of \textit{anf} is rostral to the anterior border of \textit{hoxA1}.

B) at 70% epiboly, the posterior border of \textit{otx2} (light blue) is rostral to the anterior border of \textit{hoxA1} (purple).

C,D) at 70% epiboly, the anterior border of \textit{fkd3} meets the posterior border of \textit{anf}.

E,F) at 70% epiboly, the \textit{anf} (purple) posterior border (white arrowhead) is rostral to the \textit{otx2} (light blue) posterior border (black arrowhead).

Scale bars: A-E) 125μm. F) 175μm.
Figure 3.7 By the end of gastrulation there are at least five AP boundaries in the rostral neural plate.
Embryos labelled as whole mounts by in situ hybridisation. Animal pole views with rostral to the left of 95% epiboly (B,D,H,I,K) or bud to lysis (A,C,E,F,G,J,L) stage embryos.
A) the posterior border of \textit{anf} is rostral to the anterior borders of \textit{hoxA1}.
B) the posterior border of \textit{otx2} is rostral to the anterior border of \textit{hoxA1}.
C) \textit{anf} (purple) posterior border (black arrowhead) is rostral to \textit{otx2} (red) posterior border (white arrowhead).
D) the posterior border of \textit{anf} meets the anterior border of \textit{fkd3}.
E) the posterior border of \textit{anf} meets the anterior border of \textit{flh}.
F) the posterior border of \textit{flh} meets the anterior border of \textit{wnt1}.
G) there is a small gap (white arrowhead) between the posterior border of \textit{flh} and the anterior border of \textit{pax2.1}.
H) there is a gap between the posterior border of \textit{anf} and the anterior border of \textit{wnt1}.
I) there is a gap between the posterior border of \textit{anf} (purple) and the anterior border of \textit{pax2.1} (red).
J) the posterior border of \textit{wnt1} is rostral to the anterior border of \textit{hoxA1}.
K) the posterior border of \textit{otx2} is rostral to the anterior border of \textit{fgf8}. The posterior region of \textit{otx2} appears to be organised into a stripe (arrowhead).
L) the posterior border of \textit{fgf8} meets the anterior border of \textit{hoxA1}.
Scale bar: 125µm.
next boundary was demarcated by the posterior borders of *wntl* and perhaps *otx2*, and the anterior border of *pax2.1*. However, as previous studies have found that *wntl* and *pax2.1* overlap (Lun and Brand 1998), further analysis is necessary to determine the number of AP boundaries at the end of gastrulation. A further border was defined by the posterior border of *pax2.1* and the anterior border of *fgf8*, in agreement with previous studies (Reifers et al 1998). The most caudal AP boundary identified in this study was defined by the posterior border of *fgf8* and the anterior border of *hoxA1*.

*The prospective midbrain may occupy a small region of the neural plate*

The study of AP boundaries of gene expression in the neural plate during gastrulation suggested that the prospective midbrain is initially restricted to a small part of the neural plate. For instance, there was a small space between the posterior border of *anf*, which labels the prospective mid-diencephalon, and the anterior border of *hoxA1*, which labels the prospective rhombomere3/4 border (Fig. 3.6A; Alexandre et al 1996).

To follow the relationship between *anf* and *hoxA1* during later stages, I double-labelled embryos with *anf* and *hoxA1* from early gastrulation to 2s (Fig. 3.8). The gap between *anf* and *hoxA1* remained small during early gastrulation (Fig. 3.8A), midgastrulation (Fig. 3.8B) and late gastrulation (Fig. 3.8C). From early bud stage (Fig. 3.8D) to late bud stage (Fig. 3.8E) and 2s (Fig. 3.8F) the space between the posterior border of *anf* and the anterior border of *hoxA1* increased. Thus, the space between *anf* and *hoxA1* expanded during late gastrulation.

The changes to gene expression in this region were analysed at later stages using *axial* and *wntl*. In addition to labelling the ventral midline, *axial* extends a stripe of expression dorsally which labels the prospective mid-diencephalon from midsomitogenesis stages (Strahle et al 1993). *wntl* labels the dorsal region of the prospective caudal midbrain and it also extends a stripe of expression ventrally at the caudal midbrain (Molven et al 1991). Embryos double-labelled with *wntl* and *axial* showed that the *wntl* expression domain in the dorsal neural tube gradually increased in length along the AP axis from about 4s (Fig. 3.8G). By 6s, the ventral stripe of *wntl* was visible at the caudal edge of the *wntl* expression domain (Fig. 3.8H). It could also be seen that the *wntl* expression domain in the dorsal neural tube had expanded slightly along the AP axis by 6s (Fig. 3.8H). By 10s, the anterior border of *axial* was visible in the ventral neural tube and was directly in line with the anterior border of *wntl* in the dorsal neural tube (Fig. 3.8I). At 14s, the DV stripe of *axial* from the anterior edge of the axial expression domain had begun to form and the region bordered by the dorsal stripe of *axial* and the ventral stripe of *wntl* (Fig. 3.8J) was similar to its length at 10s. At 18s, the space between the DV stripes of *axial* and *wntl* had increased (Fig. 3.8K) but did not appear to change further by 24h (Fig. 3.8L). Thus, the AP distance between the anterior border of *wntl/axial* and the posterior border of *wntl* increased gradually during somitogenesis.
Figure 3.8 The region of the neural plate including the prospective midbrain expands at the end of gastrulation.

Embryos were labelled as whole mounts by in situ hybridisation with rostral to the left. Lateral views.

A) at 60% epiboly, there is a small space between the posterior border of anf (starred), which labels the prospective mid-diencephalon, and the anterior border of hoxA1 (starred), which labels the prospective r3/r4 border.

B,C) the space between anf and hoxA1 does not significantly increase between 80% and 90% epiboly.

D,E) the region between anf and hoxA1 expands from early to late bud stage.

F) by 2s, the region is significantly larger than at early gastrulation.

G) at 4s, the region between the posterior border of wntl (starred), a caudal midbrain marker, and the anterior border of axial (starred), a mid-diencephalon marker, is small.

H,I) between 6s and 10s, the region between axial and wntl expands.

J,K) a dorsal projection of axial expression appears by 14s and the region between axial and wntl continues to expand until 18s.

L) the region does not increase further by 24h (L).

Scale bar: 125μm.
### 3.2.3 The rostral and caudal regions of the neural plate are differently organised

*During gastrulation, the ventral midline does not extend to the anterior most edge of the neural plate*

Two opposing models have been proposed to account for the organisation of the rostral neural plate (reviewed by Shimamura et al. 1995). In the non-convergent model, the ventral midline extends to the rostral tip of the neural plate (Keyser 1972, Altman and Bayer 1986, 1995, Swanson 1992). In the convergent model, the ventral midline does not extend to the rostral tip of the neural plate (Puelles 1987, Rubenstein et al. 1994). To determine how far rostrally the ventral midline of the zebrafish neural plate extends, embryos were labelled with markers of the midline (*shh* or *axial*) and markers of the edge of the neural plate (*dlx3* or *anf*).

Double-labelling with *dlx3* and *shh* (Fig. 3.9A) or with *dlx3* and *axial* (Fig. 3.9B) showed that there was a gap between the rostral tip of the midline and the edge of the neural plate at late gastrulation. Likewise, when embryos were double-labelled with *anf* and *axial* (Fig. 3.9C) or *anf* and *shh* (Fig. 3.9D) this gap was also observed.

Embryos were also analysed after gastrulation to determine if the ventral midline extends to the tip of the developing neural tube in later stages. When bud stage embryos were double-labelled with *shh* and *anf*, it was found that there was a small gap between the rostral tip of *shh* and *anf* (Fig. 3.9E,F). However, when 2s stage embryos were double-labelled with *shh* and *anf*, it was found that the *anf* and *shh* expression domains touched in the rostral neural tube (Fig. 3.9G,H). Thus, during early somitogenesis, *shh* and *anf* expression domains met.

*There is a difference in the organisation of the rostral and caudal regions of the neural plate during gastrulation*

The finding that the midline does not extend to the rostral tip of the neural plate until after gastrulation raised the possibility that the midline provides patterning information differently in rostral and caudal regions of the neural plate. Such different patterning methods might lead to differences in the regional organisation of the rostral and caudal neural plate. To compare the regional organisation in the rostral and caudal neural plate during gastrulation, the expression of several genes was studied.

It was found that genes were expressed differently in the rostral and caudal regions of the neural plate. The rostrally expressed genes *anf* (Fig. 3.10A), *pax6* (Fig. 3.10B; Krauss et al. 1991b,c), *rxB* (Fig. 3.10C; Mathers et al. 1997), *six3* (Fig. 3.10D; Seo et al. 1998), and *LH2A* (Fig. 3.10E; H. Okamoto, unpublished data) were expressed in radial arcs in the rostral neural plate. In contrast, the caudally expressed genes *wntl*
Figure 3.9 The midline does not reach the rostral tip of the neural plate at 95% epiboly.

Embryos were labelled as whole mounts by in situ hybridisation. Animal pole with rostral to the left (A-E,G) or dorsal with animal pole to top (F,H). 95% epiboly (A-D), bud (E,F) or 2s (G,H) stages.

A,B) the midline markers shh or axial do not touch the expression domain of the non-neural ectoderm marker, dlx3.

C,D) the midline markers shh and axial do not reach the expression domain of the rostral neural plate marker, anf.

E,F) at late bud stage, shh does not meet the expression domain of anf at the rostral neural plate (starred).

G,H) by 2s, the shh labelled ventral midline meets the anf labelled dorsal midline in the rostral neural tube (starred).

Scale bars: A-E, G) 125μm. F,H) 250μm.
(Fig. 3.10A), pax2.1 (Fig. 3.10B), fgf8 (Fig. 3.10D) and flh (Fig. 3.10F) were expressed in stripes. svp46 (Fjose et al 1993) was expressed in both rostral and caudal regions of the neural plate and it was expressed in a radial arc in the rostral neural plate but in stripes in the caudal neural plate (Fig. 3.10G). Thus, of the markers analysed to date, genes expressed in the most rostral neural plate were organised into radial arc expression domains, whereas genes expressed in the more caudal neural plate were expressed in striped domains.

The transition between radial and stripe organisation occurs at the mid-diencephalon

To determine which was the most rostral stripe, embryos were labelled with a number of genes expressed in either striped or radial arc-shaped domains. When embryos were double-labelled with anf, which is expressed in a crescent during late gastrulation, and flh, which is expressed in stripes, it was found that the posterior border of anf met the anterior border of flh (Fig. 3.10F). Embryos were also double-labelled with flh and the rostral marker pax6 (Fig. 3.10H) and it was found that flh overlapped the posterior region of pax6. When embryos were double-labelled with anf and pax6 (Fig. 3.10I) it was found that pax6 had a more caudal posterior border than anf. Embryos were also double-labelled with fkdl3, which has a striped anterior border, and anf (Fig. 3.10J) and it was found that the posterior border of anf and the anterior border of fkdl3 meet. Thus, the most rostral stripe in the neural plate during gastrulation appeared to be labelled dorsally by flh and ventrally by fkdl3.

3.2.4 Regulation of anf expression

The regional organisation of the rostral neural plate is likely to be regulated by signals from the same tissues that have been shown to pattern more caudal regions of the neural plate. Studies of the caudal neural plate have implicated the axial mesoderm and the non-neural ectoderm as sources of patterning information. The gene anf is an early regionally restricted marker of the rostral neural plate so it was of interest to study the regulation of anf.

The axial mesoderm may be involved in the downregulation of anf

The axial mesoderm has been shown to be important for regulating the expression of genes in the rostral neural plate, including otx2 (Ang and Rossant 1994). To examine if the axial mesoderm is involved in the downregulation of anf, embryos were double-labelled with anf and an axial mesoderm marker, axial (Strahle et al 1993), shh (Krauss et al 1993) or an antibody to Fkd2 (Warga and Nusslein-Volhard 1999). Double-labelling with axial and anf (Fig. 3.11A,B), shh and anf (Fig. 3.11C,D) or Fkd2 and anf (Fig. 3.11E,F) indicated that the axial mesoderm is beneath the anf expression domain at the
Figure 3.10 Gene expression in the rostral neural plate is organised into crescents or semi-circles and the caudal neural plate into stripes.

Embryos were labelled as whole mounts by in situ hybridisation. Animal pole views with rostral to the left of 95% to 1 somite stage embryos.

A-E) rostrally expressed markers are expressed in a crescent or semicircle shaped domain, including: anf (A); pax6 (B); rxB (C); six3 (D); and LH2A (E).

A,B,D,F) caudally expressed markers are expressed in stripes, including: wnt1 (A); pax2.1 (B); fgf8 (D); and flh (F).

G) the marker svp46 is expressed in both rostral and caudal regions are expressed in semi-circles in the rostral neural plate and stripes in the caudal neural plate.

H) the posterior border of pax6 meets and may overlap the expression domain of flh.

I) the posterior border of anf (purple) is rostral to the posterior border of pax6 (light blue).

J) the posterior border of anf meets the rostral border of fkd3.

Scale bar: 125μm.
Figure 3.11 The midline mesoderm is beneath the anf expression domain before anf is downregulated.

Embryos labelled as whole mounts by in situ hybridisation (A-D) and then with Fkd2 antibody (E,F). Animal pole views with rostral to the left at 80% epiboly (A,C,E) or 95% epiboly (B,D,F).

A,B) the axial expression domain extended beneath the anf expression domain before (A) and during (B) anf downregulation.

C,D) the shh expression domain extended beneath the anf expression domain before (C) and during (D) anf downregulation.

E,F) Fkd2 epitope is expressed beneath the anf expression domain before (E) and during (F) anf downregulation.

Scale bar: 125μm.
time when \( anf \) begins to be downregulated. Thus, the axial mesoderm is in a position to play a role in mediating \( anf \) downregulation. To analyse the role of the axial mesoderm in \( anf \) regulation, \( anf \) expression was studied in two midline mutants, \( cyc \) and \( oep \).

\textit{anf expression is similar in cyc mutant and wild type embryos}

\( cyc \) mutant embryos (Hatta et al 1991a) lack the most rostral expression of midline markers, such as \( shh \) (Krauss et al 1993), and have reduced expression of \( gsc \) (Fig. 3.12A,B; Thisse et al 1994). Therefore, \( cyc \) mutant embryos were used to analyse the role of the midline in \( anf \) regulation.

\( cyc \) mutant embryos were double-labelled with \( anf \) and \( shh \). \( shh \) was used to identify \( cyc \) mutant embryos. \( anf \) was expressed in the same way in wild type (Fig. 3.12C) and \( cyc \) mutant (Fig. 3.12D) embryos. \( anf \) downregulation also followed the same pattern in wild type (Fig. 3.12E) and \( cyc \) mutant (Fig. 3.12F) embryos except for a small patch of \( anf \) expression retained at the midline. This patch of expression was due to \( anf \) not \( shh \) as \( cyc \) mutant embryos labelled with \( anf \) also expressed it (data not shown). Thus, Cyc protein is not required to regulate \( anf \) expression at the midline.

\textit{The neural plate is the same size in cyc mutant and wild type embryos}

It is uncertain if \( cyc \) mutant embryos have wrongly specified midline cells or if they are absent (Macdonald et al 1995, Hatta et al 1991a, 1994). If the midline cells are present but wrongly specified the neural plate should be the same size in wild type and \( cyc \) mutant embryos. However, if the midline cells are absent then the neural plate should be smaller in wild type then in \( cyc \) mutant embryos. \( cyc \) mutant embryos labelled with \( anf \) (Fig. 3.12F), \( dlx3 \) (Fig. 3.13A), \( otx2 \) (Fig. 3.13B), \( six3 \) (Fig. 3.13C) and \( pax6 \) (Fig. 3.13D) showed that the neural plate is the same size in mutant and wild type embryos. This finding supports the idea that the midline is present during late gastrulation but wrongly specified.

\textit{The midline of cyc mutant embryos is mis-specified}

The midline of \( cyc \) mutant embryos express dorsal markers that are not normally present. \( svp46 \) was expressed broadly in the rostral neural plate but not the midline of wild type embryos (Fig. 3.13E). In \( cyc \) mutant embryos \( svp46 \) was also broadly expressed in the rostral neural plate but including the midline (Fig. 3.13F). \( pax2.1 \) was not expressed in the midline of wild type (Fig. 3.13G) but was in the midline of \( cyc \) mutant (Fig. 3.13H) embryos. In contrast, \( rtk1 \) (Xu et al 1994) is normally expressed in the rostral midline of wild type (Fig. 3.13G) but is not expressed in the midline of \( cyc \) mutant (Fig. 3.13H) embryos. Thus, the midline of \( cyc \) mutant embryos expressed dorsal markers such as \( svp46 \) and \( pax2.1 \) instead of ventral markers such as \( rtk1 \). Therefore, the \( cyc \) mutant midline is wrongly specified as dorsal.
Figure 3.12 cyc embryos express and downregulate anf.
Embryos labelled as whole mounts by in situ hybridisation. Animal pole views with rostral to the left. 95% epiboly (A-D) or 2s (E,F) stage embryos. A,B) wild type gsc expression is reduced in cyc embryos. C,D) shh expression extends further rostrally in wild type than cyc embryos. E,F) anf is restricted to a crescent in the rostral neural plate in wild type and cyc embryos, except at the midline (arrowhead in F). Scale bar: 125μm.
Figure 3.13 The neural plates of wild type and cyc embryos are the same size.

Embryos labelled as whole mounts by in situ hybridisation. Animal pole views with rostral to the left. Bud (A,C,D,G,H), 90% epiboly (B) or 95% epiboly (E,F) stages.

A-D) expression of neural plate markers dlx3, otx2, six3 and pax6 is indistinguishable in wild type and cyc embryos.

E,F) svp46 is not expressed across the midline (arrowhead) of wild type but is across the midline of cyc embryos.

G,H) rtkl is expressed further rostrally in wild type than cyc embryos. pax2.1 is not expressed across the midline (arrowhead) of wild type but is across the midline of cyc embryos.

Scale bar: 125µm.
**anf is expressed and downregulated in oep embryos but in a smaller domain**

-oep- mutant embryos have defects in their ventral CNS and cyclopia (Schier et al. 1996a, Solnica-Kretzel et al. 1996). Genes normally expressed in ventral midline cells in wild type embryos are absent anteriorly in -oep- mutant embryos. For example, -gsc- was expressed strongly in wild type (Fig. 3.14A) but weakly in -oep- mutant embryos (Fig. 3.14B; Schier et al. 1996a). -oep- mutant embryos were used to analyse the role of the midline in -anf- regulation.

-oep- embryos were double-labelled with -anf- and -shh-. -shh- was used to identify -oep- mutant embryos. -anf- was expressed in a broader domain in wild type (Fig. 3.14C) then -oep- mutant embryos (Fig. 3.14D). -anf- midline expression was downregulated in wild type (Fig. 3.14E) and -oep- mutant embryos (Fig. 3.14F). An ectopic patch of -anf- expression was retained in the midline of -oep- mutant embryos that was lost in wild type embryos. These cells were expressing -anf- not -shh-, as -oep- mutant embryos labelled with -anf- alone also had the ectopic staining (data not shown). Thus, -oep- mutant embryos express -anf- in a smaller domain and downregulate expression at midline apart from a small ectopic region.

**The rostral neural plate is smaller in oep mutant than wild type embryos**

-anf- labelling suggested that the rostral neural plate was smaller in -oep- mutant than in wild type embryos. -dlx3- labelling showed that the wild type rostral neural plate (Fig. 3.15A) was larger than -oep- mutant neural plate (Fig. 3.15B). The expression of -six3- (Fig. 3.15C,D) and -pax6- (Fig. 3.15E,F) was dramatically reduced in the -oep- rostral neural plate compared to wild type embryos.

The -oep- mutant caudal neural plate appeared to be less reduced than the rostral neural plate. The -pax2.1- stripes were a similar size in -oep- and wild type neural plates (Fig. 3.15G,H). The posterior region of the -svp46- (Fig. 3.15I,J) and -otx2- (Fig. 3.15K,L) expression domains were similar in size in the caudal neural plates of wild type and -oep- mutant embryos.

Thus, -oep- mutant embryos have smaller rostral neural plates which are regionally organised and almost normal sized caudal neural plates.

**The midline of oep mutant embryos expresses dorsal genes**

-oep- mutant embryos were examined to determine if the midline is absent or mis-specified as observed in cyc mutants. The midline of wild type embryos expressed -rtkl- but not -pax2.1- (Fig. 3.15G). However, -oep- mutant embryos lacked -rtkl- expression in the rostral midline but had -pax2.1- expressed across the midline (Fig. 3.15H). -svp46- was also expressed across the midline of -oep- mutant but not wild type embryos (Fig. 3.15I,J).
**Figure 3.14 oep embryos express and downregulate anf.**
Embryos labelled as whole mounts by in situ hybridisation. Animal pole views with rostral to the left. 90% epiboly (A,B), 80% epiboly (C,D) or bud (E,F) stages.

A,B) gsc expression in wild type embryos is reduced in oep embryos.
C,D) anf is downregulated in wild type and oep embryos. shh is not expressed as far rostrally in oep as wild type embryos.
E,F) anf is downregulated to a crescent in wild type and oep embryos, except for an ectopic patch of expression in oep embryos (arrowhead in F).
Scale bar: 125μm.
Figure 3.15  

*oeo* embryos have smaller rostral neural plates than wild type embryos.

Embryos labelled as whole mounts by in situ hybridisation. Animal pole views with rostral to the left. Bud (A,B,E,F), 80% epiboly (C,D,I,J), 90% epiboly (K,L) or 95% epiboly (G,H) stages.

A,B) *dlx3* is expressed in a smaller rostral domain in *oeo* than wild type embryos but the caudal expression of *dlx3* is normal in *oeo* embryos.

C-F) the rostral markers *six3* and *pax6* are expressed in smaller domains in *oeo* than wild type embryos.

G,H) the midline marker *rtk1* is expressed to a less rostral extent in *oeo* than wild type embryos. *pax2.1* is not expressed across the midline (arrowhead) in wild type embryos but is across the midline (arrowhead) of *oeo* embryos.

I,J) *svp46* is expressed across the midline of *oeo* but not wild type embryos.

K,L) *otx2* is expressed in a smaller domain in *oeo* than wild type embryos.

Scale bar: 125µm.
Thus, the midline of *oep* mutant embryos also expressed dorsal rather than ventral markers.

