Mechanisms of patterning and neurogenesis in the zebrafish forebrain

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Abstract

The vertebrate forebrain is a highly complex structure containing millions of neurons that form highly ordered and precise connections. The mechanisms that give rise to this complexity are slowly being elucidated through work on a number of developmental systems. I have investigated the organisation of the zebrafish forebrain and the mechanisms by which a simple neuroepithelial sheet becomes patterned to the more complex adult forebrain structure. Initially, I examined the development of the zebrafish CNS during later stages of development (18 somites stage to 5 days postfertilisation) through studies on gene expression, axon pathfinding, morphology and cell proliferation. Using the prosomeric model as a basis, we can begin to understand how the regions demarcated by genes, such as Dlx2 and Emx1, relate to similar regions in other organisms, such as mice, chickens and turtles.

In order to investigate the how different genes influence forebrain development, I conducted misexpression studies and have characterised zebrafish mutants. Analysis of the acerebellar mutant reveals that Ace/Fgf8.1 is responsible for correct specification of, and axon pathfinding within, the midline optic stalk territory. Furthermore Ace/Fgf8.1 is also responsible for neuronal differentiation in the dorsal forebrain and for correct specification of the olfactory bulb. Analysis of zebrafish embryos with mutations in both Ace/Fgf8.1 and Syx/Shh reveals that although patterning of the forebrain is not more severely affected in the double mutant, the growth of the ventral forebrain is greatly reduced. In zebrafish, the members of the Emx gene family are the earliest known markers for the presumptive telencephalon and loss of function studies in Drosophila and mice suggest an important role for Emx genes in development of anterior structures. To further investigate potential roles for Emx genes in forebrain development, the zebrafish homologues of Emx genes were misexpressed. However ectopic expression of Emx genes does not appear to have the capacity to promote ectopic telencephalic structures. In conclusion, my study has started to reveal how the zebrafish forebrain develops and the genes involved in growth, patterning, neurogenesis and axon pathfinding in the forebrain.
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Chapter One

Introduction

In order to understand how the vast array of vertebrate neuronal cell types utilise and influence their environment to co-ordinate virtually all physiological processes, neurobiologists are increasingly studying the mechanisms responsible for specification of these diverse cell types. Of the different central nervous system (CNS) subdivisions, the adult forebrain has been the focus of intense study and yet our knowledge about its early development remains poor. Recent advances in our understanding of forebrain development have come from studies performed in a number of different organisms, including the zebrafish. Although the zebrafish is well established as a model developmental organism (Eisen, 1996), some aspects of its development have yet to be well described. In this study, I initially characterised zebrafish telencephalic development and subsequently investigated mechanisms which may contribute to the patterning of the zebrafish forebrain. Following a brief overview of CNS development, I will address the issues of forebrain segmentation, patterning and early axon pathfinding in separate sections of this chapter.

1.1. Overview of the formation of the brain

1.1.1. Early development of the CNS.

The CNS develops from the neural plate, an ectodermal layer of pseudostratified epithelium, present during