**oep mutant embryos have a small fused eyefield**

_oep* mutant embryos have a small fused eye (Schier et al 1996a) which may be reduced because it develops from a small eyefield. The markers *six3* (Fig. 3.15C,D) and *pax6* (Fig. 3.15E,F) label the rostral neural plate, including the eyefield and diencephalon, and were expressed in larger domains in wild type than *oep* mutant embryos. The eyefield marker, *svp46* was split at the midline in wild type (Fig. 3.15I) but not in *oep* mutant (Fig. 3.15J) embryos. Thus, *oep* mutant embryos have a small eyefield which fails to separate at the midline. Therefore, the analysis of *anf* expression in *cyc* and *oep* mutant embryos showed that *anf* is relatively normally regulated in *cyc* and *oep* even though the midline is mis-specified.

**The embryonic shield does not determine the posterior border of anf**

The zebrafish embryonic shield expresses organiser specific genes such as *gsc* (Stachel et al 1993, Schulte-Merker et al 1994, Thisse et al 1994). Transplantation of the zebrafish organiser, the embryonic shield, to ectopic locations can induce secondary axes (Oppenheimer 1936, Shih and Fraser 1996, Koshida et al 1998). These secondary axes always retain the same AP orientation as the main axis (Koshida et al 1998). Secondary axes induced by transplanted shields were used to determine if signals emanating from the shield controlled the posterior border of *anf*. As the position of the ectopic shield varies along the animal to vegetal pole axis, the affect of the position of the shield on *anf* expression was assessed. If the shield controlled the posterior border of *anf*, *anf* expression would not be influenced by the position of the shield. However, if the posterior border was not controlled by the shield, the posterior border of *anf* might be the same as in the main axis.

Control sibling embryos did not express *anf* ectopically (n= 20; Fig. 3.16A,B). Shield transplants induced ectopic expression of *anf* in 73% of the secondary axes (n=27; Fig. 3.16C,D). The ectopic *anf* expression was downregulated in the midline as was the usual expression domain. The posterior border of the ectopic *anf* expression domain was the same as the normal *anf* expression domain and not affected by the position of the shield along the animal pole to vegetal pole axis. Likewise, in the secondary axes which ectopically expressed *svp46* (n=9; Fig. 3.16E,F), the ectopic domain of *svp46* expression was expressed with the same posterior border as the normal *svp46* expression. The ectopic *svp46* expression was downregulated in the midline as was the usual expression domain. Therefore, the posterior boundaries of *anf* and *svp46* expressed in the secondary axis were independent of the position of the shield.
Figure 3.16 Ectopically placed embryonic shields induce secondary axes which express *anf* and *svp46*.

Embryos, in which zebrafish shields were transplanted to ectopic locations at late dome stage, were fixed at late gastrulation for whole mount in situ hybridisation. Animal pole (A,C,E) or lateral (B,D,F) orientations with rostral to the left. All embryos 90% epiboly.

A,B) *anf* was not ectopically expressed in control embryos which did not have transplanted shields.

C,D) Shield transplants induced secondary axes which expressed *anf* (arrowheads). The ectopically induced *anf* expression domain shared the same posterior boundary as the normal *anf* expression domain. Dots indicate the boundary of *anf* expression in the primary axis.

E,F) Shield transplants induced secondary axes which also expressed *svp46* (arrowhead) with the same AP borders as in the primary axes. Dotted lines indicate the boundary of normal *svp46* expression in the primary axis.

Abbreviations: A, anterior; AP, animal pole; D, dorsal; P, posterior; V, ventral.

Scale bar: 125μm.
Hensen's node is able to induce secondary axes which express but may not downregulate anf

To study the role of the axial mesoderm in regulating anf expression, transplants of Hensen's node, the chick organiser, were used to induce a secondary axis in zebrafish embryos (Hatta and Takahashi 1996, Koshida et al 1998). It has previously been shown that Hensen's node transplants to other vertebrate species induce secondary axes which do not form axial mesoderm (Kintner and Dodd 1991, Koshida et al 1998).

Control sibling embryos did not have secondary axes or ectopically express anf (n=20; Fig. 3.17A,B). anf was expressed in 67% of the secondary axes induced by Hensen's node (n=24; Fig. 3.17C,D) in addition to the usual expression domain. The secondary axes that did not express anf did not extend anteriorly as far as the AP level where anf is expressed in the normal axis. The ectopic expression of anf did not appear to be downregulated in the midline by bud stage, the latest stage examined.

Overexpression of Xbmp4 suppresses anf

In addition to the axial mesoderm, the non-neural ectoderm may also pattern the neural plate (Liem et al 1995, Dale et al 1997). bmp4 is expressed in non neuronal ectoderm adjacent to the neural plate from the start of gastrulation (Nikado et al 1997) so may regulate anf. To determine if bmp4 regulates anf expression, Xbmp4 was ectopically expressed in zebrafish embryos (Neave et al 1997).

Control embryos, both uninjected and βgal injected siblings did not have an alteration to anf expression (n= 87; Table 3.1; Fig. 3.18A,B). However, 65% of embryos injected with Xbmp4 had reduced or a complete loss of anf expression (Fig. 3.18C,D; Table 3.1; n= 96). Therefore, Xbmp4 is capable of suppressing anf expression in the neural plate.

dino mutant embryos express anf in a smaller region

The secreted protein chordin is expressed within the neural plate during early development and may regulate the dorsoventral axis by antagonising bmp4 (Sasai et al 1994, Piccolo et al 1996). dino mutant embryos (Hammerschmidt et al 1996) which have a mutation in chordin that may be null (Schulte-Merker et al 1997) were used to test if chordin regulates anf expression levels. anf and hoxA1 expression in wild type (Fig. 3.19A) embryos was reduced in dino mutant (Fig. 3.19B) embryos but retains the same spatial boundaries. In the same way, otx2 and hoxA1 expression in wild type (Fig. 3.19C) embryos was reduced in dino mutant (Fig. 3.19D) embryos whilst retaining the same spatial boundaries. Thus, anf, otx2 and hoxA1 are expressed in reduced domains in dino mutant embryos.
Figure 3.17  Hensen's node induced secondary axes which expressed anf.

Chicken organiser, the Hensen's node (HN), was transplanted into zebrafish embryos at dome stage and these embryos were fixed at late gastrulation for whole mount in situ hybridisation. Animal pole (A,C) or lateral orientations (B,D) with rostral to the left. All embryos 90% epiboly.

A,B) control embryos which did not have HN transplants did not express anf ectopically C,D) HN induced secondary axes which expressed anf (arrowhead). In the induced axes, anf was expressed with the same posterior border as in the primary axes. Scale bar: 125μm.
Figure 3.18 Xbmp4 suppresses anf expression.
Embryos injected at 2-4 cell stage with Xbmp4 or βgal and fixed for whole mount in situ hybridisation. Animal pole views with rostral to the left. A,B,C) 90% epiboly or (D) 70% epiboly stages.
A,B) anf is expressed normally in uninjected and βgal injected (B) control embryos.
C,D) anf expression is either reduced or abolished in Xbmp4 injected experimental embryos.
Scale bar: 125μm.
Table 3.1 Expression of anf after Xbmp4 overexpression

<table>
<thead>
<tr>
<th></th>
<th>Reduced anf expression no. of embryos</th>
<th>Normal anf expression no. of embryos</th>
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<tbody>
<tr>
<td>X-bmp4 injected</td>
<td>96 65%</td>
<td>53 35%</td>
</tr>
<tr>
<td>Uninjected control</td>
<td>0 0%</td>
<td>65 100%</td>
</tr>
<tr>
<td>βgal injected control</td>
<td>0 0%</td>
<td>22 100%</td>
</tr>
</tbody>
</table>
**Figure 3.19** *anf* expression domain is reduced in *dino* embryos.

Embryos labelled as whole mounts by in situ hybridisation. Animal pole views with rostral to the left. 80% epiboly (A,B) or bud stage (C,D) embryos.

A,B) wild type *anf* and *hoxA1* expression is reduced in *dino* embryos.

C,D) wild type *otx2* (light blue) and *hoxA1* (purple) expression is reduced in *dino* embryos.

Scale bar: 125μm.
3.3 Discussion

3.3.1 anf is an early rostral neural plate marker

My studies found that anf is expressed in the rostral neural plate from early gastrulation and by 24h anf is expressed in the telencephalon and prospective anterior pituitary. The expression of anf in the zebrafish neural plate and forebrain was consistent with the expression of homologs of anf in other species, including XANF1 in Xenopus (Zariasky et al 1995) and hesxl in mouse (Hermesz et al 1996, Thomas and Beddington 1996). However, hesxl is also expressed in the extra-embryonic anterior visceral endoderm (AVE) before neural plate expression is induced (Thomas and Beddington 1996). The AVE has been shown to be required for anterior patterning and may act by inducing an anterior organiser (Ruiz i Altaba 1998). The zebrafish does not have an AVE but it has been proposed the YSL performs the same function (Houart et al 1998). My study of the YSL showed that the YSL expresses the AVE marker hex and thus supports the theory that the AVE and YSL are functionally equivalent. However, my finding that anf is not expressed in the YSL suggests either anf does not perform the same function as the murine AVE marker hesxl or there may be another anf related gene which is expressed in the YSL. My characterisation of a fish line which does not express anf supports this second possibility (Chapter 4).

3.3.2 AP boundaries in the neural plate

I have found there are at least three AP boundaries in the zebrafish neural plate by early gastrulation. The finding that AP patterning is present in the neural plate from early gastrulation is earlier than suggested by commitment studies (Ho and Kimmel 1993, Woo and Fraser 1997) but in agreement with fate and specification studies (Woo and Fraser 1995, Grinblat et al 1998). The reason for the different findings of these studies may be because they analyse different steps in the regionalisation process. Commitment studies examine the later steps of regional organisation but gene expression, fate maps and tissue specification studies look at the earlier steps leading to these commitment decisions. The finding that gene expression and fate mapping studies agree when AP pattern is first assigned may be because neural plate cells do not mix much after the onset of gastrulation (Warga and Kimmel 1990) so cells of similar potential stay together even before they have begun to make fate decisions.

The prevailing model of AP patterning, the Two Signal Model (reviewed by Doniach 1995), maintains that during gastrulation prospective posterior neuroectoderm is initially specified as anterior and subsequently respecified as posterior (Eyal-Giladi 1954, Sive et al 1989, Ang and Rossant 1994). My finding that at early gastrulation anterior
(anf, otx2) but not posterior (hoxA1, fkd3) markers were expressed in the rostral, but not caudal, neural plate suggests the entire neural plate has at least partial AP pattern by early gastrulation. As the two signal model predicts the neural plate should begin as all anterior my findings do not support the two signal model. This does not rule out the possibility that the caudal neural plate is specified as anterior before gastrulation, but it is unlikely as genes such as hoxA1 are regionally expressed at the very onset of gastrulation (Alexandre et al 1996). To be more certain of the AP patterning at early gastrulation requires the analysis of further early markers with restricted expression patterns, but at the time of the study, further markers were not available.

My finding that there are fewer AP boundaries at early gastrulation then late gastrulation suggest AP identity is acquired gradually. The precise number of AP boundaries identified in my gene expression study must be regarded as provisional, as there are certainly more markers yet to be identified, which may delineate additional AP boundaries. The fine detail of the AP pattern of the neural plate appears to be induced in distinct phases as genes are expressed either at the start of gastrulation, such as otx2 and anf, or near the end, such as pax6 or rxB. The induction of organising centres may be responsible for patterning the neural plate particularly during later stages, as demonstrated for the midbrain (Crossley et al 1996).

To understand the importance of early AP boundaries to subsequent development requires labelling them at early gastrulation and examining these embryos at 24h to determine their fate. An identification of boundaries that persist into later stages and correlate with morphology would suggest these early boundaries are of importance to neural development.

It is not known how the AP boundaries are established, but studies of AP patterning of the hindbrain suggest rhombomeric boundaries are regulated by eph signalling mediated cell sorting (Xu et al 1999, Mellitzer et al 1999). Overexpression studies of rtkl suggest eph signalling is involved in defining the diencephalon and eyefield territories (Xu et al 1996). Overexpression of eph ligands or blocking eph signalling in local regions of the rostral neural plate during early gastrulation, as done in the hindbrain (Xu et al 1999), should reveal if eph signalling is involved in establishing the AP boundaries during early gastrulation. However, at present there are no eph genes with an appropriate early expression pattern in the neural plate.

3.3.3 Development of the prospective midbrain

Using gene expression domains, I identified a region of the neural plate during gastrulation bordered by the prospective mid-diencephalon and prospective rostral hindbrain. On the basis of gene expression, this region was thought to contain the
prospective midbrain. I also defined a similar region after gastrulation using gene expression boundaries which was thought to contain the prospective midbrain.

I found that this region of the neural plate remained small during gastrulation and expanded during early to mid-somitogenesis stages. Fate mapping of the zebrafish neural plate during gastrulation has shown that the presumptive midbrain occupies a small region which expands along its AP axis due to the cell movements of dorsal convergence (Woo and Fraser 1995). However, such fate mapping studies do not show when the prospective midbrain is induced. There is evidence that the midbrain is induced by an interaction between the hindbrain and forebrain (Nieuwkoop and Albers 1990). Thus, it is interesting to speculate that the reason the region of the neural plate containing the prospective midbrain expands after gastrulation is because this is when the midbrain is induced. However, borders of gene expression cannot be used as fate markers. Therefore, to determine if the gene expression borders are adjacent to the presumptive midbrain requires fate-mapping the borders to a stage when morphological criteria can be used. To determine if midbrain induction involves an interaction between the forebrain and midbrain the prospective forebrain or hindbrain could be ablated at early gastrulation to determine the effect on midbrain development. An alternative explanation to why the prospective midbrain expands is that there is a localised increase to cell division increases. The role of cell division in neural plate development is examined in Chapter 5.

3.3.4 The rostral and caudal regions of the neural plate are differently organised

*The ventral midline does not extend to the rostral tip of the neural plate.*

The ventral midline has been shown to be important for DV patterning of the caudal neural plate in previous studies (reviewed by Tanabe and Jessel 1996). However, it is not clear how far the ventral midline extends, and thus is able to pattern, the rostral neural plate (reviewed by Shimamura et al 1995). In various models it has been proposed that the midline either extends to the tip of the rostral neural plate (non convergent model; Keyser 1972) or stops somewhere short of the tip of the rostral neural plate (convergent model; Puelles 1995). The different outcomes affect how the forebrain is patterned along the DV axis and may contribute to the differences in the organisation of the forebrain compared with more caudal regions of the neural plate. My finding that the ventral midline does not extend to the rostral tip of the neural plate during gastrulation supports a convergent model of rostral neural plate patterning. During gastrulation, the midline may send patterning signals radially in the rostral neural plate and orthogonally in more caudal regions (Fig. 3.20A). Studies of neural plate patterning in mice and chick have come to similar conclusions (Shimamura et al 1995, Puelles 1995).
Figure 3.20 The rostral neural plate is differently to the caudal neural plate.
Schematic animal pole views of the neural plate at the end of gastrulation with rostral to the left.

A) Gene expression studies suggest the midline of the neural plate does not extend to the rostral tip of the neural plate. The most rostral midline may send patterning information (red arrows) in a radial manner to the rostral neural plate and the caudal midline send patterning information orthogonally in the caudal neural plate.

B) Genes are expressed in a different way in the most rostral neural plate than in more caudal regions of the neural plate. Many genes including anf, pax6, rxB, six3 and LH2A are expressed in crescent or curved shaped domains (radial, red) in the rostral neural plate. In caudal regions, genes including, wnt1, pax2.1, fgf8 and flh are expressed in striped domains (blue).
Studies of the rostral patterning of the chick have suggested the PCP mediates DV patterning in the rostral neural plate in an analogous way to the notochord more caudally (Dale et al 1997, 1999). The signals involved have been shown to be shh and BMP7 produced by the PCP (Dale et al 1997). Recent studies suggest rostral midline cells are initially specified as caudal midline and respecified when chordin is downregulated in the PCP (Dale et al 1999). In chick, it has been shown that the ventral midline migrates rostrally after lateral regions of the neural plate have migrated so the rostral ventral midline cells may arrive at an anterior position too late to be influenced by the anterior organising (Dale et al 1999). A similar late migration rostrally of the ventral midline has also been shown in zebrafish (Woo and Fraser 1995, Heisenberg and Nusslein-Volhard 1997) and I found that the shh labelled ventral midline extends to the tip of neural plate after gastrulation. Therefore, it is likely that similar mechanisms are used to regulate rostral ventral midline in zebrafish and other vertebrates and that the rostral midline is initially specified as caudal and respecified as rostral during later stages.

The rostral neural plate is organised into radial arcs and caudal regions is organised into stripes

My study showed that rostral to the mid-diencephalon the neural plate is organised into radial domains whereas it is striped in caudal regions (Fig. 3.20B). Previous studies have suggested that the neural plate rostral to the mid-diencephalon is different to caudal regions. For instance, transplantation experiments have shown that neural plate caudal to the mid-diencephalon is competent to form midbrain but rostral to the mid-diencephalon it cannot (Martinez et al 1991, Crossley et al 1996). Furthermore, in mbl mutant embryos, the rostral neural plate is respecified as diencephalon with the characteristics of the mid-diencephalon and flh expression suggests the rostral neural plate retains a radial organisation (Masai et al 1997). Other studies which block eph tyrosine kinase receptor signalling (Xu et al 1996) have found that defects occur rostral, but not caudal, to the mid-diencephalon. The differences between the neural plate rostral and caudal to the mid-diencephalon may be because the anterior neural plate is patterned by an anterior organising (Houart et al 1998). It is unclear why different mechanisms of organisation should operate in rostral and caudal regions of the neural plate or if these lead to differences in the CNS during later development.

3.3.5 Regulation of AP patterning

anf induction

anf is one of the earliest regionally restricted markers of the rostral neural plate. I used anf to analyse the signals involved in the induction and regulation of early rostral neural plate markers. My study found that the midline mesoderm, including the PCP,
not needed to induce \textit{anf} expression and there may be a prepattern in the ectoderm which controls AP pattern of the neural plate.

It has been suggested that the rostral neural plate is patterned by the rostral axial mesoderm (Ang and Rossant 1994, Blitz et al 1995) and it has recently been shown that the notochord has AP pattern (Prince et al 1998c). My analysis of the expression of \textit{anf} and other rostral neural plate markers in the mutant \textit{cyc}, which lacks the rostral axial mesoderm (Hatta et al 1991a, 1994), suggested the axial mesoderm is not needed to induce rostral markers.

It has also been suggested the PCP is involved in rostral neural plate patterning (for instance, Dale et al 1999). As \textit{oep} embryos do not form a PCP (Schier et al 1996a) these were used to study the role of the PCP in rostral neural plate patterning. Rostral neural plate markers were found to be expressed at normal levels in \textit{oep} embryos although their expression domains were smaller than normal. The size of the rostral neural plate was analysed in both \textit{oep} and \textit{cyc} embryos and it was found to be normal in \textit{cyc} but smaller in \textit{oep} embryos. As \textit{oep} is expressed before gastrulation (Zhang et al 1998), the reduced domain of \textit{anf} expression is probably caused by the size of the neural plate rather than the absence of the PCP. Previous studies have shown the rostral neural plate marker \textit{opl} is expressed without the PCP (Grinblat et al 1998).

To further analyse the influence of the midline on \textit{anf} induction, secondary axes were induced in wild type embryos which lacked the axial mesoderm. Heterospecific transplants of the chick organiser, Hensen's node, into zebrafish embryos induced secondary axes which lacked the axial mesoderm, including the PCP (Kintner and Dodd 1991, Koshida et al 1998). These axes induced \textit{anf} expression, which agreed with previous studies of \textit{otx2} induction by ectopic Hensen's node (Koshida et al 1998), and with the \textit{cyc} and \textit{oep} results. Therefore, the expression of rostral neural plate markers, such as \textit{anf}, does not depend on the rostral axial mesoderm or PCP.

Transplants of the embryonic shield showed that the expression of \textit{anf} is induced by the embryonic shield, in agreement with previous studies of \textit{otx2} induction (Koshida et al 1998). These studies also indicated AP pattern does not depend on the shield but may be the consequence of a prepattern in the epiblast. Transplanted shields or Hensen's nodes induced \textit{anf} which was expressed with an AP identity independant of the inducing shield or node. The posterior border was at equivalent latitude to the endogenous expression in the dorsal side of the embryo. A possible source of the AP prepattern is the non-axial mesoderm germ ring which may act by providing posterior identity (Woo and Fraser 1997, Koshida et al 1998). It is not certain how much of the AP pattern in the neural plate is due to the prepattern or if this provides an approximate pattern which is refined by signals from other sources, such as an anterior organiser (Houart et al 1998).

\textit{Downregulation of anf expression}
anf expression is likely to be tightly regulated as anf is downregulated in a complex way. The most likely sources of regulatory signals are the underlying axial mesoderm, which expresses the signalling molecule shh (Krauss et al 1993), and the adjacent non-neural ectoderm, which expresses the signalling molecule bmp4 (Liem et al 1995, Dale et al 1997). The roles of the axial mesoderm and the non-neural ectoderm in rostral neural plate patterning were studied by overexpressing bmp4, and analysing midline mutants.

Double-labelling embryos with markers of the axial mesoderm and anf indicated that the axial mesoderm is in a position to play a role in mediating anf downregulation. However, the finding that most of anf expression was downregulated normally in cyc and oep mutants suggested the rostral axial mesoderm was not needed to downregulate anf. The exception to this was a small region of anf expression was retained at the midline of cyc and oep embryos. The reason for ectopic anf in the midline of cyc and oep embryos is uncertain. To determine what these cells form at later stages may be useful to elucidate why they do not downregulate anf and could be done by labelling them at late gastrulation and analysing embryos at 24h. It has been shown that cyc embryos have defective midline cell migration (Varga et al 1998) so the ectopic anf may be caused by a failure of a number of anf expressing cells to migrate.

Transplants of the chick organiser, Hensen's node, induced secondary axes which lacked the axial mesoderm. anf expression was not downregulated in these secondary axes by the end of gastrulation which is suggests that the axial mesoderm is needed to downregulate anf in contrast to the results from midline mutants. However, the development of the Hensen's node induced secondary axis is likely to be slower relative to the normal axis and may have led to the apparent absence of anf downregulation. To examine this further, Hensen's node transplants should be carried out at earlier stages or left to develop for longer before examining anf expression.

In addition to axial mesoderm, it is likely that anf is also regulated by the non-neural ectoderm adjacent to the neural plate. Overexpression of Xbmp4 in Xenopus and zebrafish has previously been shown to expand ventral tissues and reduce neural plate markers (Neave et al 1997, Nikado et al 1997) and was found in this study to reduce or abolish anf expression. BMPs interact with chordin to regulate neural plate induction (Piccolo et al 1996) and the dino phenotype is caused by a mutation to chordin (Hammerschmidt et al 1996, Schulte-Merker et al 1997). The dino mutant phenotype is similar to the effects of bmp4 overexpression (Hammerschmidt et al 1996) and this study found that anf and other neural plate markers, such as otx2, are expressed in smaller domains then usual in dino embryos. Thus, bmp4 and chordin appear to interact to mediate neural plate patterning. A number of studies have suggested BMPs act as diffusible long-range signals (reviewed by Smith 1996b) and are involved in patterning the lateral, but not medial, neural plate (Liem et al 1995, Dale et al 1997). Thus, the
finding that BMP signalling was able to downregulate anf across the entire neural plate was unexpected as anf is only normally downregulated in the medial neural plate which is adjacent to the bmp4 expressing non-neural ectoderm (Nikado et al 1997). However, bmp4 is weakly expressed in the PCP in addition to the non-neural ectoderm (Nikado et al 1997) so may act locally to downregulate anf in the overlying medial rostral neural plate. It would be interesting to examine BMP signalling in the rostral axial midline of mutants, such as cyc and oep, to determine if bmp4 is expressed and able to provide DV patterning information in the rostral neural plate.

A further potential source of downregulatory signals is the Row 1 anterior organiser (Houart et al 1998) which may be required to downregulate anf expression. Row 1 is likely to be retained in midline mutants, for example oep embryos express the telencephalic marker emx1 (data not shown), which is regulated by Row 1 (Houart et al 1998). The role of Row 1 in regulating anf expression was not tested but could be done by ablating Row 1 at mid-gastrulation and determining if anf expression is downregulated normally towards the end of gastrulation.

3.3.6 Comparison between gene expression, fate maps and specification studies

My gene expression study identified three sharp AP boundaries during early gastrulation (Fig. 3.21A). Fate mapping studies of the zebrafish neural plate at early gastrulation (Kimmel et al 1990, Woo and Fraser 1995; Fig. 3.21B) also find that the regions are in the correct AP order but they overlap considerably. Specification studies of the zebrafish neural plate (Grinblat et al 1998) suggest the AP order is present at mid-gastrulation but, as with the fate maps, these regions overlap. In contrast, gene expression suggests that the neural plate has acquired several sharp AP boundaries by early gastrulation (Fig. 3.21A). Fate and specification studies also find AP pattern at early gastrulation but do not find such sharp AP boundaries. The reason for this difference may be because although early AP boundaries are sharp, the regions they define are subdivided by late gastrulation. For instance, the anf expression domain at early gastrulation is likely to encompass the prospective telencephalon, at least part of the prospective diencephalon and the eyefield. Fate maps show where cells for these regions are at a given time point, but do not say when they are finally determined. Specification studies give similar information to gene expression studies as they say when cells begin to acquire regional identity. Thus, the AP boundaries of gene expression at early gastrulation, are probably the initial divisions of the neural plate and further divisions are made before fate is specified.

The brain regions defined by gene expression at early gastrulation are approximations based on where the genes studied are expressed at later stages. These
Figure 3.21  Gene expression and fate maps define similar regions within the rostral neural plate.
Summaries of gene expression at early (A) and late (C) gastrulation. Summaries of fate maps of early (B) and late (D) gastrulation.

A,B) at early gastrulation, gene expression suggests the most rostral neural plate contains a mixture of prospective telencephalon (red) and retina (green). The fate map identifies a separate region of telencephalon rostrally with a mix of retina and telencephalon caudally (B). The diencephalon (black) and midbrain (orange) cannot be resolved with the available markers, so are shown as overlapping. The caudal hindbrain (purple) and the midbrain cannot be resolved with the available markers, so are shown as overlapping.

C,D) at late gastrulation, the telencephalon and retina are mostly separate domains in the gene expression (C) and fate map (D) studies. The AP order of the brain regions is defined with fairly sharp boundaries in both studies. The diencephalon extends into the rostral midline in the retina in both studies.

Key to colours: red, telencephalon; green, retina; black, diencephalon; orange, midbrain; purple, hindbrain. Arrows label the midline in (B) and (D).

B,D) are adapted from Woo and Fraser (1995).
gene expression boundaries may change during subsequent development or the regions assigned may be inaccurate. For instance, although results from my gene expression study at early gastrulation suggest the retina overlaps with the telencephalon (Fig. 3.21A), a number of studies suggest the retina arises from the diencephalon, perhaps mediated by eph signalling (Xu et al 1996). Thus, further markers are required which are present during early gastrulation to define the brain regions more precisely.

I did not find evidence of DV patterning of the rostral neural plate at early gastrulation with any of the markers analysed. Fate maps of zebrafish (Kimmel et al 1990, Woo and Fraser 1995; Fig. 3.21B) suggests that an approximate DV pattern is present at early gastrulation with dorsal always lateral to ventral. As with AP patterning, it may be that the apparent DV pattern in these fate maps is established because cells do not mix much in the neural plate (Warga and Kimmel 1990) so appear to be regionally organised before they have acquired any regional identity (Wilson et al 1993, Woo and Fraser 1995) as reported in Xenopus (Keller 1975; 1976). To clarify when DV regional identity is established requires combined fate mapping and analysis of gene expression, ideally in living embryos. These experiments would show the relationship between the expression of regionally restricted markers in the DV axis, such as flh, and the acquisition of the appropriate position in the neural plate.

By the end of gastrulation, the gene expression study and fate mapping studies agree that AP and DV pattern is established (Fig. 3.21C,D; Woo and Fraser 1995). Along the AP axis, the main regions are defined and in the appropriate order in both gene expression (Fig. 3.21C) and fate mapping (Fig. 3.21D) studies. There is less overlap between regions in the fate map and the gene expression study shows the regions defined at early gastrulation have become subdivided. Recent studies of zebrafish mutants which affect the patterning of the hindbrain, such as valentino (val; Moens et al 1996), have shown there is a similar gradual acquisition of AP identity in the hindbrain. val mutants fail to form rhombomeres r5 and r6 but form a precursor rX instead which has some features of both r5 and r6 (Moens et al 1996). Analysis of hox gene expression has shown that val is needed to subdivide the prospective r5/r6 territory and regulated AP identity through the control of hox gene expression (Prince et al 1998b). It is likely that genes acting like val subdivide AP regions in the rostral neural plate.

DV patterning is present in both gene expression and fates maps by the end of gastrulation (Fig. 3.21C,D). The axial mesoderm has been shown to be an important source of DV information (reviewed by Tanabe and Jessell 1996). In zebrafish the axial mesoderm migrates beneath the neural plate during gastrulation (Kimmel et al 1995) so the migration of the axial mesoderm probably affects the timing of DV pattern. In the most rostral regions of the neural plate, it has been found that the PCP provides ventral patterning information (Dale et al 1997) and the PCP migrates beneath the rostral neural plate during gastrulation (Kimmel et al 1995). However, studies showing the rostral
ventral midline acquires DV identity late (Dale et al 1999) and my finding that the ventral midline of zebrafish does not reach the tip of the neural plate until after gastrulation, suggests the DV pattern in the rostral neural plate is only partially established by the end of gastrulation. Indeed, both my gene expression study and the fate map (Woo and Fraser 1995) at late gastrulation, suggest the midline of the prospective diencephalon does not extend to the rostral tip of the neural plate. Therefore, the results of fate maps and commitment studies agree with my gene expression study that the DV regional organisation begins to be established by the end of gastrulation.

In summary, during my analysis of the regional organisation of the rostral neural plate I have shown that sharp AP gene expression boundaries are present in the neural plate at early gastrulation. I have also found that there are differences in the organisation of the rostral and caudal regions of the neural plate. I have also studied the regulation of regional gene expression using the transcription factor anf as an example of an early rostral neural plate marker.
CHAPTER 4

CHARACTERISATION OF THE ANFLESS FISH LINE

4.1 Introduction

One of the most useful tools to study developmental biology has been the analysis of mutant lines. In zebrafish, the technology to generate mutations to specific genes is not yet available (Schier et al 1996b). Many groups have therefore taken the approach of random mutagenesis followed by positional cloning to find novel developmental genes (see Chapter 6; Driever et al 1996, Haffter et al 1996). In addition, many useful mutations, have occurred spontaneously, such as flh (Talbot et al 1995). Thus, chemically induced and spontaneously occurring mutations have been useful for studying zebrafish development.

A difficulty of studying development by mutagenesis screening is that genes with redundant functions which produce a partial phenotype or no phenotype at all, are not isolated with this strategy (Haffter et al 1996). Gene redundancy can be caused by a number of reasons, including gene duplication which generates two copies of a gene with overlapping, or identical, expression patterns and functions. However, duplicate genes will only be retained if they confer a selective advantage to the organism.

One mechanism which causes duplicate genes to be retained is when the duplicate copy of a gene becomes mutated leading to a change to its expression pattern or function. For instance, in mammals there are several forms of the globin gene which have an essential function in the embryonic, fetal or adult blood. These related forms of the globin gene are thought to have arisen by tandem gene duplication from a single ancestral globin gene. The globin genes have been maintained because they are expressed and function at different times. However, not all duplicated genes acquire a new function. For instance, in the globin gene clusters there are several globin pseudogenes, with close homology to functional globin genes, but which are disabled by mutations that prevents their expression. It is not uncommon to find pseudogenes as not every random gene duplication event leads to new functional genes (Wolpert et al 1998).

Members of the hox gene complex provide a clear example of gene duplication. hox genes contain a characteristic 180 nucleotide motif, the homeobox, which encodes a helix-turn-helix domain involved in transcriptional regulation. hox genes are characterised by being organised into clusters and are expressed along the AP axis in register with their order in the hox gene complex (Wolpert et al 1998). Studies of hox genes in a number of species suggest that there was a set of as many as ten genes in the single hox cluster in the ancestor of all metazoan species (de Rosa et al 1999, Martindale and Kourakis 1999). Amphioxus, the closest surviving relative to the ancestor of
chordates, has one *hox* cluster but mammals have four clusters of *hox* genes, each on a different chromosome. These four *hox* clusters probably arose from a single ancestral cluster through two genome duplications. Recently, it has been shown that there are seven *hox* clusters in zebrafish which are thought to have arisen by an additional gene duplication in the ancestor of teleost fish (Postlethwait et al 1998). These additional zebrafish *hox* genes may have acquired new roles which may have led to the diversity of the morphology of modern teleosts (Wittbrodt et al 1998). Thus, gene duplication has been shown to be an important mechanism for the evolution of novel genes.

In this chapter I characterise a line of fish that do not express the homeobox gene *anf*. These *anf* minus embryos did not appear to have an early phenotype so I examined them for late defects. However, after an extensive analysis I have been unable to find any defects. The reasons for the absence of a phenotype are unknown but may be related to the finding of extra copies of many genes in teleost fish in comparison with mammals.

### 4.2 Results

#### 4.2.1 *anf* is not expressed in a sub-population of *oep* embryos

*anf* expression was studied in the midline mutant *oep*. In one cross, three different expression patterns were found when embryos were labelled with *anf* (Fig. 4.1; Chapter 3). Embryos either expressed *anf* in the wild type expression pattern (Fig. 4.1A; 43%, n=13), they expressed *anf* in a reduced domain (Fig. 4.1B; 26%, n=8) or they did not express *anf* at all (Fig. 4.1C; 30%, n=9). As *oep* has been reported to be a recessive mutation (Schier et al 1996), the finding that *anf* was either expressed in a reduced domain or not expressed at all in 56% of embryos produced by a cross between *oep* carrier fish was unexpected.

To determine if the embryos that did not express *anf* were wild type siblings or *oep* mutant embryos, an independent method to distinguish between wild type and *oep* mutant embryos was needed. It has been shown that the axial mesoderm marker *shh* (Krauss et al 1993) is absent in the anterior midline of *oep* embryos (Chapter 3, Schier et al 1997). Thus, *shh* expression was used to unambiguously identify *oep* mutant embryos. When embryos produced by crosses between pairs of *oep* carrier fish were double-labelled with *anf* and *shh* at several developmental stages, four patterns of expression were found. In all stages examined, 59% (n=87) expressed both *anf* and *shh* in the wild type patterns (Fig. 4.1D), 24% (n=35) expressed both *anf* and *shh* in a reduced pattern (Fig. 4.1E) and 17% (n=25) did not express *anf* but expressed *shh* in either the wild type (Fig. 4.1F) or *oep* pattern (Fig. 4.1G). Thus, as expected from a cross between a pair of *oep* carrier fish, a quarter of the embryos had a reduced *shh* expression pattern and so were likely to be *oep* mutant embryos. However, in addition to the embryos with a
Figure 4.1 *anf* is not expressed in a subset of *oep* embryos

Embryos labelled as whole mounts by in situ hybridisation with *anf* (A-C) or *anf* and *shh* (D-G). Animal pole views with rostral to the left of 80% epiboly stage embryos.

A-C) *oep* carrier fish generated embryos with wild type, *oep* mutant or no *anf* expression.

D-G) double-labelling of embryos from *oep* carrier fish gave: wild type *anf* and *shh*; *oep anf* and *oep shh* which has a less rostral anterior extent; wild type *shh* without *anf*; or *oep shh* without *anf* patterns.

Scale bar: 125\(\mu m\).
reduction in anf expression, a proportion of wild type and oep mutant embryos did not express anf at all.

To confirm that a quarter of the embryos generated by crosses between pairs of oep carrier fish were oep mutants, embryos from the crosses were allowed to develop to 24h. At 24h, it was found that a quarter (24%, n=25) of the embryos had the oep phenotype and the remaining embryos were wild type. Thus, the reduced expression pattern of anf observed in both the anf single labelling (Fig. 4.1B) and the anf and shh double-labelling (Fig. 4.1E,H) correlated with the number of embryos with the oep phenotype. Therefore, the absence of anf expression in a proportion of the embryos from a pairing between oep carrier fish was probably not due to the oep mutation. As only a quarter of the embryos from pairings between oep carrier fish had a phenotype at 24h, the absence of anf expression did not appear to correlate with an early developmental defect.

4.2.2 Boston oep alleles do not express anf

To determine if the failure to express anf was caused by oep, or due to the background line in which the oep fish had been outcrossed, it was necessary to study anf expression in the same oep alleles maintained in another facility. Two oep alleles were obtained from the facility at New York University, these were the point mutation oep^tz257, (which was the same as the KCL oep allele) and a deletion mutant oep^z1. Embryos from crosses between oep^z7 and oep^tz257 carrier fish were double-labelled with anf and shh (Fig. 4.2).

In oep^tz257, three patterns of anf expression were found, with 45% (n=14) embryos expressing anf and shh normally (Fig. 4.2A), 19% (n=6) expressing anf and shh in a reduced domain (Fig. 4.2B) and 35% (n=11) not expressing anf at all but expressing shh in the wild type pattern (Fig. 4.2C). Therefore, the oep^tz257 line lacked anf expression when maintained at either NYU or KCL. Unexpectedly, the same result was found in the oep^z1 with 39% (n=26) expressing anf and shh normally, 14% (n=9) expressing anf and shh in a reduced domain, and 47% (n=31) not expressing anf at all but expressing shh in the wild type pattern. Therefore, both of the oep alleles analysed, did not express anf. These results suggested that the absence of anf expression in oep was not due to a background mutation but may be linked to the oep mutation. To determine if anf expression was only lost in oep alleles, anf expression was analysed in several other mutant lines.

4.2.3 anf expression is lost in several mutant lines

To determine if the failure to express anf was confined to the oep mutant line, anf expression was analysed in other mutant lines isolated during various screens. silberblick
Figure 4.2 anf is not expressed in a subset of oep embryos from NYU, slb, ace or kas embryos.


A-C) oep^{z257} embryos, from NYU, have wild type, oep mutant or no anf expression.
D,E) slb homozygote mutants either express or do not express anf.
F,G) ace'' embryos either express or do not express anf.
H,I) kas'' embryos either express or do not express anf.

Scale bar: 125μm.
(slb) is a mutation affecting dorsal convergence identified during the Tubingen mutagenesis screen (Heisenberg and Nusslein-Volhard 1997). When some crosses of homozygote slb embryos were double-labelled with anf and ntl (Schutle-Merker et al 1994), 55% (n=48) of embryos expressed anf in a slightly abnormal pattern (Fig. 4.2D) and 45% (n=39) embryos failed to express anf at all (Fig. 4.2E). As these embryos were all slb homozygotes, an additional mutation was probably causing the absence of anf. anf expression was also analysed in acerebellar (ace; Brand et al 1996) embryos and knollnase (kas; Heisenberg et al 1996) embryos which were isolated during the Tubingen screen. As with slb, it was found that a small proportion of embryos from crosses between ace (Fig. 4.2F,G) or kas (Fig. 4.2H,I) heterozygous carriers lacked anf expression but that these embryos were not ace or kas mutants. Therefore, anf expression was lost in the Tubingen mutant lines slb, ace and kas.

Although anf was absent in slb, ace and kas, not all Tubingen mutant lines showed an absence of anf expression. Analysis of anf expression in the mbl (Heisenberg et al 1996) mutant line isolated in the Tubingen screen, showed that anf was expressed in mbl mutant and wild type sibling embryos (Fig. 4.3A,B). Furthermore, the mutants squint (sqt; Heisenberg and Nusslein-Volhard 1997; Fig. 4.3C,D) and dino (Hammerschmidt et al 1996; Chapter 3) which were identified during the Tubingen screen, also expressed anf in all of the embryos analysed. anf was also found to be expressed in the mutant and wild type embryos of knypek (kny; Solnica-Krezel et al 1996; Fig. 4.3E,F) and trilobite (tri; Solnica-Krezel et al 1996; Fig. 4.3G,H) lines which were identified during the Boston mutagenesis screen (Dreiver et al 1996). The mutant cyc (Hatta et al 1991a, 1994) which was identified during the Oregon mutagenesis screen and maintained at KCL also expressed anf normally in both wild type and mutant embryos (Fig. 3.2L,M). Therefore, anf expression was lost in some, but not all, Tubingen mutant lines and anf was expressed in two mutants generated during the Boston screen.

To examine the possibility that the loss of anf expression was due to a background mutation present in the wild type lines used to outcross fish at KCL, or during the earlier mutagenesis screens, anf expression was studied in the available wild type lines.

### 4.2.4 anf is expressed in all wild type lines analysed

To determine if anf was expressed in the wild type lines used within the KCL facility for outcrosses or during mutagenesis at KCL and elsewhere, anf expression was determined in several wild type lines. For this analysis, embryos were labelled with anf and either shh or ntl to provide an internal control which showed the labelling had worked. A preliminary analysis of anf expression was carried out in embryos from two pairings of *AB (Fig. 4.4A; n=43), Ekwill (Fig. 4.4B; n=40), tue (Fig. 4.4C; n=43), tup1l (Fig. 4.4D; n=41), KCWT (Fig. 4.4E; n=100) wild type lines and in all cases anf was
Figure 4.3  \( anf \) is expressed in some Tubingen and Boston mutants.  
Embryos labelled as whole mounts by in situ hybridisation.  Animal pole views with rostral to the left.  A,B) 95\% epiboly.  C-H) bud.
A,B) \( anf \) is expressed in wild type and mutant \( mbl \) embryos from Tubingen screen.  
C,D) \( anf \) is expressed in wild type and mutant \( sqt \) embryos from Tubingen screen.  
E,F) \( anf \) is expressed in wild type and mutant \( kny \) embryos from Boston screen.  
G,H) \( anf \) is expressed in wild type and mutant \( tri \) embryos from Boston screen.  
Scale bar: 125\( \mu \)m.
Figure 4.4  *anf* is expressed in all wild type lines analysed.

Embryos labelled as whole mounts by in situ hybridisation with *anf* and *ntl* (A-D) or *anf* and *shh* (E). Animal pole views with rostral to the left. A) 80% epiboly. B) 60% epiboly. C-E) 75% epiboly.

A) *anf* is expressed in *AB* wild type line embryos.
B) *anf* is expressed in *ekwill* wild type line embryos.
C) *anf* is expressed in *tues* wild type line embryos.
D) *anf* is expressed in *tup* wild type line embryos.
E) *anf* is expressed in *KCWT* wild type line embryos.

Scale bar: 125μm.
expressed normally. Thus, anf was expressed normally in all of the wild type fish studied in the preliminary analysis.

4.2.5 A line of fish which do not express anf was established independent of the oep mutation

The absence of anf expression did not appear to be caused by the oep mutation as anf was not expressed in several other mutant lines. Thus, it was likely that it would be possible to separate the oep and loss of anf genotypes. First, fish from within the oep background which were not carriers of the oep mutation were identified. These oep "non carriers" were paired and embryos double-labelled with anf and shh to find pairs of fish which were not heterozygous for the oep mutation but lacked anf expression. The first such pair of fish was identified and found to lack anf expression in 30% (n=9) of its embryos. These were then used to establish a new line of fish called anflessU32 which lacked anf expression. Further pairs of fish not expressing anf were eventually identified from within the oep background and these were included in the new line. To be certain that the carriers of the anfless mutation were not oep heterozygotes, each of the anfless fish was crossed against a known oep carrier. These crosses established for certain that the anfless mutation was separate from the oep mutation and were used for further analysis.

4.2.6 Homozygous and heterozygous carriers were established in the anfless line

Both homozygous and heterozygous carriers were identified for the anfless line. Crosses between homozygous and heterozygous carriers gave the proportions of embryos with and without anf expression predicted for a recessive mutation (Table 4.1). As both heterozygous and homzygous carriers were viable and fertile adults, a severe early developmental defect was not caused by the absence of the expression of the anf gene.

As anf is expressed early in a large domain of ectoderm, it was surprising to find that the anfless line had no early defect. However, a number of genes expected to give an early phenotype when knocked-out in mice, such as gsc (Yamada et al 1995, Rivera-Perez et al 1995), have not given as severe or as early a phenotype as predicted from the expression pattern, perhaps due to redundancy (reviewed by Jacobson and Anagnostopoulos 1996). Therefore, the anfless embryos were analysed carefully to determine if they had a subtle phenotype. The anfless mutants were also analysed to determine if genes which may be upstream of anf were expressed normally.
TABLE 4.1: Analysis of *anf* expression in the *anfless* line

<table>
<thead>
<tr>
<th></th>
<th>homozygote x homozygote</th>
<th>homozygote x heterozygote</th>
<th>heterozygote x heterozygote</th>
<th>heterozygote x wild type</th>
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</thead>
<tbody>
<tr>
<td><em>anf</em> expressing</td>
<td>0</td>
<td>376</td>
<td>309</td>
<td>323</td>
</tr>
<tr>
<td><em>anf minus</em></td>
<td>713</td>
<td>407</td>
<td>118</td>
<td>0</td>
</tr>
<tr>
<td>% <em>anf minus</em></td>
<td>100%</td>
<td>52%</td>
<td>28%</td>
<td>0%</td>
</tr>
</tbody>
</table>
4.2.7 *The yolk syncitial layer expresses hex and gta6 in anfless mutant embryos*

It has been suggested that the yolk syncitial layer (YSL) may have a role in inducing anterior gene expression (Houart et al. 1998, Beddington 1998). To determine if an alteration to the genes expressed in the YSL correlates with the absence of *anf* expression in the *anfless* line, two genes, *gta6* and *hex*, which are normally expressed in the YSL, were analysed.

*Gta6* is normally expressed in the YSL (J. Broadbent, A. Rodaway and R. Patient, unpublished data) and this pattern was observed in *anfless* mutants (Fig. 4.5A). *Hex* is normally expressed in the YSL (Chapter 3; R.K. Ho, R. Beddington, D. Stainier, unpublished data) and was found to be normally expressed in the YSL of *anfless* embryos (Fig. 4.5B). Therefore, the expression of the YSL markers *gta6* and *hex* was unaffected in *anfless* embryos.

4.2.8 *The neural plate of anfless mutant embryos is patterned normally*

*Anf* is normally expressed in the anterior neural plate during gastrulation and early somitogenesis (Chapter 3). The expression of a number of neural plate markers was analysed in *anfless* mutant embryos produced by a cross between pairs of heterozygous carriers to determine if anterior neural plate patterning is affected by the absence of *anf* expression.

As expected, when *anfless* embryos were double-labelled with *shh* and *anf*, a proportion of embryos expressed *anf* and *shh* normally (Fig. 4.6A, Table 4.1) and a quarter of the *anfless* embryos did not express *anf* but they all expressed *shh* normally (Fig. 4.6B, Table 4.1). Other neural plate markers analysed included *fkd3* (Fig. 4.6C), *otx2* (Fig. 4.6D), *pax6* (Fig. 4.6E), *six3* (Fig. 4.6F), *svp46* (Fig. 4.6G) and *tailless* (*til*; Fig. 4.6H; Hollemann et al. 1998). The expression of these markers in *anfless* mutant embryos and wild type siblings was indistinguishable. Thus, according to the expression of the neural plate markers analysed, *anfless* mutant embryos appear to have normally patterned neural plates.

4.2.9 *The telencephalon and epiphysis of anfless mutant embryos is patterned normally*

To understand if telencephalon patterning is disrupted in the absence of *anf*, *anfless* mutant embryos were labelled with markers of the dorsal and ventral telencephalon, *dlx2* (Akimenko et al. 1994) and *emx1* (Morita et al. 1995) and a marker of the epiphysis, *flh*.
Figure 4.5 *gta6* and *hex* are expressed normally in the yolk syncitial layer of *anfless* embryos.

Embryos labelled as whole mounts by in situ hybridisation with rostral to the left. Animal pole views with rostral to the left of 70% epiboly homozygote embryos.

A) the YSL marker *gta6* is expressed normally in *anfless* embryos.

B) the YSL marker *hex* is expressed normally in *anfless* embryos.

Scale bar: 125μm.
Figure 4.6 *anfless* embryos express neural plate markers normally. Embryos labelled as whole mounts by in situ hybridisation. Animal pole views with rostral to the left. A-C) 90% epiboly. D,E,H) bud. F,G) 95% epiboly.

A,B) *anf* is expressed normally in three-quarters and absent in a quarter of *anfless* embryos.

C-H) several neural plate markers are expressed normally in *anfless* embryos.

Scale bars: 125μm.
**dlx2** (Fig. 4.7A,B) and *emx1* labelling of the telencephalon showed that wild type and *anfless* embryos (Fig. 4.7C,D) were indistinguishable. Thus, the expression of *emx1* and *dlx2* suggests the telencephalon is patterned in the same way in *anfless* mutant embryos as in wild type embryos (S. Shanmugalingham and S.W. Wilson unpublished, Fernandez et al. 1998).

As the posterior expression border of *anf* meets the anterior border of *flh* in the neural plate (Chapter 3) *anf* may regulate the anterior limit of *flh* and therefore, the position of the epiphysis. When *anfless* embryos were double-labelled with *flh* and *dlx2* (Fig. 4.7A,B) it was found that both *dlx2* and *flh* were expressed normally. Thus, the epiphysis was not expanded or positioned incorrectly in the absence of *anf* expression.

### 4.2.10 The anterior pituitary is induced normally in the *anfless* mutant embryos

At 24h, *anf* is also expressed in the prospective anterior pituitary, so gene expression was used to determine if the prospective anterior pituitary is patterned normally in *anfless* embryos.

The prospective anterior pituitary gland expresses *anf* from about 24h (Chapter 3) and fate mapping studies suggest that the anterior pituitary may arise from within the rostral edge of the neural plate (Eagleson and Harris 1990, Couly and LeDouarin 1985). To assess the effect of the loss of *anf* expression on anterior pituitary patterning, the anterior pituitary was labelled using *lim3* (Fig. 4.8A,B; Glasgow et al. 1997), *nk2.2* (Fig. 4.8C,D; Barth and Wilson 1995) and *six3* (Fig. 4.8E,F). *lim3*, *nk2.2* and *six3* were found to be expressed in the same pattern in *anfless* and wild type embryos. Thus, from the markers analysed, the anterior pituitary of *anfless* mutant embryos appears to be the same as the anterior pituitary in wild type embryos.

This analysis of gene expression suggested that the three tissues which express *anf* in the wild type (anterior neural plate, dorsal telencephalon and anterior pituitary) were patterned normally in *anfless* mutant embryos. To discover if subtle defects appeared during later development, further analysis was conducted.

### 4.2.11 The olfactory bulb develops normally in *anfless* embryos

Fate mapping studies have mapped the olfactory bulbs to the anterior edge of the rostral neural plate (Eagleson and Harris 1990) which is one of the regions where *anf* is normally expressed (Chapter 3). Therefore, the olfactory bulbs and their associated nerves were analysed at 48h to determine if *anf* is needed for olfactory development. The antibody *zns2* labels an epitope within the olfactory bulb, olfactory nerves and olfactory neuroepithelium (Whitlock and Westerfield 1998) so *zns2* was used for this study.
Figure 4.7 *anfless* embryos express telencephalon markers *emxl* and *dlx2* normally.

Embryos labelled as whole mounts by in situ hybridisation. Lateral (A,C) or dorsal (B,D) views with rostral to the left of 24h embryos.

A,B) the ventral telencephalon marker *dlx2* and the epiphysial marker *flh* are expressed normally in *anfless* embryos.

C,D) the dorsal telencephalon marker *emxl* is expressed normally in *anfless* embryos.

Abbreviations: dd, dorsal diencephalon; e, eye; h, hypothalamus; mb, midbrain; t, telencephalon.

Scale bar: 50µm.
Figure 4.8 *anfless* embryos express anterior pituitary markers *lim3*, *nk2.2* and *six3* normally.

Embryos labelled as whole mounts by in situ hybridisation. Ventral (A,C,E) or lateral (B,D,F) views with rostral to the left of 33h (A,B) or 27h (C-F) embryos.

A,B) anterior pituitary marker *lim3* is expressed normally in *anfless* embryos.

C,D) ventral CNS marker *nk2.2* is expressed normally adjacent to the anterior pituitary in *anfless* embryos.

E,F) anterior pituitary and rostral CNS marker *six3* is expressed normally in *anfless* embryos.

Abbreviations: ap, anterior pituitary; dd, dorsal diencephalon; hyp, hypothalamus; mb, midbrain; t, telencephalon.

Scale bar: 50μm.
*anfless* mutant embryos were generated from crosses between heterozygous *anfless* carriers and it was found that zns2 labelling in the olfactory bulb, olfactory nerve and olfactory epithelium (Fig. 4.9) was indistinguishable between wild type and *anfless* mutant embryos. Therefore, the loss of *anf* expression did not appear to affect olfactory development as determined by zns2 labelling.

**4.2.12 The anfless mutant embryos have normal brain morphology**

To elucidate if there was any alteration to the overall brain morphology possibly due to an early patterning role of *anf* in the neural plate, the axon tracts were labelled using two different immunohistochemical methods.

Heterozygous *anfless* embryos were double-labelled with *anf* by in situ hybridisation and then with HNK1 antibody. At 28h, the post optic commissure of *anfless* mutant embryos (Fig. 4.10A,B) was indistinguishable from the commissures of wild type sibling embryos (Fig. 4.10C,D).

To determine if the axon tracts were normal during later stages of development, the axon tracts of *anfless* mutant embryos were labelled with anti-acetylated tubulin (Macdonald et al 1997) at 3 days of development (Fig. 4.11). This labelling revealed the axon tracts more clearly than after in situ at 28h with HNK1. Anti-acetylated tubulin labelling showed that structures including the telencephalon, nose and epiphysis were indistinguishable in *anfless* mutant and wild type embryos. Thus, the loss of *anf* did not appear to affect the morphological development of the axons of the brain.
**Figure 4.9 anfless embryos have a normal olfactory system.**
Embryos labelled as whole mounts by labelling with zns2 antibody. Dorsal views with rostral to the top of 48h embryos.
A,B) zns labelling is normal in anfless embryos.
Abbreviations: dd, dorsal diencephalon; e, eye; ob, olfactory bulb; oe, olfactory epithelium; on, olfactory nerve; t, telencephalon.
Scale bar: 300µm.
Figure 4.10 *anfless* embryos have normal a post-optic commissure.

Embryos labelled as whole mounts by in situ hybridisation with *anf* (blue) and then with HNK1 antibody (brown). Dorsal (A,C) or lateral (B,D) views of 28h embryos. A,B) wild type *anfless* sibling embryos express *anf* and have normal HNK1 labelling of the post-optic commissure.

C,D) *anfless* embryos do not express *anf* but have normal HNK1 labelling of the post-optic commissure.

Abbreviations: dd, dorsal diencephalon; e, eye; h, hypothalamus; mb, midbrain; poc, post-optic commissure; t, telencephalon.

Scale bars: 50μm.
Figure 4.11 *anfless* embryos have normal brain morphology.

Embryos labelled as whole mounts with anti-acetylated tubulin antibody (brown). Ventral (A), dorsal (B-D) or lateral (E) views of embryos at the third day. Rostral to top (A-D) or left (E).

A) the optic stalk and eyes are normal in *anfless* embryos.
B) the olfactory bulbs are normal in *anfless* embryos.
C) the anterior commissure and olfactory epithelium are normal in *anfless* embryos.
D) the midbrain and epiphysis are normal in *anfless* embryos.
E) the main features of the brain are normal in *anfless* embryos.

Abbreviations: ac, anterior commissure; cb, cerebellum; e, eye; ep, epiphysis; hb, hindbrain; hy, hypothalamus; ob, olfactory bulb; oe, olfactory epithelium; os, optic stalk; pc, posterior commissure; soc, supra-optic commissure; t, telencephalon; te, tectum.

Scale bars: A,D,E 50μm. (B,C) 30μm.
4.3 Discussion

Unexpectedly, the loss of anf expression in anfless fish does not appear to cause any deleterious phenotype in the tissues where anf is normally expressed or elsewhere in the embryo. The anterior neural plate, telencephalon, anterior pituitary, olfactory system and axon tracts were examined and all found to be normal in anfless embryos. As anf is expressed and downregulated in a complex pattern (Chapter 3), it seems likely that anf is tightly regulated. Therefore, it was expected that anf would be carrying out an important function during early development. The lack of a phenotype in anfless fish raises the question of why the absence of anf expression causes no phenotype and how this may arise.

4.3.1 The anfless genotype may have arisen during large-scale mutagenesis screens

anf expression was lost in several mutant lines from Tubingen, including oep and ace, and both single and double mutants occurred. The only mutant line analysed lacking anf expression, which was not from Tubingen, was the deletion mutant oep\(^{2.1}\). The Tubingen mutants have point mutations generated by chemical mutagenesis (Haffter et al 1996). Thus, it is possible that anf was already absent in one of the 49 Tubingen founder males (Haffter et al 1996) and then passed onto a proportion of the mutants identified in the F2 screen, including ace and oep. As the anfless genotype does not lead to a deleterious phenotype, the anfless mutants would not have been detected with the morphological screening protocol (Haffter et al 1996). An extensive analysis of the records at Tubingen may be sufficient to determine if the mutants that do not express anf were derived from a common founder fish. However, many more Tubingen mutant lines need to be analysed than have been done so far, as nearly 2000 mutant lines were isolated during the Tubingen mutagenesis screen (Haffter et al 1996). Alternatively, it is possible that the anfless genotype was introduced from one or more of the fish used to outcross the mutant lines in Tubingen, or later, at KCL.

It is unlikely that the anfless genotype occurred in the background at KCL because oep\(^{2.257}\) line generated during the Tubingen screen, but obtained from the NYU facility, also failed to express anf suggesting Tubingen is the source of the anfless genotype. In addition, the analysis of KCL wild type lines did not find any embryos with the anfless genotype although embryos from only a few crosses were analysed. Analysis of the deletion mutant oep\(^{2.1}\) showed that it did not express anf.

The loss of anf expression in oep\(^{2.1}\) is intriguing as this line has a deletion which has been generated independently from the mutant screens at Boston and Tubingen. The finding that anf is not expressed in oep\(^{2.1}\) suggests either that anf is physically close to
oep, or anf is particularly prone to mutation so was mutated independently of the deletion, or that the NYU facility wild type lines had an anfless genotype or that fish were mixed or cross bred in NYU. To determine if anf is physically close to oep requires mapping anf and comparing its position with oep on the zebrafish linkage map (Postlethwait et al 1998, Knapik et al 1998). On the basis of the analysis of the available lines, it seems that the anfless genotype was introduced during the Tubingen but not the Boston or Oregon screens. The loss of anf expression in the anfless line may be a mutation to the anf promoter, the coding region, or to an upstream gene.

4.3.2 The yolk syncitial layer of anfless embryos does not have a major defect

It is not clear why anf expression was absent in the anfless line. Studies of mice have suggest the regulation of the murine homolog of anf, hesx1, is regulated by the anterior visceral endoderm (AVE; Thomas and Beddington 1996). It has also been shown that the AVE is capable of inducing hesx1 expression in ectopic locations in mouse embryos and the AVE can induce the chick homolog of anf, GANF, in ectopic locations in chick embryos (reviewed by Beddington and Robertson 1999). As it has been suggested that the teleost YSL is equivalent to the AVE (Houart et al 1998), it is possible that the YSL controls anf expression and so may be defective in anfless embryos. However, the expression of two YSL markers was normal in anfless embryos, suggesting there is not a major defect in the YSL of anfless embryos. An analysis of additional YSL markers will be necessary to be able to draw strong conclusions about the normality of the anfless YSL. However, as several neural plate markers were normally expressed in anfless embryos, it is unlikely that a defect to the YSL would cause the specific loss of anf expression in anfless embryos. Alternatively, a mutation affecting an upstream regulator of anf could cause the loss of anf expression in anfless embryos. However, the apparent specificity of the mutation to anf and absence of other defects implies that the anf locus is affected directly.

4.3.3 anfless mutants may have mutations to the coding or regulatory region of anf

Alterations to the DNA, affecting coding, non coding regions, or both could explain the loss of anf in anfless fish. The most straightforward way to determine if the coding region of anf is mutated in anfless embryos is to use PCR to amplify the anf coding regions from anfless and wild type embryos, sequence and compare them. However, point mutations to the coding region of genes would rarely be expected to lead
to complete loss of mRNA expression at all stages as observed for anf in the anfless line. Thus, it might be more likely that there is a deletion of the coding region of anf.

The deletion of the coding region of anf in the anfless line could account for the complete absence of anf expression at all stages. A short deletion may cause the loss of the anf coding region without affecting neighbouring genes. To determine if the anf coding region is deleted, PCR or southern analysis could be carried out on the genomic DNA of anfless embryos.

anf is likely to be under tight regulatory control as anf has a complex and dynamic expression pattern (Chapter 3). To determine if a promoter/enhancer region is affected by mutation in the anfless line, the anf promoter would need to be cloned from anfless genomic DNA. Analysis of the anf promoter would determine if it is altered and if such changes are sufficient to prevent anf expression altogether. The most extreme mutation to the anf promoter could be the deletion of the entire promoter in anfless embryos. The results of the anfless analysis, particularly the absence of any anf mRNA, are consistent with a mutation affecting the anf promoter.

4.3.4 anfless fish may have a subtle phenotype

It may be that anf has a function which is lost in anfless fish but the analyses carried out were not sufficient to detect it. "A trip to the opera," as suggested by Wolpert (Cooke et al 1997) as a way to evaluate a mutant which has no obvious phenotype argues that a gene which serves an important role in the natural habitat may, under laboratory conditions, not be required. For instance, a gene which subtly affects predation may not cause a noticeable phenotype in the laboratory as it will not be selected against, however in the natural environment this would cause a reduction in viability. The chances of finding a genotype that causes a small reduction of fitness is small (Brookfield 1992). If a genotype causes a 5% viability disadvantage relative to wild type, more than 20,000 animals must be examined to have a 95% chance of finding significant evidence for this difference. For a 1% viability disadvantage, 600,000 animals need to be examined. Yet such selection coefficients are powerful in terms of evolution. Therefore, a subtle role of anf may not be detected.

There are a number of other genes which were expected to have important roles in early development but null mutants had no obvious phenotype. For example, myf5 and myoD (reviewed by Ordhal and Williams 1997) were thought to be crucial for early muscle cell specification but when null mutants of both myf5 and myoD were analysed, it was found that the muscle cells develop robustly despite some morphological irregularities (Rudnicki et al 1992, Braun et al 1992). The finding that double mutants for myoD and myf5 causes the loss of differentiated muscle as well as myoblast pools (Rudnicki et al 1993) led to the suggestion that these two genes collaborate in a redundant
regulatory network (Weintraub 1993, Molkentin and Olson 1996, Lassar and Munsterberg 1994). However, a subsequent analysis revealed a subtle phenotype in myf5 and myoD single mutants showed that myf5 and myoD are required to specify different muscle lineages (Kablar et al 1997). In the same way, anf may cause a subtle phenotype which affects behaviour or a small subset of neurons that is not readily detected.

4.3.5 There may be a duplicate copy of anf

There may be a duplicate copy of anf with enough functional overlap between the genes to prevent a phenotype in anfless embryos. There are several reasons that could explain how a duplicate copy of anf might be retained rather than lost through selection (Nowak et al 1997, Cooke et al 1997).

Firstly, an additional copy of anf could act to ensure fidelity in anf function. Such an additional copy would need to be maintained at little or no selective cost (Wittbrodt et al 1998). An example of this is found in the G1 cyclin genes of yeast which probably form a functionally redundant family of genes which regulate progression of the cell cycle from G1 to S phase (Thomas 1993). In this family, loss of function of CLN1 or CNL2 has little or no phenotype (Hadwiger et al 1989) and loss of CLN3 causes a slight delayed entry to S phase (Cross 1988). Double mutants for combinations of these genes have more severe phenotypes (Hadwiger et al 1989, Cross 1988, Lew et al 1992) and triple mutants are permanently arrested in G1 (Hadwiger et al 1989). Thus, these genes ensure a robust high-fidelity control of the cell cycle (Nasmyth and Dirick 1991). Therefore, duplicates of anf may be maintained if they form part of a robust network for important steps in early neural development.

Secondly, a duplicate copy of anf could be retained if it has a separate function which is selected for but can also compensate for the loss of the anf. For instance, the expression pattern of a duplicate copy of anf may overlap with the expression pattern of anf but also be expressed in additional places or at additional stages which are crucial and selected for. An example of when this has been shown is the engrailed-1 (En1) and engrailed-2 (En2) genes which are expressed in the prospective midbrain and hindbrain and can compensate for each other's function (Joyner 1996). Null mutants for En2 have a mild phenotype because En1 overlaps with En2 expression early but not late. Therefore, the En1- and En2- phenotypes are seen in places where En1 and En2 are uniquely expressed such as the patterning of the folia of the cerebellum by En2 and the limb and sternum by En1 (Joyner 1996). Therefore, both genes are selected for but either can act where they overlap so they are probably functionally interchangeable (Joyner 1996). If this situation applies to anf, the duplicate would be selected for in its own right, but it would also be able to perform the role of anf. To determine if there is a gene which is
closely related to anf, RT-PCR could be used to amplify anf related genes from mRNA derived from anfless embryos which are free of anf mRNA.

Thirdly, anf may be a pseudogene which is not expressed in anfless embryos but performs no function in wild type embryos. The best characterised examples of pseudogenes are found in the globin gene clusters (reviewed by Wolpert et al 1998). These globin pseudogenes have similar sequences to functional genes but are not transcribed. Thus, anf may be a pseudogene which has a redundant function but, unlike globin pseudogenes, is transcribed. However, studies of mice with null mutations affecting the murine homolog of anf, hesx1 (Dattani et al 1998), suggest anf has an important role in anterior development. Furthermore, overexpression studies of the anf homolog in Xenopus, XANF1 (Ermakov et al 1999a,b), suggest anf may be required during neurogenesis. Perhaps, the best way to determine if anf has a function during zebrafish development may be through gain of function experiments.

Recent studies which have revealed teleost genomes contain extra copies of many genes, including the hox genes, provide indirect evidence for how duplicate copies of anf could have arisen.

4.3.6 Duplicate copies of anf may arise during genome duplications

Gene duplication followed by divergence is thought to be a common mechanism driving evolution (Ohno 1970). Genomic studies suggest there may have been two chordate genome duplications before fish and tetrapods diverged and a further genome duplication after the teleosts separated from their last common ancestor with the tetrapods (Postlethwait et al 1998). Thus, whereas mice have a single copy of the anf related gene, hesx1, teleosts may have "extra" copies of anf related genes. Thus, there is a theoretical basis to explain how a duplicate of anf could arise but, to date, such a gene has not been demonstrated in zebrafish. In the meantime, it is uncertain if anf is functionally redundant, performs a subtle role or is a pseudogene.
CHAPTER 5

CELL DIVISION IN THE ZEBRAFISH NEURAL PLATE

5.1 Introduction

My analysis of gene expression in the neural plate raised a number of questions regarding how patterning affects morphology. For instance, it was not clear what drives the expansion of the region of the neural plate which is likely to contain the prospective midbrain. The apparent expansion may be mediated by alterations in regional gene expression, cell migration, cell division or other mechanisms. Furthermore, mutants, such as cyc, may lack regions of the forebrain due to reduced rates of cell division. Studies of Drosophila suggest a close link between patterning and cell division, perhaps mediated through cdc25/string related genes (reviewed by Edgar and Lehner 1996). Therefore, in this chapter I set out to investigate the relationship between patterning and cell division in the rostral neural plate.

A number of studies of cell division during vertebrate development have been carried out. In the Xenopus spinal cord, there is thought to be a posterior to anterior wave of mitosis across the neural plate at the end of gastrulation (Hartenstein 1989). Further studies of Xenopus suggest that cell division is not crucial for either the development of the structure of the CNS or cell fate determination within the CNS (Harris and Hartenstein 1991). However, other studies indicate that critical events in fate determination occur at or just prior to a cell's final mitosis (Eisen 1991, Chenn and McConnell 1995, Ericson et al 1996). Of the studies conducted in zebrafish, the results indicate that cell division is highly regulated spatially (Papan and Campos-Ortega 1994) and that morphogenesis may be regulated in a cell cycle dependant manner (Kimmel et al 1994).

The aims of this chapter were firstly, to characterise a new immunohistochemical method to label dividing cells. A problem regarding studies of cell division has been the limited usefulness of bromodeoxyuridine (BrdU) or thymidine incorporation for studying patterns of cell division in three dimensions. Therefore, I used an antibody to a phosphorylated form of histone H3 (PH3; L. Mahadevan unpublished data, Hendzel et al 1997) to label the G2 and M phases of the cell cycle in whole embryos. Secondly, I studied the earliest cell divisions which are thought to be synchronous (Kimmel and Law 1985) but might lead to different fates (Strehlow and Gilbert 1993). Thirdly, I analysed the pattern of cell division in the rostral neural plate and forebrain to identify zones of proliferation. I also analysed the expansion of the prospective midbrain to determine if it involves cell division and studied the midbrain noi mutant as it may lack this cell division. Finally, I made a preliminary analysis of cell division in the cyc and oep
mutants which have smaller brains so may have less cell division. I also made a preliminary analysis of cell division in mbl mutants as these embryos have abnormally patterned forebrains and so allowed me to ask if cell division was linked to forebrain regionalisation.

5.2 Results

5.2.1 A new method for studying cell division

BrdU incorporation is of limited value to analyse cell proliferation in whole mount embryos

Previously published work has used BrdU to label dividing cells (for example, McConnell and Kaznowski 1991). In this project I wished to look at very early stages of development but found that BrdU was inappropriate. Firstly, BrdU labelling requires harsh treatments which resulted in heavy losses of embryos during processing. Secondly, the neural plate is present for only a short time during development and is made up of rapidly dividing cells. BrdU labelling requires a minimum incubation period which spanned a stage in development and so is inappropriate for seeing transient patterns of cell division (Fig. 5.1A). An alternative method for labelling dividing cells is radioactive thymidine incorporation but although whole tissues are labelled they need to be serially sectioned which is technically difficult. Therefore, a new method was developed to follow cell proliferation in whole mount embryos.

An antibody to phosphorylated histone H3 labels proliferating cells

Through a collaboration with L. Mahadevan I obtained an antibody, PH3, which labels dividing cells exclusively. The PH3 antibody recognises an epitope present on a phosphorylated histone H3 protein which is present during the late G2 and mitosis phases of the cell cycle (L. Mahadevan, unpublished data, Hendzel et al 1997). This epitope is highly conserved (Hendzel et al 1997) and the antibody labels cells throughout the zebrafish embryo body (Fig. 5.1B).

The PH3 antibody epitope is specific to dividing cells

Confirming in vitro studies suggesting that the antigen is only present in proliferating cells (L. Mahadevan, unpublished data), the PH3 antibody labelled cells in zebrafish embryos in regions known to be proliferating. For instance, the ventricular zone of the developing nervous system is known to be a major site for cell proliferation (Rakic, 1971) and was labelled in whole mount embryos (Fig. 5.1B) and in thick sections (Fig. 5.1C). In addition, mitotic bodies were stained clearly (Fig. 5.1D).
Figure 5.1 Proliferating cells labelled with BrdU and PH3.
Embryos labelled as wholemounts with BrdU antibody (A) or PH3 antibody (B-D). A,B,D) dorsal views with rostral to the top and (C) is a 30-50um transverse section. A-C) 24h and (D) 16 cell stage.
A) BrdU labels many dividing cells in forebrain and eyes.
B) PH3 labels regions of division in ventricular zone and eyes.
C) PH3 labels dividing cells in ventricular marginal zone of neural keel.
D) PH3 labels mitotic bodies.
Abbreviations: e, eye; nk, neural keel; v, ventricle.
Scale bars: A,B,C) 25μm. D) 5μm.
The PH3 antibody cross-reacts with other species

As the PH3 epitope is highly conserved, the antibody was likely to cross react with different species. This was important as in many systems the PH3 technique will be more useful than current methods for studying cell division. To test this, labelling was carried out on tissue from a number of different species.

The PH3 antibody labelled dividing cells in ascidian (Fig. 5.2A), *Drosophila* (Fig. 5.2B) and chick (Fig. 5.2C) embryos. Therefore, the conserved epitope detected by the PH3 antibody enables this method to label dividing cells in all species tested.

### 5.2.2 Synchrony in early cell divisions

**Cell division is synchronised during 2, 8 and 16 cell stages**

Cell division happens every 15 minutes during the cleavage period (Kimmel et al 1995). Whilst it has been reported that these divisions are synchronised (Kimmel et al 1995) conflicting results have been reported about the fate of these cells (Kimmel and Law 1985, Strehlow and Gilbert 1993). Previous studies have shown asymmetric fates of neural cells can be linked to asymmetric division (for instance, Guo et al 1996). As these cells are dividing quickly, it is possible that a transiently asymmetric division, which might lead to different fates, could be missed. To elucidate if the earliest divisions are synchronised embryos were labelled with PH3.

In 2, 8 and 16 cell stage embryos, either all the nuclei were labelled (Fig. 5.3A,C,E; Table 5.1) or none were labelled (Fig. 5.3B,D,F; Table 5.1). Labelling of a portion of the cells was never observed (n=58). Therefore, cell divisions were synchronous at 2, 8 and 16 cell stages. As the entire cell cycle is only 15 minutes at these stages of development (Kimmel et al 1995), the PH3 epitope must only be present for a few minutes.

**Cell division is not synchronised in all 4 cell stage embryos**

Cell proliferation was not always synchronised at the 4 cell stage (Table 5.1). Embryos were either labelled in no cells (Fig. 5.4A; 46%), two cells (Fig. 5.4B; 9%), three cells (Fig. 5.4C; 5%) or four cells (Fig. 5.4D; 41%). Therefore, cell proliferation during early development is synchronised during all of the early divisions except at the 4 cell stage.

### 5.2.3 Patterns of cell division in the neural plate

PH3 labelling, unlike BrdU labelling, does not require a harsh incorporation step, the embryo is simply fixed and processed for antibody labelling as described (Macdonald
Figure 5.2 PH3 labels proliferating cells in ascidian, *Drosophila* and chick embryos.

Embryos labelled as wholemounts with PH3 antibody. A) is a lateral view of larval stage ascidian with rostral to the left. B) is dissected larval stage Drosophila gut C) is transverse 30-50µm section of E3.5 stage chick.

A) PH3 labels dividing cells (arrowheads) in a larval stage ascidian embryo.
B) PH3 labels dividing cells (arrowheads) in gut of *Drosophila* embryo.
C) PH3 labels dividing cells in hindbrain of chick embryo.

Abbreviations: fp, floor plate; n, notochord; rp, roof plate; sg, sticking gland; sv, sensory vesicle; v, ventricle.

Scale bars: A,B) 150µm. C) 40µm.
Figure 5.3 PH3 labelling of 2, 8 and 16 cell stage embryos.
Embryos labelled as wholemounts with PH3. A,B) lateral or animal pole (C-F) views.
A,B) at 2 cell stage, PH3 epitope (arrowhead) either in both or neither cell.
C,D) at 8 cell stage, PH3 epitope (arrowhead) in all or no cells.
E,F) at 16 cell stage, PH3 epitope (arrowhead) in all or no cells.
Scale bar: 125μm.
Figure 5.4 PH3 labelling of 4 cell stage embryos.
Embryos labelled as wholemounts with PH3. Animal pole views of 4 cell stage embryos.
A) some embryos do not express PH3 epitope.
B) a proportion of embryos express the PH3 epitope in two cells (arrowheads).
C) occasionally, the PH3 epitope is expressed in three cells (arrowhead).
D) some embryos express the PH3 epitope in all cells (arrowhead).
Scale bar: 125μm.
Table 5.1 Patterns of early cell divisions.

<table>
<thead>
<tr>
<th>Stage</th>
<th>All labelled</th>
<th>None labelled</th>
<th>Some labelled*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 cell</td>
<td>8</td>
<td>22</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>4 cell</td>
<td>9</td>
<td>10</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>8 cell</td>
<td>13</td>
<td>5</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>16 cell</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

* Two embryos at the 4 cell stage had 2 cells labelled. One embryo at the 4 cell stage had 3 cells labelled.
Figure 5.5  Schematic showing orientations used to analyse PH3 labelling during gastrulation.
Embryos labelled as wholemounts with PH3. A) is an animal pole view with ventral to the left. B) is a lateral view with ventral to the left.
Abbreviations: V, ventral half of animal pole; D, dorsal half of animal pole; AV, ventral half of animal pole; AD, dorsal half of animal pole; PV, posterior ventral half; VD, dorsal half of vegetal pole.
Scale bars: 125μm.
Figure 5.6 PH3 labelling during gastrulation.

Embryos labelled as wholemounts with PH3. A,C,E,G) are animal pole views with ventral to the left. B,D,F,H) are lateral views with ventral to the left.

A,B) at 30% epiboly, the PH3 epitope expressed evenly across the embryo.

C,D) at 80% epiboly, the PH3 epitope is expressed strongly in the dorsal and weakly in the ventral side of the embryo.

E,F) at 90% epiboly, the PH3 epitope is strongly expressed in the dorsal side of the embryo.

G,H) at bud stage, PH3 labelling is mostly in the main embryonic axis.

Scale bar: 125μm.
Table 5.2 Analysis of the pattern of cell division during gastrulation.

<table>
<thead>
<tr>
<th>Region</th>
<th>80% epiboly mean no. divisions ± s. d. (n=10)</th>
<th>90% epiboly mean no. divisions ± s. d. (n=7)</th>
<th>bud mean no. divisions ± s. d. (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AV ventral</td>
<td>25±12 13%</td>
<td>19±6 9%</td>
<td>16±6 8%</td>
</tr>
<tr>
<td>animal pole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD dorsal</td>
<td>71±17 38%</td>
<td>74±8 36%</td>
<td>64±11 33%</td>
</tr>
<tr>
<td>animal pole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PV posterior</td>
<td>38±13 20%</td>
<td>44±10 21%</td>
<td>33±8 17%</td>
</tr>
<tr>
<td>ventral</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VD dorsal</td>
<td>54±9 29%</td>
<td>70±9 34%</td>
<td>84±24 43%</td>
</tr>
<tr>
<td>vegetal pole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>188±22</td>
<td>207±20</td>
<td>197±29</td>
</tr>
</tbody>
</table>
Figure 5.7 Schematic showing the trend of PH3 labelling during mid to late gastrulation.

A) at 80% epiboly, most dividing cells are in the dorsal half of the animal pole.
B) at 90% epiboly, the dorsal half of the vegetal and animal poles have similar number of dividing cells.
C) at bud stage, most dividing cells are in the dorsal half of the vegetal pole.

Abbreviations: AP, animal pole; D, dorsal; V, ventral; VP, vegetal pole.
bud stage

90% epiboly

80% epiboly
and Wilson 1997). Thus, PH3 labelling was suitable for analysing patterns of cell division in the zebrafish neural plate during gastrulation when the embryos are fragile.

**Cell division is not regionalised before gastrulation**

The number of dividing cells was counted from lateral or animal pole orientations (Fig. 5.5). Pre-gastrulation embryos labelled with PH3 showed a uniform distribution of dividing cells across the blastula (Fig. 5.6A,B).

**The rate of cell division does not change between mid to late gastrulation**

Mid to late gastrulation stage embryos were labelled with PH3 and the numbers of dividing cells counted and their distribution analysed (Table 5.2). There was no significant difference in the number of dividing cells between 80% epiboly (P<0.1; Fig. 5.6C,D), 90% epiboly (P<0.1; Fig. 5.6E,F) and bud (P<0.1; Fig. 5.6G,H) stages.

**More dividing cells are in the dorsal half than the ventral half of the embryo**

There were significantly more dividing cells in the dorsal than ventral halves of the embryo between mid to late gastrulation (Table 5.2). Significantly more divisions occurred in the dorsal than ventral half at 80% epiboly (67%; P>0.001; Fig. 5.6C,D), 90% epiboly (70%; P>0.001; Fig. 5.6E,F) and bud (76%; P>0.001; Fig. 5.6G,H) stages.

**The region of most cell division shifts from the dorsal animal pole to the dorsal vegetal pole**

There was a significant shift in the pattern of cell division with the region of highest cell divisions shifting from the dorsal animal pole to the dorsal vegetal pole during gastrulation (Table 5.2). At 80% epiboly, significantly more divisions occurred in the dorsal animal pole (38%; P>0.001) than elsewhere. At 90% epiboly there was no significant difference in the numbers of dividing cells in the dorsal animal pole (36%) and dorsal vegetal pole (34%; P<0.1). At bud stage significantly more divisions occurred in the dorsal vegetal pole (43%; P>0.02) than elsewhere. Thus, the trend of cell division from mid to late gastrulation was from the dorsal animal pole to the dorsal vegetal pole (Fig. 5.7).

**Dividing cells in the neural plate are often arranged into strings**

In the wild type neural plate, it was found that PH3 labelled cells are often arranged into short chains or strings (Fig. 5.8A,B). The length of the strings ranged from 3 cells to 6 cells, with an average length of about 4 cells. Previously, it has been reported that cells divide within clonal strings in the neural plate (Kimmel et al 1994). More recently, it has been shown that most cell divisions in the neural plate are aligned along the AP axis during gastrulation (Concha and Adams 1998) so may provide a mechanism
Figure 5.8 Strings of PH3 labelled cells during gastrulation.
Embryos labelled as wholemounts with PH3 antibody. A) is a lateral view with dorsal to the left. A,C) 80% epiboly. B,E) 90% epiboly.
A) strings of PH3 labelled cells (arrowheads) occur across the neural plate.
B) a high power view of a string of PH3 labelled cells.
C,D) an example of one pair of PH3 labelled strings of cells with a schematic indicating a possible sequence of cell divisions (numbered 1 to 4) that may have led to it.
E) example of a string of clonally related cells.
Scale bars: A) 250µm. B-D) 10µm. E) 200µm.

E) adapted from Kimmel et al 1994.
for the formation of clonal strings. Although this study did not examine the clonal relationship of cells within the neural plate, there was an intriguing similarity between the strings of PH3 labelled stings of cells (Fig. 5.8C,D) and the clonal strings (Fig. 5.8E; Kimmel et al 1994).

**Dividing cells in the neural tube become concentrated at the midline before neurocoel formation**

Zebrafish embryos undergo secondary neurulation whereby a solid neural keel develops into a hollow neural tube by cavitation (Papan and Campos-Ortega 1994). In vertebrates undergoing primary neurulation, such as mammals, the dividing cells in the neural tube become organised into a ventricular marginal zones at the midline (Rakic 1971). A similar organisation has been noted in zebrafish when studied using sectioned material (Papan and Campos-Ortega 1994). The PH3 antibody labelling enabled the three dimensional pattern of division to be analysed during neural tube formation.

A range of stages of embryos from the end of gastrulation to one day of development were labelled with PH3. At 2s, dividing cells were distributed widely and evenly across the neural plate (Fig. 5.9A). By 6s (Fig. 5.9B), cell division became focused at the midline although this organisation did not extend to the rostral tip of the neural keel. Comparison with an embryo labelled with anf (Fig. 5.9C), which has a posterior border at the prospective mid-diencephalon (Chapter 3), indicated the midline organisation of dividing cells continues as far rostrally as the caudal forebrain at the 6s stage. By 10s, most dividing cells were organised at the midline and this organisation extended to the rostral tip of the neural keel (Fig. 5.9D). The midline was organised into a distinct ventricular marginal zone (VMZ) of dividing cells by 24h (Fig. 5.9E).

To assess the pattern of division, embryos between 4s and 24h were labelled with PH3 and sectioned through the forebrain. At 4s, cells were found to be dividing at the midline (Fig. 5.10A) as well as more laterally (Fig. 5.10A). Occasionally, mitotic bodies were seen of cells that appeared to divide across the midline (Fig. 5.10A) which was also seen in previous studies (Papan and Campos-Ortega 1994, Kimmel et al 1994). At 10s, most dividing cells were found at the midline, although a few dividing cells were also found in more lateral positions (Fig. 5.10B). At 18s, all dividing cells were at the midline (Fig. 5.10C) and by 24h dividing cells were arranged into a VMZ (Fig. 5.10D). Thus, dividing cells became organised at the VMZ by 18s.

**5.2.4 There is increased cell division in dino mutant embryos**

*chordin may play a role in the regulation of cell division*

It has been shown that *chordin* has an important role in patterning the ectoderm (Sasai et al 1994; Piccolo et al 1996). However, its role regarding the regulation of cell
Figure 5.9  PH3 labelled cells converge at the midline during early somitogenesis.

Embryos labelled as whole mounts with PH3 antibody or by in situ hybridisation with anf. A-D) animal pole or (E) dorsal views with rostral to the left.

A) PH3 labelled cells are not at the midline at 2s.

B) PH3 labelled cells converge at the midline by 6s except in rostral forebrain. The rostral limit of the midline aggregation of dividing cells is indicated with the arrowhead. C) at 6s the posterior border of anf expression (arrowhead) is at a similar position as the rostral extent of PH3 midline labelling.

D) PH3 labelled cells are organised along the entire midline (arrowhead) by 14s.

E) PH3 labelled cells are organised into a ventricular marginal zone (arrowhead) by 24h.

Scale bars: A-D) 125µm. E) 10µm.
Figure 5.10 Prior to neurocoel formation, PH3 labelled are increasingly located at the midline.
Embryos were labelled as whole mounts with PH3 antibody and 30-50μm transverse sections made at the level of the forebrain.
A) at 4s most PH3 labelled cells are not at the midline (black arrowhead) but a few PH3 labelled are at the midline (white arrowhead).
B) at 10s, many PH3 labelled cells are at the midline (white arrowhead).
C) by 18s, most PH3 labelled cells are found at the midline (white arrowhead).
D) at 24h, PH3 labelled cells are organised into a ventricular marginal zone (white arrowhead).
Abbreviations: e, eye; m, midline; v, ventricle.
Scale bars: 25μm.
division has not been tested. The zebrafish mutant *dino* (Hammerschmidt et al 1996) is a consequence of a mutation to the *chordin* gene (Schulte-Merker et al 1997), thus it provides a tool to directly address the role of *chordin* in the regulation of cell division. Midgastrulation *dino* mutant embryos were labelled with PH3 to determine if there is an alteration to the level of cell division in mutant embryos (Fig. 5.11).

**Midgastrulation *dino* embryos have more dividing cells**

In agreement with previous studies (Hammerschmidt et al 1996), gene expression showed that the neural plates of wild type embryos (Fig. 5.11A) were larger than *dino* embryos (Fig. 5.11B). The total number of dividing cells was significantly higher (P=>0.001) in *dino* than wild type embryos. There was a mean of 165 divisions per wild type (n=10; Fig. 5.11C,E) and 223 per *dino* (n=10; Fig. 5.11D,F) embryo. Thus, there were 35% more dividing cells in *dino* mutant than wild type embryos.

There was an increased rate of cell division in both dorsal and ventral halves of *dino* embryos. In the dorsal half of wild type (n=10) embryos a mean of 118 cell divisions occur whereas in *dino* (n=10) embryos a mean of 155 dividing cells occur. Thus, there were 31% more divisions in the dorsal half of *dino* than wild type embryos. The number of dividing cells was significantly higher (P=>0.001) in *dino* than wild type embryos. Therefore, there was an increase in the level of cell division in the dorsal half of *dino* mutant embryos.

There was also a rise in the mean number of cell divisions in the ventral half of *dino* embryos. There was a mean of 47 cell divisions in the wild type (n=10) and 68 in *dino* mutant (n=10) embryo ventral halves. Thus, there was a 45% rise in the number of dividing cells in the ventral half of *dino* than wild type embryos. The number of dividing cells was significantly higher (P=>0.001) in *dino* than wild type embryos. Therefore, unexpectedly, there was an increase in the number of dividing cells in both the dorsal and ventral halves of *dino* mutant embryos which suggests *chordin* may negatively regulate cell division.

**5.2.5 Elevated levels of cell division do not play an obvious role in midbrain expansion**

*The midbrain anlage expands rapidly during development*

During my study of neural plate patterning, it was noticed that the prospective midbrain may develop from a small region of the neural plate (Chapter 3). This expansion could be driven by cell proliferation, cell migration or be a result of the induction of the midbrain by an interaction between the forebrain and hindbrain (Nieuwkoop and Albers 1990). To examine the role of cell division, embryos of various
**Figure 5.11 PH3 labelling in midgastrulation* dino mutant embryos.**

Embryos labelled as whole mounts by in situ hybridisation or with PH3 antibody with ventral to the left. Animal pole (A-D) or lateral (E,F) views with rostral to the left of 75% epiboly stage embryos.

A,B) *otx2* (pale blue) and *hoxA1* (purple) expression is reduced in *dino* mutant embryos.

C-F) PH3 labelling of wild type embryos increases in *dino* mutant embryos.

Scale bar: 125μm.
stages were labelled with PH3 and the pattern of cell division in the prospective midbrain analysed using the results of the gene expression study to define its position.

Division in the prospective midbrain region was spread across the neural plate at 2s and apparently not organised into an AP or DV domains at the prospective midbrain (Fig. 5.12A,B). By 12s, dividing cells in the prospective midbrain were mainly at the midline but there did not appear to be a localised region of cell division in the prospective midbrain (Fig. 5.12C,D). At 20s, dividing cells at the midline were organised into ventricular zones but there was not more cell division in the prospective midbrain then elsewhere (Fig. 5.12E,F). By 24h, the dividing cells in the prospective midbrain were organised into a ventricular marginal zone which extended medially and dorsally along the edge of the midbrain-hindbrain boundary (Fig. 5.12G,H). Thus, at 24h there appeared to be more dividing cells in the prospective midbrain than at earlier developmental stages. To address the role of cell division in the development of the midbrain further, cell division was analysed in the midbrain mutant noi.

noi mutant embryos have reduced cell division in the midbrain but have a normal sized midbrain

Strong alleles of the noi mutation lack the dorsal midbrain, midbrain-hindbrain boundary and cerebellum (Brand et al 1996). noi is linked is the pax2.1 gene and may involve an anterior transformation of the prospective midbrain (Brand et al 1996). It has been shown that there is an increase in the level of cell death in noi mutant embryos (Brand et al 1996) but I wanted to determine if a reduction to the level of cell division was also involved in the phenotype of noi embryos.

noi mutant embryos were labelled with PH3 and the number of dividing cells in the prospective midbrain counted by camera lucida (Fig. 5.13E,F). In 33h wild type embryos, a mean of 118 cell divisions occurred in the prospective midbrain region (Fig. 5.13A,C; n=5). However, in the equivalent region of noi mutant embryos of the same stage, a mean of 75 cell divisions occurred (Fig. 5.13B,D; n=6). Thus, there was a significant (P=>0.001) reduction to the number of dividing cells in the midbrain region of noi mutant embryos.

In a preliminary study to address if the reduction of cell division in noi embryos affected the size of the prospective midbrain, its length was measured using the posterior of the eye and rostral hindbrain as landmarks (Fig. 5.13C,D). No significant difference, in the length of the prospective midbrain in wild type and mutant noi embryos was found (P=<0.5). Therefore, although there was less cell division in noi mutant embryos, the region equivalent to the prospective midbrain attains the same length as the prospective midbrain in wild type embryos.

5.2.6 The relationship between gene expression and cell division
Figure 5.12  PH3 labelling is not focused at the prospective midbrain during somitogenesis.

Embryos labelled as whole mounts with PH3 antibody. Dorsal (A,C,E,H) or lateral (B,D,F,G) views with rostral to the left. A,B) at 2s stage, the prospective midbrain (starred) does not have a focus of PH3 labelled cells. C,D) at 12s stage, there is no PH3 labelling focus at the prospective midbrain territory (starred). E,F) at 20s stage, gene expression suggests the prospective midbrain has expanded (starred) but there is no focus of PH3 labelled dividing cells. G,H) by 24h, PH3 labelled cells are organised into ventricular marginal zones, but no focus of labelled cells is apparent at the prospective midbrain (starred). Scale bars: A-F) 125µm. G,H) 75µm.
Figure 5.13 The pattern of cell proliferation in the midbrain of *noi* mutant embryos.

33h embryos labelled as whole mounts with PH3 antibody. Dorsal (A,C) or lateral (B,D) views with rostral to the left.

A,B) stars indicate the approximate AP length of the prospective midbrain in a wild type embryo.

C,D) stars indicate the approximate AP length of the prospective midbrain in a *noi* embryo.

E,F) the number of PH3 labelled cells was counted using camera lucida and an example of wild type and *noi* embryo counts are shown.

Scale bar: 75μm.
As PH3 labelling can be combined with in situ hybridisation, PH3 was used to study the relationship between cell division and the expression of markers of cell differentiation.

**pax6 expressing cells are mitotically active**

To address the relationship between cell fate determination and cell division, embryos were double-labelled with PH3 and the neuronal differentiation markers atonal or pax6. It has previously been shown that pax6 is expressed in proliferating cells in the eye (Hitchcock et al 1996). Therefore, double-labelling embryos with pax6 and PH3 was used to show that PH3 gave consistent results with other methods for identifying dividing cells.

Embryos double-labelled with pax6 and PH3 showed that many pax6 expressing cells were also labelled by PH3 (Fig. 5.14A). Therefore, in agreement with previous studies (Hitchcock et al 1996), pax6 is expressed in proliferating cells.

**A proportion of atonal expressing cells are mitotically active**

In *Drosophila*, atonal is a positive regulator of neurogenesis which is expressed in proneural clusters of the chordotonal organs and within the developing eye (for example, see Daniel et al 1999). PH3 was used to determine if a retinal specific homolog of *Drosophila* atonal (I. Masai and S.W. Wilson, unpublished) is expressed in dividing or postmitotic neurons.

Embryos were double-labelled with atonal and PH3. In some instances, cells were not double-labelled (Fig. 5.14B). However, in other cases, cells were double-labelled with atonal and PH3 (Fig. 5.14C) indicating that these cells are mitotic. Therefore, a proportion of atonal positive cells are in the G2 or M phases of the cell cycle.

**5.2.7 Cell division in the forebrains of mutant embryos**

Several patterning mutations lead to alterations to the growth of regions of the CNS. The midline mutants *oep* and *cyc* have a reduced ventral central nervous system and *mbl* embryos lack the telencephalon and eyes. Therefore, the analysis of cell division in these mutants was likely to be useful in elucidating the link between patterning and cell division. A preliminary characterisation of the pattern of cell division in *oep*, *cyc* and *mbl* mutant embryos was carried out.

*Cell division in oep neural plate*
Figure 5.14  \textit{pax6} and \textit{atonal} are expressed in mitotic cells.
Embryos labelled with \textit{pax6} or \textit{atonal} (purple) as whole mounts by in situ hybridisation and with PH3 antibody (brown). Dorsal view with rostral to top (A) or to left (B,C). C) 10\textmu m plastic section.
A) \textit{pax6} and PH3 are co-expressed (arrowed) in the diencephalon at 24h.
B) \textit{atonal} and PH3 are not co-expressed in the retina at 27h.
C) \textit{atonal} and PH3 are co-expressed in a small number of cells (arrowhead) in the retina at 48h.
Scale bars: A,B) 50\textmu m. C) 25\textmu m.
Gene expression analysis showed that the *oepl* mutant neural plate was smaller and narrower than the wild type neural plate (Chapter 3). However, it was not certain if the smaller neural plate of *oepl* embryos led to the growth defects which occur during subsequent development. To address this, it was necessary to compare the level of cell division in the neural plates of *oepl* mutant and wild type embryos.

*oepl* mutant embryos were labelled with PH3 and the number of dividing cells counted. As with the gene expression study, the size of the neural plate in wild type embryos (Fig. 5.15A) was reduced in *oepl* mutant embryos (Fig. 5.15B). There was no significant difference in the level of cell division in wild type and *oepl* mutant embryos (P=0.5; n=5). Neither did there appear to be any difference in the distribution of dividing cells across the neural plate. Therefore, differences in the size of the CNS of *oepl* embryos are likely to be caused by the smaller neural plate rather than differences in the rate of cell division.

**Cell division in the forebrain of *oepl* embryos**

It was found that the forebrains of wild type embryos (Fig. 5.15C) have 38% more, though not significantly more (P=0.05; n=3), dividing cells than *oepl* mutant (Fig. 5.15D) embryos. The dividing cells were organised into a VMZ in wild type (Fig. 5.15E) and *oepl* mutant (Fig. 5.15F) embryos. To determine if there were less dividing cells in the smaller eyes of *oepl* mutant embryos the number of dividing cells was counted using camera lucida. There were significantly (P=0.001; n=3) more dividing cells in wild type (Fig. 5.15G) than *oepl* mutant (Fig. 5.15H) eyes. Therefore, the eyes, and perhaps the forebrain of *oepl* mutant embryos have fewer dividing cells than wild type embryos.

**Cell division in cyc neural plate**

Gene expression analysis showed that *cyc* mutant embryos, unlike *oepl* mutants, have the same sized neural plate as wild type embryos (Chapter 3). The pattern of cell division was also the same in *cyc* mutant embryos as in wild type siblings at the end of gastrulation. Bud stage *cyc* embryos were labelled with PH3 and the pattern of cell division analysed (Fig. 5.16A,B). Mutant and wild type siblings could not be distinguished on the basis of their pattern of cell division. Therefore, at bud stage cell division is not altered by the *cyc* mutation.

**Cell division in cyc forebrain**

As *cyc* mutant embryos have smaller ventral forebrains the number of dividing cells was counted using camera lucida. There was a mean of 234 dividing cells in the forebrains of wild type (Fig. 5.16C; n=2) and 171 dividing cells in *cyc* mutant (Fig. 5.16D; n=3) embryos. Thus, there were 37% more dividing cells in wild type then *cyc*
Figure 5.15 The pattern of PH3 labelling in the neural plate and neural tube of oep embryos.
Embryos labelled as whole mounts with PH3 antibody. Lateral (A,B,G,H) or dorsal (C,D) views with rostral to the left. E,F) transverse 30-50µm sections through the midbrain.
A,B) PH3 labelling is in a smaller area in oep mutant embryos at 80% epiboly.
C,D) PH3 labelling is reduced in oep mutant embryos at 24h.
E,F) PH3 labelled cells are organised at the midline of the neural tube in both wild type and oep mutant embryos at 24h.
G,H) there are fewer PH3 labelled cells in the retina of oep than wild type embryos.
Lateral views of PH3 labelling in wild type (A) and oep mutant.
Abbreviations: mb, midbrain; n, notochord; nt, neural tube; t, telencephalon.
mutant forebrains. At 24h, the cells were organised into ventricular zones in both wild type (Fig. 5.16E) and cyc mutant (Fig. 5.16F) embryos.

To determine if there were less dividing cells in the eyes of cyc mutant embryos the number of dividing cells was counted using camera lucida. There was a mean of 319 dividing cells in both eyes of wild type embryos (Fig. 5.16G; n=2) and 203 dividing cells in both eyes of cyc embryos (Fig. 5.16H; n=3). Thus, there were 57% more dividing cells in the eyes of wild type embryos than in cyc embryos. Therefore, cyc embryos appear to have less dividing cells in their eyes and forebrain.

Cell division in mbl neural plate

To determine if the alteration to neural plate patterning in mbl embryos (Masai et al. 1997) affects cell division, embryos were labelled with PH3 and the pattern of cell division analysed. In this preliminary study, it was found that wild type embryos (Fig. 5.17A) did not contain the same cluster of dividing cells which was in the rostral neural plate of mbl embryos (Fig. 5.17B). Therefore, there may be a different pattern of cell division in the rostral neural plates of mbl mutant embryos.

Cell division in mbl forebrain

The forebrain of wild type embryos has a mean of 175 dividing cells (n=3; Fig. 5.17C,E) and mbl mutant embryos a mean of 231 dividing cells (n=3, Fig. 5.17D,F). The forebrain including the eyes of wild type embryos has a mean of 296 dividing cells (n=3). The cells are organised into a VMZ in both wild type (Fig. 5.17G) and mbl mutant (Fig. 5.17H) embryos. Therefore, the total number of dividing cells in the forebrain and eyes of mbl mutant embryos is less than of wild type embryos.
Figure 5.16  The pattern of PH3 labelling in the neural plate, forebrain and eyes of cyc embryos.
Embryos labelled as whole mounts with PH3 antibody. Animal pole (A), lateral (B,G,H), dorsal (C,D) or ventral (E,F) views with rostral to the left.
A,B) PH3 labelling is the same in wild type and cyc embryos at bud stage.
C,D) PH3 labelled cells are organised into ventricular zones in wild type and cyc embryos at 24h.
E,F,G,H) PH3 labelled cells occur in the brain and eyes of wild type and cyc embryos at 24h.
Abbreviations: e, eye; mb, midbrain; t, telencephalon.
Scale bars: A,B) 125µm. C-H) 50µm.
Figure 5.17  The pattern of PH3 labelling in the neural plate and forebrain of mbl mutant embryos.

Embryos labelled as whole mounts with PH3 antibody. Animal pole (A,B), dorsal (C,D) or lateral (E,F) views with rostral to the left. 30-50μm transverse sections through the midbrain (G,H).

A) at 80% epiboly, mbl mutant embryos have an ectopic patch of PH3 labelled cells (arrowhead) in the rostral neural plate absent in wild type embryos.

C-F) at 24h, PH3 labelled cells are arranged into ventricular marginal zones in wild type and mbl embryos.

G,H) PH3 labelling at the midline (arrowhead) in mbl and wild type embryos at 24h.

Abbreviations: fb, forebrain; mb, midbrain.

Scale bars: A-D) 125μm. E-H) 25μm.
5.3 Discussion

5.3.1 PH3 labels dividing cells

PH3 labelling was detected in the nuclei of G2 and M phase dividing cells (Hendzel et al 1997). PH3 staining was detected in regions of known proliferation and mitotic bodies were visible so PH3 was labelling dividing cells. The conservation of the PH3 epitope also allowed the antibody to be used to stain dividing cells in other species.

5.3.2 Early divisions are synchronous except at the 4 cell stage

The early cleavage divisions were synchronous except at the 4 cell stage. There have been conflicting reports over the timing of the formation of the major axes as different studies suggest they are formed either early (Strehlow and Gilbert 1993) or late (Kimmel and Law 1985). As the early divisions appear to be synchronous (Kimmel et al 1995) the timing of fate decision was not expected to be linked to asymmetric division as found in other systems such as Drosophila (Guo et al 1996) and mouse (Chenn and McConnell 1995). However, PH3 labelling reveals that at the 4 cell stage, the cells divide asynchronously raising the possibility of this being a cell fate decision point.

5.3.3 There are more dividing cells in the dorsal half of the embryo

In agreement with previous studies (Concha and Adams 1998), PH3 labelling revealed extensive levels of cell division throughout the epiblast. During mid to late gastrulation, most dividing cells are within the dorsal half of the embryo and the distribution shifts from the animal to vegetal pole. The dorsal ectoderm mostly comprises the neural plate whereas the ventral ectoderm is mainly the epidermis (Kimmel et al 1990). Therefore, there are more dividing cells in the neural plate then the non neural ectoderm. As more dividing cells occur in the neural plate, the shift in the majority of divisions is likely to be caused by dorsalward migration of these cells which is distinct from the dorsal convergence movements of later development (Concha and Adams 1998). The difference in the numbers of dividing cells in the dorsal and ventral halves of the embryo may be caused by differences in the distribution of cells in these regions. Previous studies of cell movements during epiboly in zebrafish embryos have shown at early gastrulation, cells are equal in size in all regions of the epiblast (Concha and Adams 1998). However, from about bud stage the cells on the ventral side of the embryo become more spread out as cells migrate dorsally (Concha and Adams 1998). Therefore, the increased numbers of dividing cells on the dorsal side of the embryo observed using PH3 labelling may be caused by the increased density of dorsal cells. However, previous
studies suggest the difference in cell size between ventral and dorsal sides of the embryos, only happens at the end of gastrulation (Concha and Adams 1998). As I find a significant difference in the number of dividing cells between dorsal and ventral halves of the embryo as early as 80% epiboly, it is likely that a change to cell density is not the only factor affecting the levels of division. Perhaps, the neural plate and non-neural ectoderm cells have differences in their levels of mitotic activity.

It was noted that chains of PH3 labelled cells occur in the neural plate reminiscent of clonal strings (Kimmel et al 1994). Although, it was not tested if the cells in these chains were clonally related, it was possible that they represented the clonal strings previously reported as cells in such clonal strings divide in tandem (Kimmel et al 1994). It could be determined if the cells labelled by PH3 were clonally related by lineage labelling cells at the beginning of gastrulation and labelling them with PH3 at later stages.

In agreement with previous studies, it was found that dividing cells aggregate at the midline long before neurocoel formation. The accumulation moved from rostral to caudal but did not extend to the rostral forebrain. Perhaps, this is because the eyes are evaginating at this time (Macdonald et al 1995) so a midline domain of cell division cannot be established. Examination of the midline of cyc and oep mutant embryos showed that midline signalling is not needed to establish a ventricular marginal zone during later development.

Unexpectedly, it was found that dino embryos have significantly more dividing cells in both dorsal and ventral halves of the embryo than wild type embryos. As chordin is mutated in dino embryos (Schutte-Marker et al 1997) this result suggests a role for chordin in directly regulating cell division in the neural plate where it is expressed. chordin may also influence cell division in the non neural ectoderm through an interaction with bmp4 (Piccolo et al 1996) and the ability of chordin to induce secondary axes (Sasai et al 1994) may involve the regulation of cell division. Such a link between chordin and cell division would support the idea that there is a close link between patterning and cell division (reviewed by Edgar and Lehner 1996), perhaps mediated through string and cdc25 (Edgar and O'Farrell 1989, Edgar et al 1994) as happens in Drosophila. Recent studies of vertebrates have shown the patterning gene shh regulates cerebellar granule neuron cell division (Hatten 1999, Wechsler-Reya and Scott 1999). Therefore, there are precedents for patterning genes regulating cell division during both invertebrate and vertebrate development.

5.3.4 Expansion of the prospective midbrain may not involve increased cell division

The region of the neural plate that, from gene expression patterns (Chapter 3), includes the prospective midbrain was not associated with regionally localised cell
division. The results of the PH3 study were consistent with a previous study of *Xenopus* which showed that cell division is not critical for development of the neurons or structures of the CNS (Hartenstein 1989). However, as PH3 labels a very short part of the cell cycle, other methods which label slightly longer periods of the cell cycle may reveal a pattern to cell division at the midbrain. Fate mapping studies have addressed the role of cell migration in the development of the prospective midbrain during gastrulation (Woo and Fraser 1995) and these studies suggest that the cells fated to form the midbrain move to the midline during gastrulation. As the expansion observed with gene expression (Chapter 3) happens after gastrulation when this dorsalward cell migration is complete at the level of the midbrain (Woo and Fraser 1995), other mechanisms are likely to drive the expansion. Perhaps the expansion of the prospective midbrain territory after gastrulation is actually the induction of the midbrain and the midbrain is not present before this stage. It has been suggested that the midbrain forms due to an interaction between the forebrain and hindbrain (Nieuwkoop and Albers 1990) so such a mechanism may cause the expansion of the prospective midbrain region. Alternatively, the gene expression boundaries may change during gastrulation and not show the actual size of the midbrain territory. To determine to what extent cell migration or cell division are required for the apparent expansion of the midbrain territory, each could be blocked to determine the effect on the midbrain at later stages. In addition, the analysis of mutants affecting midbrain development is likely to be useful in elucidating the role of cell division in midbrain development.

Previous studies of noi mutants have shown that the midbrain is induced but fails to develop properly (Brand et al 1996). I analysed noi embryos at late stages of development and found that, although the midbrain region was the correct size along the AP axis, there were fewer dividing cells. This finding suggests cell division is not required during later developmental stages to determine the correct AP length of the midbrain region in noi embryos. However, to determine the role of cell division using noi embryos requires examining earlier stages with PH3 and looking at gene expression boundaries. As noi embryos undergo increased levels of cell death at late stages of development (Brand et al 1996) this may cause the reduced number of dividing cells which could lead to a false impression of less cell division in noi mutants. To determine if the reduction to the numbers of dividing cells is secondary to the failure to maintain the midbrain or an indication that noi regulates cell division requires examining earlier stages of development.

5.3.5 *atonal* expressed in cells before their final division

*atonal* was found to be coexpressed with PH3 in a small number of cells in the eyes. The relationship between the timing of the final cell division and cell fate
assignment has been studied extensively (Eisen 1990, Ericson et al 1996, Chenn and McConnell 1995) and important steps have been shown to happen just prior to the final mitosis. The expression of *atonal* in dividing cells suggests that this transcription factors is part of the decision making process happening just prior to the final mitosis. A greater number of stages should be examined to determine if *atonal* is coexpressed with PH3 at all stages of eye development as only a small number of cells coexpressed PH3 and *atonal*.

5.3.6 Cell division in *oep, cyc and mbl* mutant embryos

The neural plates of the midline mutants *oep* and *cyc* have similar levels of cell division as wild type embryos. As both *oep* (Schier et al 1996a) and *cyc* (Hatta et al 1991a) have defects to the ventral CNS it was possible that these were caused by earlier reductions to cell division. However, no evidence to support this hypothesis was found, although the neural plates of *oep* mutant embryos were smaller than their wild type counterparts. Therefore, the reduction to the levels of cell division observed at 24h was either due to an earlier patterning defect or a later role of the midline in regulating cell division. Recent studies indicate that *shh* regulates cell division of cerebellar granule neurons (Wechsler-Reya and Scott 1999) and growth defects occur in the absence of *shh* signalling (Chiang et al 1996). Thus, the absence of *shh* in the ventral forebrain of *cyc* (Krauss et al 1993) and *oep* (Schier et al 1997) mutant embryos may cause a reduction of cell division. However, as both *oep* and *cyc* affect the nodal pathway (Blader and Strahle 1998), the reduction in cell division may be because nodal signalling is involved in regulating cell division in the ventral CNS.

The neural plate of *mbl* mutant embryos had ectopic cell division at the rostral tip of the neural plate which may be caused by the misspecification of the rostral neural plate previously reported in *mbl* embryos (Heisenberg et al 1996). Such a finding would be interesting as it would support a link between neural plate patterning and cell division. However, as the number of embryos and stages examined was low it is necessary to repeat the analysis. The forebrain of *mbl* embryos at 22h had fewer dividing cells than wild type embryos of the same stage. As *mbl* embryos do not form eyes, there are fewer regions of cell proliferation in *mbl* than in wild type embryos which may indirectly reduce the number of dividing cells rather than be due to *mbl* directly regulating cell division. As with the neural plate study, the analysis of the *mbl* forebrain needs to be repeated at more stages and with more embryos.

It has been reported that cell division is not required for normal development of the CNS in *Xenopus* (Hartenstein 1989) yet the results of PH3 labelling suggest division is organised during early development and may be regulated by patterning genes. A reason for this apparent contradiction might be that cell division must be organised so it
does not oppose morphogenesis rather than driving it (Concha and Adams 1998). To
determine the role of cell division, it would be interesting to study the effects on brain
morphology of localised increases to cell division in the neural plate.

In summary, PH3 is a useful method for studying cell division in three dimensions
during development in several systems. As early divisions are not all synchronous, the
asynchrony may be connected with the development of different cell fates. The DV
differences in division across the embryo during gastrulation may be partly due to cell
density but analysis of dino mutants suggests that chordin may regulate also cell division
in this axis. The expansion of the prospective midbrain after gastrulation does not appear
to be due to cell division as a localised region of cell division could not be found.
CHAPTER 6

THE YELLOWMAN MUTATION AFFECTS THE DIFFERENTIATION OF SEVERAL Neural Crest Derived TISSUES

6.1 Introduction

A major step in the evolution of vertebrates was a shift from a passive to an active mode of predation and a key element of this transition was the development of the neural crest (Gans and Northcutt 1983). The neural crest is a transient population of cells which are thought to have been a vertebrate innovation although studies of primitive chordates, such as the ascidian, suggest that there may have been neural crest precursors prior to the evolution of vertebrates (for example see Wada et al 1998). Nevertheless, the development of the neural crest was important to vertebrate evolution and the specification and differentiation of neural crest has been intensively studied (reviewed by Wolpert et al 1998).

The neural crest may be induced by an interaction between the neural plate and the adjacent presumptive ectoderm (Selleck and Bronner-Fraser 1995, Mancilla and Mayor 1996). Recent studies of zebrafish mutants suggest neural crest induction involves the swirl/bmp2b pathway (Nguyen et al 1998). Uncommitted neural crest cells arise from the ectoderm and are recognisable as individual cells after they emerge from the neural tube. These neural crest cells migrate from either side of the neural epithelium to give rise to many different cell types including: much of the skeleton of the head; neurons and glia of the peripheral nervous system; endocrine cells; and all pigmented cell types. Thus, the neural crest forms both ectodermal and mesodermal cell types. The differentiation of neural crest cells has been studied using both in vivo and in vitro techniques.

The fate of neural crest cells does not appear to be determined at the time when the neural crest cells leave the neural tube (reviewed by Wolpert et al 1998). For instance, labelling single neural crest cells soon after their emergence from the neural tube has shown that the cells are multipotent (Bronner-Fraser and Fraser 1988, 1989). Studies which have altered the position of neural crest cells before they migrate have shown that the neural crest cells have a greater developmental potential than revealed by fate mapping (Le Douarin et al 1975). Furthermore, studies of single neural crest cells cultured in vitro have shown that they will differentiate into several different cell types (Le Douarin et al 1993). Recently, it has been shown that there are multipotent neural crest stem cells (Morrison et al 1999). Thus, the neural crest remain multipotent until migration begins. The characterisation of mutants affecting genes involved in neural crest
development will be useful in understanding when neural crest cells become specified and the steps involved in neural crest cell differentiation.

The aims of this study were to describe the characterisation of a mutant affecting neural crest development, yellowman. yellowman was identified during a screen for mutations affecting early neural development. The yellowman mutation affects the development of the pigmentation pattern, the jaw, the ear, the fins and the peripheral nervous system.

6.2 Results

6.2.1 A pigment mutant line isolated in a random mutagenesis screen

An F2 mutant screen was conducted at KCL by ENU treatment of 20 males which were subsequently crossed with untreated females (Chapter 2). After screening over 100 families, a range of mutants was isolated including a recessive lethal affecting melanophore development, yellowman\textsuperscript{K14a}. The overall size and morphology of yellowman embryos was normal and they were indistinguishable from wild type siblings before pigmentation development at about 28h. Embryos died by six days of development perhaps due to the failure to form a swim bladder.

6.2.2 yellowman embryos have delayed melanophore differentiation

The yellowman mutation affected the development of one of the earliest pigmented cell types, the melanophore. The melanophores synthesise black pigment, melanin, and arrange themselves into a characteristic pattern which includes a set of four stripes along the body (Milos and Dingle 1978). The dorsal stripe and ventral stripe run from the head to the tail, the yolk sac stripe runs from under the heart to the anus, and the lateral stripe runs beside the horizontal myoseptum of somites 6 to 26 (Fig. 6.1; Kelsh et al 1996).

Melanophores were present in yellowman embryos but these melanophores did not differentiate properly. Melanophores were prominent black star shaped cells in wild type embryos by 48h (Fig. 6.2A,E) but were reduced to black spots in yellowman embryos by 48h (Fig. 6.2B,F). Similar numbers of melanophores appeared to be present in wild type and yellowman embryos. For instance, in the lateral region of the head there were about 9 melanophores in both wild type and yellowman embryos.

By 6 days, normal sized melanophores appeared in yellowman mutant embryos. In wild type embryos, large melanophores were more numerous and were darker (Fig. 6.3A,B) than in yellowman embryos (Fig. 6.3C,D). The occasional pale melanophores in yellowman embryos were organised into the normal stripes along the entire length of the
Figure 6.1 Schematic drawing of pigmentation pattern in posterior trunk

The left panel shows a transverse section (dorsal to the top), whilst the right panel shows a lateral view (dorsal to the top, rostral to the left), both to the same scale. Melanophores are shown as black shapes and form four stripes, the dorsal, lateral, ventral and yolk sac stripes in dorsoventral order. Iridophores (pale blue) are associated with the dorsal, ventral and yolk sac stripes. Xanthophores (green) populate the sides of the body, and are more abundant in dorsal regions.

Abbreviations: NT, neural tube; NO, notochord; S, somite; HG, hindgut.

Figure 6.2  At 48h, yellowman embryos have fewer melanophores than wild type embryos

Living embryos at 48h from dorsal (A,B) or lateral (C-F) orientations with rostral to the left.

A,B) wild type embryos have extensive black melanophores in the dorsal, lateral, ventral and yolk sac stripes but yellowman embryos have little melanophore pigmentation in any of the stripes.

C,D) the pigmented retinal epithelium is prominent in the eyes of wild type and yellowman embryos.

E,F) melanophores (arrowhead) are large and irregular in shape in wild type embryos but small in yellowman embryos.

Abbreviations: d, dorsal stripe; l, lateral stripe; ov, otic vesicle; pre, pigmented retinal epithelium; v, ventral stripe; y, yolk sac stripe; ys, yolk sac.

Scale bars: A-D) 250 μm. E,F) 100 μm.
body, including the dorsal stripe which is the first to form and the lateral stripe which is the last to form during normal development (Kimmel et al 1995). At higher power, it was apparent that the melanophores of wild type (Fig. 6.3E) and yellowman (Fig. 6.3F) embryos were similar in shape and size. There were similar amounts of pigmented retinal epithelium in wild type (Fig. 6.3C) and yellowman (Fig. 6.3D) embryos. Therefore, the yellowman mutation did not affect the synthesis of melanin. These data suggest that melanophores formed in the same number and migrated in the same way in wild type and yellowman embryos but failed to differentiate appropriately in yellowman embryos.

6.2.3 Iridophores and xanthophores form normally in yellowman mutant embryos

The neural crest derived iridophores are reflective pigment cells that appear silver under incident light or gold when viewed through the overlying xanthophores (Kelsh et al 1996). Iridophores differentiate from about 36h, beginning in the eyes, and eventually occupy three of the melanophore stripes, the dorsal, ventral and yolk sac stripe as well as lateral patches (Fig. 6.1; Kimmel et al 1995).

Iridophores formed normally in yellowman embryos in the eyes (Fig. 6.4A,B), dorsal stripe (Fig. 6.4C,D), lateral patches, ventral stripe and yolk stripe (Fig. 6.4E,F). Therefore, iridophore development was unaffected by the yellowman mutation.

Xanthophores are also neural crest derived, and confer yellow colouration to the dorsal regions of zebrafish larvae from about 42h (Fig. 6.1; Kimmel et al 1995). The distribution of xanthophores across the dorsal and lateral regions of the embryo was the same in wild type (Fig. 6.5A) and yellowman (Fig. 6.5B) embryos. By 5 days, wild type embryos were mostly dominated by black colouration due to melanophores (Fig. 6.5C) but in yellowman embryos the predominant colouration at 5 days was yellow (Fig. 6.5D). Xanthophores did not appear to expand beyond their wild type pattern (Fig. 6.5E) in yellowman embryos or to fill the spaces caused by the absence of differentiated melanophores (Fig. 6.5F). Thus, xanthophore development was unaffected by the yellowman mutation.

Thus, both xanthophore and iridophore pigment cells were unaffected by the yellowman mutation whereas the melanophores failed to differentiate properly.

6.2.4 yellowman mutant embryos have a shorter and narrower jaw

The jaws and branchial arches comprise a basic segmented feature of the head (Schilling 1997). In zebrafish, seven arches develop which mainly give rise to the cartilaginous skeleton. The lower jaw is formed from the first two branchial arches, the
Figure 6.3  A small number of pale melanophores form in yellowman embryos by the sixth day.
Living embryos on the sixth day from dorsal (A,B,E,F) or lateral (C,D) orientations. Rostral to the left (A-D) or up (E,F).
A,B) prominent dark melanophores are present in all of the stripes of wild type embryos.
C,D) yellowman embryos have a small number of pale melanophores in the stripes and lateral patches (arrowhead in D).
E,F) high power view shows that melanophores (arrowhead) have a similar size and shape in wild type and yellowman embryos.
Abbreviations: d, dorsal stripe; l, lateral stripe; pre, pigmented retinal epithelium; v, ventral stripe; y, yolk sac stripe.
Scale bars: A-D) 250μm. E,F) 100μm.
Figure 6.4 Iridophores develop normally in *yellowman* embryos by the sixth day.

Living embryos on the sixth day from lateral (A,B,E,F) or dorsal (C,D) orientations with rostral to the left. A,B) when illuminated with transmitted light, iridophores appear golden in the retina of wild type and *yellowman* embryos. C,D) iridophores (arrowhead) are visible in the dorsal stripe of wild type and *yellowman* embryos.

E,F) iridophores form in the lateral patch (arrowhead), ventral stripe and yolk sac stripe also occur in wild type and *yellowman* embryos.

Abbreviations: v, ventral stripe; y, yolk sac stripe.

Scale bar: 175 µm.
Figure 6.5  Xanthophores develop normally in yellowman embryos by the sixth day.

Living embryos on the sixth day from lateral (A,B) or dorsal (C-F) orientations. Rostral to the left (A-D) or the top (E,F).

A,B) xanthophores confer a yellow cast to a similar sized dorsal region of wild type and yellowman embryos.
C,D) from a dorsal view, black melanophores provide the predominant colouration in wild type embryos but xanthophores confer a yellow cast to the entire yellowman embryo.
E,F) xanthophores are separated by black melanophores in wild type embryos and are separated by unpigmented regions (arrowhead) in yellowman embryos.

Abbreviations: ov, otic vesicle; pre, pigmented retinal epithelium.

Scale bars: A,B) 250μm. C,D) 500μm. E,F) 100μm.
Figure 6.6 *yellowman* embryos develop a phenotype affecting the jaw by the fourth day. Living embryos on fourth day from lateral views with rostral to the left. A,B) wild type embryos do not have the curled down lower jaw (arrowhead) which develops in *yellowman* embryos. Scale bars: 250μm
mandibular and hyoid, and the upper jaw by the ethmoid plate of the neurocranium (Fig. 6.7A, 6.8A,B).

Analysis of living yellowman mutant embryos revealed an abnormality to the jaws which was first detectable at around four days. The lower jaw appeared to be affected as it seemed to be curled downwards in yellowman embryos (Fig. 6.6). To analyse the jaws in more detail, the cartilage of wild type and yellowman embryos was stained with alcian blue.

At 3 days, the number, shape and size of the cartilaginous elements of the jaw were the same in wild type (Fig. 6.7B, Fig. 6.8C) and yellowman (Fig. 6.7C, Fig. 6.8D) embryos. By 5 days, Meckel's and the palatoquadrate cartilage were smaller in yellowman than wild type embryos (Fig. 6.8E,F) but this almost recovered by 6 days (Fig. 6.8G,H). The distance between the basihyal cartilage and Meckel's cartilage was reduced in yellowman mutant embryos at 5 days (Fig. 6.8E,F) and 6 days (Fig. 6.8G,H). The basihyal cartilage was lifted dorsally at 6 days in yellowman mutants (Fig. 6.7F,G). The ceratohyal cartilage was bent medially at 5 days (Fig. 6.8E,F) and 6 days (Fig. 6.8G,H) making the jaw shorter and narrower. The overall number and identity of elements in the jaw was normal at all stages examined.

The ethmoid plate (upper jaw) was bent dorsally by 5 days (Fig 6.7D,E) and remained so at 6 days (Fig. 6.7F,G) and may have contributed to the reduction of the lower jaw.

Therefore, yellowman mutant embryos have a shorter and narrower jaw which is likely to be due to alterations to the neural crest derived cartilaginous elements that form the jaw.

6.2.5 yellowman mutant embryos have smaller posterior otoliths

The ear of the zebrafish comprises a series of three semi-circular canals with a pair of otoliths and sensory macula and cristae (Fig 6.9A; Whitfield et al 1996, Haddon and Lewis 1996).

The yellowman embryos did not have any alteration to the semi-circular canals at any stage examined (Fig. 6.9B,C). The anterior otolith is smaller than the posterior otolith in the wild type ear (Fig. 6.9A) and although this was still the case in yellowman embryos, the posterior otolith was reduced in size. At all stages examined, the anterior otolith of yellowman embryos was the same size as in wild type embryos (Fig. 6.9D,E). However, the posterior otolith was smaller from about 3 days of development and remained smaller from then onwards (Fig. 6.9F,G).

To determine if the yellowman otolith phenotype reflected a defect during earlier patterning, the expression of two genes expressed in the ear anlage was analysed. The markers pax2.1 and dlx3 are both expressed early on during ear development and were
Figure 6.7 Alcian blue staining of the pharangeal arches and head skeleton of yellowman mutant embryos.

Embryos were labelled as whole-mounts by alcian blue staining. Lateral views with rostral to the left.

A) schematic of the head showing the elements of the pharangeal skeleton.

B,C) wild type and yellowman mutant embryos at 3 days when the number, shape and size of the elements was the same in yellowman and wild type embryos.

D,E) by the fifth day, the ethmoid plate was bent dorsally (arrowhead) in yellowman embryos compared to wild type embryos.

G,H) at the sixth day, the elements were different when comparing wild type with yellowman embryos. The basihyal (bh) was lifted dorsally and the ethmoid plate (e) was bent dorsally (arrowhead in G).

Abbreviations: abc, anterior basicranial commissure; ac, auditory capsule; bb, basibranchial; bh, basihyal; c, cleithrum; cb, ceratobranchial; ch, ceratohyal; e, ethmoid plate; hs, hyosymplectic; m, Meckel's cartilage; pc, parachordal; pq, palatoquadrate; t, trabeculae cranii.

Scale bars: 175μm.

A) From Schilling et al 1996.
Figure 6.8 Alcian blue staining of the pharangeal arches and head skeleton of yellowman mutant embryos.

Embryos were labelled as whole-mounts by alcian blue staining. Dorsal views with rostral to the left.
A,B) schematic of the head showing elements of pharangeal skeleton and neurocranium.
C,D) wild type and yellowman mutant embryos at 3 days when the elements were not affected by the yellowman phenotype.
E,F) wild type and yellowman mutant embryos at 5 days when the palatoquadrate cartilage and Meckel's cartilage were smaller in yellowman than wild type embryos. The distance between the basihyal and Meckel's cartilage was smaller in yellowman than wild type embryos.
G,H) wild type and yellowman embryos at 6 days revealed the distance between basihyal and Meckel's cartilage was smaller than wild type. The ceratohyal cartilage was bent in middle in yellowman but not wild type embryos.

Abbreviations: abc, anterior basicranial commissure; ac, auditory capsule; bb, basibranchial; bh, basihyal; cb, ceratobranchial; e, ethmoid plate; hb, hypobranchial; hs, hyosymplectic; ih, interhyal; m, Meckel's cartilage; pc, parachordal; pq, palatoquadrate; t, trabeculae cranii.
Scale bars: 175μm.

A,B) From Schilling et al 1996.
Figure 6.9  Ear phenotype in living yellowman mutant embryos at 96 hours.

A) Cut away lateral view of a wild type zebrafish ear at 96 hours. Epithelial projections within the ear form hubs of the developing semicircular canal (curved arrows). Each canal (anterior, lateral and posterior) is associated with a small sensory patch or crista, while each otolith overlies a larger sensory patch or macula. The smaller (anterior) otolith lies in a lateral position; the larger (posterior) otolith lies medially.

B,C) the shape and number of semi-circular canals was the same in wild type and yellowman embryos.

D,E) the size and shape of the anterior otolith was the same in wild type and yellowman embryos.

F,G) the posterior otolith was larger in wild type than yellowman embryos.

Abbreviations: ac, anterior canal; lc, lateral canal; pc, posterior canal; dls, dorsolateral septum; ep, epithelial projections; kc, kinocilia; sc, stereociliary bundles.

Scale bars: B-G 75μm.

A) From Whitfield et al 1996.
analysed in *yellowman* embryos at 24 hours development. However, both *pax2.1* (Fig. 6.10A) and *dlx3* (Fig. 6.10B) were found to be expressed normally in the ear anlage of *yellowman* embryos. Therefore, some markers of early ear development are expressed normally in *yellowman* embryos.

### 6.2.6 *yellowman* mutant larvae have defects in the differentiation of a neural crest derivative in their pectoral fins

As the neural crest may play a role in fin development (Smith et al 1994), the cartilage in the pectoral fins of *yellowman* embryos was analysed using alcian blue staining. The paired pectoral fins develop from the limb bud and are thought to be homologous with the forelimbs of tetrapods (Sordino et al 1995). The epithelial blade of the fin develops from the apical epidermal ridge and is strengthened by collagenous fibres, the actinotrichia. By 5 days, the fin has developed a compact mesenchymal condensation which will form the supportive girdle of the fin. In this region, the cartilage begins to differentiate and the chondrocytes are arranged into cobblestones (Kimmel et al 1995). The dermal skeleton of the pectoral fins includes the lepidotrichia, which forms within the fin fold (Sordino et al 1995). The origin of the lepidotrichia is uncertain, but they are thought to arise from the neural crest (Smith et al 1994).

At 6 days of development, the morphology of the blade of the pectoral fins of *yellowman* mutant embryos was the same as wild type embryos (Fig. 6.11A,B) suggesting the apical epidermal ridge forms normally. However, *yellowman* mutant embryos were found to lack the majority of the differentiating lepidotrichia cells which were present in the fins of wild type siblings (Fig. 6.11C,D). The strengthening collagenous fibres of the actinotrichia were unaffected in *yellowman* mutant embryos (Fig. 6.11C,D). Therefore, the fins of *yellowman* mutant embryos appear to have a delay in the development of the neural crest derived lepidotrichia but not other elements of their structure.

### 6.2.7 Touch response

Responsiveness to tactile stimulation develops from about 24h and an escape response at 36h (Kimmel et al 1995). Previous studies have found defects in touch sensitivity in pigment mutants (Granato et al 1996) so the response to tactile stimulation was tested in *yellowman* embryos. Sensory responsiveness was tested by gently touching the nose, back and tail ten times with a pair of forceps.

At 2 days, wild type siblings responded to 90% of the tactile stimuli and *yellowman* mutant embryos responded to 40% of the tactile stimuli. At 4 days, wild type embryos responded to 82% of the tactile stimuli and *yellowman* mutant embryos responded to 32% of the tactile stimuli. By 6 days, both wild type and *yellowman*
Figure 6.10  *pax2.1* and *dlx3* expression is normal in *yellowman* mutant ears.

Embryos were labelled as whole mount by in situ hybridisation. Rostral to the left and dorsal to top. 
A,B) the markers of early ear development, *pax2.1* and *dlx3*, were expressed in the same way in wild type and *yellowman* embryos at 24h.

Scale bars: 50μm.
Figure 6.11 The differentiation of the neural crest derived lepidotrichia is delayed in *yellowman* pectoral fins at the sixth day. Embryos were labelled as whole mounts with alcian blue.

A,B) the pectoral fin blade and coraco-scapular cartilage (cs) develop in the same way in wild type and *yellowman* embryos.

C,D) higher power views show that numerous lepidotrichia (arrowheads) develop in the wild type pectoral fin but few lepidotrichia (arrowhead) develop in the *yellowman* pectoral fin by the sixth day.

Abbreviations: cs, coraco-scapular cartilage.

Scale bars: A,B) 50µm. C,D) 25µm.
embryos responded to 100% of the tactile stimuli. Therefore, yellowman mutant embryos initially had reduced sensory response which recovered by 6 days.

6.2.8 Neural crest cell numbers and migration are not obviously abnormal in yellowman embryos

The tissues affected by the yellowman mutation all require neural crest cells for their development. Two markers of neural crest were used to directly determine if there were obviously fewer pre-migratory neural crest cells and if neural crest migration is disturbed by the yellowman mutation. forkhead-6 (fkd6; Odenthal and Nusslein-Volhard 1998) labels the neural crest cell population before migration. yellowman embryos labelled with fkd6 were indistinguishable from their wild type siblings at all stages analysed (Fig. 6.12A-C). Therefore, the numbers and distribution of pre-migratory neural crest was not significantly affected by the yellowman mutation.

To determine if the migration of neural crest was affected in yellowman mutant embryos, a population of the migratory neural crest was labelled using an antibody to Pax7 (Kawakami et al 1997). Labelling with Pax7 antibody did not reveal any difference in the pattern or numbers of neural crest cells migrating in wild type (Fig. 6.12D,E) or yellowman (Fig. 6.12F,G) embryos at all stages examined. For instance, in dorsal regions of the head at 48h the same number of Pax7 positive neural crest cells was found in wild type (mean = 82; n=3) and yellowman (mean = 83; n=3) embryos. From Pax7 labelling, the neural crest cells appeared to be distributed in the same way in wild type and yellowman embryos. For instance, the most dorsal stream over the eyes was present in both wild type (Fig. 6.12D) and yellowman (Fig. 6.12F) embryos. Therefore, the number and distribution of migrating neural crest cells was unaffected by the yellowman mutation.

6.2.9 Axonal tracts in the brain are unaffected by the yellowman mutation

A number of zebrafish pigmentation mutants have been found to have alterations to their axon tracts when analysed after 3 days of development (C.-P. Heisenberg, T.F. Schilling, C. Houart, R.E. Macdonald and S.W. Wilson, unpublished data). To ascertain if the yellowman mutation leads to any changes to the axon tracts in the brain, three day old embryos were labelled with an antibody to acetylated tubulin. These embryos were also double-labelled with an antibody to opsin which labels photoreceptor cells, most notably in the epiphysis.

Careful analysis of tubulin and opsin double-labelled embryos did not find any difference in the overall morphology or in any specific region in yellowman mutant
Figure 6.12  Two neural crest markers are expressed normally in *yellowman* embryos.

Embryos were labelled as whole mount by in situ hybridisation with *fkdb* (A-C) or with Pax7 antibody (D-G). A,C,E,G) lateral and (B,D,F) dorsal views. Rostral to the left at 24h. A-C) the pre-migratory neural crest marker *fkdb* was expressed normally in *yellowman* embryos along the main axis and in the head. D-G) Pax7 labelling of migratory neural crest was the same in wild type and *yellowman* embryos. The same streams of migrating neural crest were visible in wild type and *yellowman* embryos, such as across the eyes (arrowhead in D,E).

Scale bars: A) 250μm. B,C) 175μm. D-G) 125μm.
Figure 6.13  Axon tracts are normal in *yellowman* mutant embryos at the third day.

Embryos were labelled as whole mounts with antibodies to acetylated tubulin and opsins. Dorsal views with rostral to the top.
A-D) the axon pathways were the same in both wild type and *yellowman* embryos, including the anterior commissure and optic chiasm.
E,F) the epiphysis developed in the same way in wild type and *yellowman* embryos. G,H) the midbrain and hindbrain also appeared to be the same in wild type (E,G) and *yellowman* embryos (F,H).
Abbreviations: ac, anterior commissure; c, cerebellum; ep, epiphysis; ob, olfactory bulb; oc, optic chiasm; oe, olfactory epithelium; poc, post optic chiasm; te, tectum.
Scale bars: A,B) 200μm. C-H) 100μm.
embryos compared with wild type embryos (Fig. 6.13). Structures including the optic nerve, optic chiasm, anterior commissure and post-optic commissure were examined and found to be the same in wild type (Fig. 6.13A) and yellowman (Fig. 6.13B) embryos. Similarly, the olfactory epithelium and olfactory bulbs (Fig. 6.13C,D), the epiphysis and tectum (Fig. 6.13E,F) and cerebellum (Fig. 6.13G,H) were all the same in wild type and yellowman embryos. Therefore, the yellowman mutation did not appear to cause any defects to the axonal tracts of the brain.

6.3 Discussion

Neural crest cells form many different cell types and so the development of the neural crest is likely to be complex and involve many genes (Kelsh et al 1996). The combination of defects observed in yellowman mutant embryos suggested yellowman is needed during neural crest development.

6.3.1 Pigmentation phenotype

The pigmentation phenotype of yellowman suggests that it affects either neural crest specification or, the later event of melanophore differentiation. Previous screens (Kelsh et al 1996, Streisinger et al 1986) have identified mutants that have similar, but not identical, phenotypes to yellowman, these are sparse (spa), touch down (tdo), colourless (cls) and puzzle (puz).

yellowman mutant embryos have delayed melanophore differentiation leading to a few pale melanophores. spa mutant embryos also have fewer melanophores (Streisinger et al 1986, Kelsh et al 1996). However, unlike yellowman mutants, spa mutants accumulate melanophores behind the otic vesicle and by the sixth day, the melanophores are abnormally shaped. Thus, yellowman does not have the same phenotype as spa.

tdo is more similar to yellowman than spa and many alleles of tdo were found during the Tubingen screen (Kelsh et al 1996). tdo mutants, like yellowman, initially have fewer melanophores and those present are very small. Both tdo (Kelsh et al 1996) and yellowman mutant embryos also have a transient reduction to touch response, perhaps due to a delayed differentiation of the touch response circuitry. tdo partly recovers its pigmentation phenotype by sixth day, although the melanophores which form are pale and small as in yellowman mutants. There are several differences between the yellowman and tdo phenotypes. yellowman embryos degenerate at about the sixth day and have additional defects, including jaw and ear phenotypes, which are not present in tdo. However, the similarities in the pigmentation and sensory phenotypes suggest yellowman either acts in the same pathway as tdo or is an allele of tdo. To determine between these
possibilities requires additional studies, such as complementation testing between \textit{tdo} and \textit{yellowman}.

The \textit{yellowman} mutation causes defects to a number of neural crest derivatives so \textit{yellowman} is probably needed during the specification or differentiation of a subpopulation of the neural crest. Mutations affecting neural crest specification are likely to have more severe and possibly more widespread effects than mutations affecting differentiation of particular types of neural crest cells. For instance, \textit{tdo} affects pigmentation and sensory neural crest derivatives and it has been proposed that \textit{tdo} is required for melanophore differentiation (Kelsh et al 1996). The most severe neural crest specification mutant identified in the Tubingen screen, \textit{cls}, had defects affecting all chromatophores (Kelsh et al 1996). \textit{cls} mutant embryos, like \textit{yellowman} and \textit{tdo}, have a reduced number of melanophores that are small and fail to develop. In \textit{cls}, it has been shown that most neural crest cells fail to migrate, which is not found in \textit{yellowman} embryos. The melanophores which form in \textit{cls} mutants, are pale but the xanthophores and iridophores have a normal phenotype, which is the same in \textit{yellowman} and \textit{tdo}. \textit{cls} mutants also have small ears and small otoliths (Whitfield et al 1996) which is similar to the \textit{yellowman} phenotype. The \textit{tdo} mutation has not been shown to affect inner ear development, like \textit{cls} and \textit{yellowman}, but as the ear phenotype is relatively slight in \textit{yellowman}, it may be present in \textit{tdo} but be missed or absent in a weak allele of \textit{tdo}. Therefore, \textit{cls} has a defect in neural crest migration, melanophore differentiation and inner ear development. Thus, \textit{yellowman} is similar to \textit{cls} as it also affects melanophore differentiation, otolith development but does not affect neural crest migration so perhaps acts after \textit{cls}. Therefore, \textit{cls} and \textit{yellowman} may act in the same pathway affecting neural crest development.

A further pigment mutant, \textit{puz}, affects similar tissues as \textit{yellowman}. \textit{puz} mutant embryos have a phenotype affecting jaw and ear development but, unlike \textit{cls}, degenerate by about the fifth day (Kelsh et al 1996). A difference between \textit{puz} and \textit{yellowman} is that the melanophores which form in \textit{puz} embryos don't appear as tiny spots. Therefore, \textit{puz} may act in a related pathway during neural crest development as \textit{yellowman}.

The interactions between \textit{yellowman}, \textit{tdo}, \textit{cls} and \textit{puz} are likely to be complex as they affect several overlapping functions. To determine the relationship requires complementation testing between \textit{yellowman} and the other mutants. The generation of double mutants will also be useful in discerning the hierarchical relationship between the genes.

\textbf{6.3.2 Cartilage phenotype}

The jaw phenotype of \textit{yellowman} mutant embryos was restricted to the anterior structures. The elements of the anterior two arches were smaller in \textit{yellowman} embryos.
than in wild type siblings but almost recovered by the sixth day. The defects to the position and shape of the elements in yellowman embryos was consistent with the effect of a delay to jaw cartilage differentiation.

The elements of the anterior arches, the mandibular and the hyoid, and the anterior neurocranium elements, the ethmoid plate and trabeculae, were affected in yellowman embryos. There have been previous examples of mutants which specifically affect the first two arches (Piotrowski et al 1996) and it has been proposed that the patterning of the first two arches is linked (Rijli et al 1993). Furthermore, the anterior elements of the neurocranium are often affected in jaw mutants (Schilling et al 1996) and are neural crest derived, whereas posterior elements of the neurocranium are mesodermally derived (Noden 1988, LeDouarin 1982) and not affected by yellowman mutation. Therefore, the yellowman jaw phenotype is restricted to anterior branchial arches and neurocranium.

It is not unprecedented for pigmentation mutants to affect cartilage, as in low mutants (Schilling et al 1996), and fate mapping suggests the cartilage and pigmentation cells have a similar origin (Schilling and Kimmel 1994). Jaw mutants have also been found which affect the pectoral fin, as in yellowman, such as the jaw mutant dak (Schilling et al 1996, van Eden et al 1996). Thus, yellowman may have a general role in differentiation of a subset of neural crest derivatives.

yellowman mutant embryos did not develop the cartilaginous leptodotrichia normally found in the pectoral fins by 6 days. A number of other mutants which affect branchial arch development and pectoral fin development have been characterised, including hammerhead (ham; van Eden et al 1996, Schilling et al 1996). The yellowman gene is probably needed for differentiation of the neural crest derivatives in the pectoral fin, such as the lepidotrichia, in the same way as yellowman seems to be needed for neural crest differentiation in other tissues.

### 6.3.3 Ear phenotype

yellowman mutants have smaller posterior otoliths and some other pigment mutants, such as pus and cts, also affect inner ear development and have small ears and otoliths (Whitfield et al 1996). More generally, neural crest abnormalities often correlate with inner ear defects which may be due to an interaction between neural crest mesenchyme and otic epithelium or because genes act in both tissues. The neural crest have been shown to contribute to the cartilaginous otic capsule (Noden 1988) and mouse mutants at the W and SI loci affect melanocyte and cochlea development (Steel and Brown 1994). Therefore, the ear phenotype in yellowman may be due to a defect in differentiation of a sub-population of neural crest derivatives.

### 6.3.4 Touch response phenotype
A number of mutants have been identified which affect sensitivity, including \textit{tdo}, which has a transient reduction to tactile stimuli (Granato et al 1996). In the head, a subset of trigeminal ganglion cells and in the trunk and tail, Rohon-Beard cells in the dorsal part of spinal cord, initially mediate the response to touch (Kimmel et al 1991, Metcalfe et al 1990). During later stages, the Rohon-Beard cells die by apoptosis (J. Williams, N. Holder and S.W. Wilson, unpublished data) and tactile sensitivity is provided by neural crest derived dorsal root ganglia. These sensory inputs activate a specific class of interneuron which in turn excite motor neurons to complete the reflex circuitry (Sillar and Roberts 1988). In wild type embryos, the transition between Rohon-Beard and dorsal root ganglia may cause the dip in tactile sensitivity at 4 days. However, in \textit{yellowman} embryos there is a greater reduction to sensitivity at 4 days than happens in wild type embryos which may be because the Rohon-Beard cells die before the dorsal root ganglia have differentiated in \textit{yellowman} embryos. Once the dorsal root ganglion cells differentiate in \textit{yellowman} embryos, the touch response phenotype is lost. To determine if the transient reduction of tactile sensitivity in \textit{yellowman} embryos is caused by the delayed differentiation of the dorsal root ganglia, the development of axons by the dorsal root ganglia and the timing of apoptosis in the Rohon-Beard cells needs to be examined.

\textbf{6.3.5 Future directions}

The phenotype of \textit{yellowman} embryos suggests \textit{yellowman} is required for the differentiation of a subset of neural crest derivatives. The reason that so many different types of tissue are affected may be because they share a common lineage which is disrupted. Alternatively, fate mapping suggests neural crest lineages arise early (Schilling and Kimmel 1994), so the cells may experience a similar environment which is itself disturbed and affects their differentiation. To determine if \textit{yellowman} affects intrinsic or extrinsic events, transplants of labelled neural crest between mutant and wild type embryos could be carried out. Such experiments would show if the melanophore phenotype was rescued by a wild type environment. Furthermore, complementation testing between \textit{yellowman} and other mutants, particularly \textit{tdo}, will show if \textit{yellowman} is a previously described mutant.

A study of the shared features between neural crest mutants identified by random mutagenesis screens, such as \textit{yellowman}, is likely to be of great benefit in understanding how these genes affect multiple steps in neural crest determination.
CHAPTER 7

GENERAL DISCUSSION

In this study, I set out to examine the regionalisation of the zebrafish anterior neural plate. The investigation examined the expression of molecular markers to determine when regions were first established and tissue transplantation, RNA overexpression and mutant analysis examined the mechanisms that regulate these regions. Although this study focused on zebrafish, previous studies suggest similar regions are present in the early neural plates of other vertebrates, such as *Xenopus* (reviewed in Eagleson 1996). The results of my analysis supports a model of neural plate patterning in which an initial pattern at early gastrulation is refined by signals from within and outside the neural plate during late gastrulation.

7.1 There are two phases of gene expression in the rostral neural plate

My study of the rostral neural plate suggests the neural plate is patterned in at least two phases. In the first phase during early gastrulation, at least three AP boundaries are established in the neural plate which define broad regions. My tissue transplantation experiments support previous studies (Koshida et al 1998) which suggest early gastrulation AP boundaries are established by a prepattern within the epiblast. Analysis of gene expression patterns suggests DV pattern is not present in the rostral neural plate at early gastrulation. In the second phase of gene expression at late gastrulation, the regions defined by the early gastrulation AP boundaries are subdivided. In addition, DV pattern begins to be established during late gastrulation with the expression of dorsally restricted genes, such as *flh*, and ventrally restricted genes such, as *shh*.

7.2 Brain region specific genes are expressed from late gastrulation

During the second phase of gene expression, markers of the prospective midbrain, such as *pax2.1*, start to be expressed. Examination of gene expression suggested the region of the neural plate containing the prospective midbrain territory, expands from a small region of the neural plate along its AP axis from the end of gastrulation. Analysis of cell division and previous fate mapping studies of the zebrafish neural plate (Woo and Fraser 1995) suggest that neither cell division nor cell migration drives the expansion of the prospective midbrain territory. Previous studies have suggested the midbrain is induced by an interaction between the forebrain and the hindbrain (Nieuwkoop and Albers 1990). In addition, it has been shown that the midbrain organiser, the isthmus, can
induce genes, such as en2 (Crossley et al 1996), which are normally induced at late gastrulation. The isthmus is an AP boundary in the rostral neural plate (Crossley et al 1996). Thus, the finding that the prospective midbrain territory expands and midbrain specific gene expression begins from late gastrulation is consistent with the induction of the midbrain happening at late gastrulation.

Telencephalon specific genes are expressed during the second phase of gene expression. The anterior organiser, Row 1, appears to be required for telencephalon gene expression (Houart et al 1998), and thus Row 1 may act during late gastrulation. For instance, it has been shown that Row 1 can ectopically induce genes normally expressed at late gastrulation, such as dlx2, and that ablation of Row 1 abolishes the expression of telencephalic specific genes, such as enxl but does not affect the gross morphology of the forebrain. Thus, Row 1 may act as part of a second phase of gene expression to induce telencephalic markers but not early AP markers of the neural plate. The Row 1 organising centre is located at a boundary which is thought to be important sources of signals during neural plate regionalisation (Ruiz i Altaba 1998).

The expression of eye specific genes, such as rxB and LH2A, did not occur until late gastrulation during the second phase of gene expression. When expression was initiated, eye specific genes were expressed within the most rostral territory defined during early gastrulation and, along with telencephalic specific genes, subdivided the region. Thus, brain region specific gene expression in the neural plate did not commence until late gastrulation.

7.3 DV pattern is established by late gastrulation

DV pattern appears to be established at late gastrulation during the second wave of gene expression. Perhaps DV patterning information emanates from the midline mesoderm and non-neural ectoderm. The division of the most rostral region of the early gastrulation neural plate into telencephalon and retina may be caused by the acquisition of DV pattern. DV patterning information is likely to emanate from the midline mesoderm and non-neural ectoderm. The midline mesoderm may also be involved in AP patterning.

7.4 The midline and anterior organiser may refine the AP pattern in the neural plate

The midline mesoderm may have a role in defining the AP pattern in the neural plate at late gastrulation. The finding that the midline mutants oep and cyc have an almost normal AP pattern without the rostral midline mesoderm and that null shh mutant mice have a normal AP pattern (Chiang et al 1996) suggests the midline mesoderm is not needed to establish anteroposterior pattern. However, it has been shown that the mesoderm can
induce anterior neural plate AP markers, such as \textit{en}1 and \textit{en}2 (Ang and Rossant 1993), and that the posterior mesoderm suppresses anterior gene expression (Ang et al 1994). In addition, the finding that \textit{oep} and \textit{cyc} have small alterations to the downregulation of \textit{anf} expression, suggests some involvement of the midline mesoderm in AP patterning. Perhaps the midline mesoderm mediates refinement of the AP pattern posterior to the mid-diencephalon and the anterior organiser, Row 1, regulates the refinement of anteroposterior pattern rostral to the mid-diencephalon. Such a regional difference in how AP pattern within the neural plate is refined could explain why the midbrain organiser, the isthmus, is unable to induce late phase midbrain markers, such as \textit{En}1, rostral to the mid-diencephalon (Crossley et al 1996). Therefore, the midline is likely to play roles in establishing DV and refining AP neural plate patterning.

7.5 The neural plate behaves as an embryonic field

My studies of neural plate patterning supports the suggestion that the neural plate is organised as an embryonic field (Ruiz i Altaba 1998, Meinhardt 1983). Meinhardt proposed that the boundaries established by the primary organisation of the developing embryo act as patterning centres for secondary embryonic fields (Meinhardt 1983). The initial patterning leads to the specification of different populations of cells in adjacent territories which are separated by sharp borders. These two populations co-operate to produce a signal which provides more detailed secondary patterning. This idea of borders as sources of patterning information has received support from studies of embryonic segmentation (Ingham and Martinez-Arias 1992), wing and leg imaginal disc development in \textit{Drosophila} (Basler and Struhl 1994, Diaz-Benjumea et al 1994) and the vertebrate midbrain (Crossley et al 1996, reviewed by Ang 1996).

Therefore, in the neural plate, the primary organisation of the neural plate by a prepattern in the epiblast which confers a general anteroposterior pattern to the neural plate. A secondary level of neural plate organisation is due to signals emanating from boundaries and adjacent tissues, such as the non-neural ectoderm and the underlying midline mesoderm. Thus, the regional character of the neural plate is gradually imposed with an early gastrulation phase and at least one subsequent phase at late gastrulation. Recent studies of primitive chordates provide clues about how the regional organisation of the neural plate has arisen during evolution.

7.6 Evolution of neural plate patterning

It has been suggested that the earliest sites of specific gene expression will be those associated with the oldest evolutionary role (Cooke et al 1997). For instance, the axial organisation of the amphibian dorsal blastopore lip organiser occurs before the
subdivision of the brain rudiment (Cooke et al 1997). Although ontogeny does not
simply recapitulate phylogeny, during development general features are thought to arise
before specific ones (Butler and Hodos 1996). Thus, it is interesting to speculate that the
initial anteroposterior subdivisions of the rostral neural plate at early gastrulation in the
zebrafish may represent a more general level of organisation which may have evolved
before the features which arise during late gastrulation.

It is possible that neural plate regionalisation has evolved with the imposition of
secondary levels of patterning onto an initial organisation. For instance, gene expression
suggests that the telencephalon, and perhaps also the midbrain, arise during a second
phase of gene expression so it would be interesting if either, or both, the midbrain and
telencephalon, were absent from a vertebrate mutant or ancestor. The mbl mutation
causes the loss of the telencephalon because the rostral neural plate adopts a diencephalic
identity (Masai et al 1997). In noi mutant embryos, the midbrain is absent and gene
expression indicates that the midbrain is induced but not maintained (Brand et al 1996).
Thus, there are mutants which specifically affect the development of regions, such as the
telencephalon or midbrain, which apparently form during the second phase of gene
expression.

The rostral neural plate of ancestral vertebrates appears to lack some structures
associated with the second phase of gene expression. For instance, the forebrain of
amphioxus, which is thought to be homologous to the diencephalon of craniate
vertebrates, may lack the telencephalon and, perhaps also the midbrain (Lacalli et al 1994,
Holland and Holland 1998). However, only a relatively small number of genes have been
studied in the amphioxus forebrain which makes it difficult to draw strong conclusions
about what homologous structures are present or absent. Nevertheless, it would be
interesting if it could be shown that a primitive vertebrate, such as amphioxus, or a
primitive chordate, such as the ascidian, lacked features associated with the second phase
of gene expression. Such a finding would indicate these features arose independently of
the features associated the first phase, such as the diencephalon.

7.7 Future directions

The regions defined by gene expression during early gastrulation may represent
cell lineage restrictions. To determine if cells within a region behave the same way
individual cells, or small groups of cells, could be transplanted to ectopic locations within
the same region and to other regions, to see if the cells share the same identity. The
expression of region specific markers, such as anf or otx2, could be used to assess identity
after transplantation. These experiments would show when the cells within a region
become specified and if there is a restriction to cell fate in these early regions. Studies of
the chick neural plate show there is a boundary in the mid-diencephalon rostral to which
midbrain cannot be ectopically induced (Crossley et al 1996). These experiments would need to be carried out during early gastrulation before the second phase genes are expressed.

As boundaries are thought to be important sources of patterning information (Meinhardt 1983, Ruiz i Altaba 1998), it would be interesting to determine if the early AP boundaries have any signalling ability. This could be tested by transplanting the boundary cells to an ectopic location in the neural plate and studying whether they induce changes to gene expression. The method used in recent studies of eph signalling in the zebrafish hindbrain (Xu et al 1999, Mellitzer et al 1999) could be used. In these studies, mosaic expression of ephs was used to create ectopic boundaries. In a similar way, expression of early neural plate markers, such as anf, could be used to impose ectopic boundaries in the neural plate and the effect of these analysed.

Analysis of mutants in this study provide contrasting but complementary examples of why mutant analysis is a powerful tool for understanding developmental biology. On the one hand, the characterisation of the anfless line showed that gene expression is not a reliable guide to function and that gene redundancy is likely to feature in zebrafish as in other vertebrates. yellowman shows that mutants are useful in identifying the steps involved in the development of tissues which are likely to involve a complex interaction of many genes. A screen for zebrafish mutants which affect specific early neural plate boundaries could be carried out. The isolation of a mutation that causes the loss of a particular boundary would be useful for analysing how that boundary affects regionalisation.

Thus, a number of experiments could be conducted to determine the significance of neural plate boundaries which arise during early and late gastrulation. A combination of approaches, using methods such as gene expression analysis, fate mapping and commitment analysis, is likely to be needed in a single organism at several stages for a fuller understanding of rostral neural plate regionalisation.
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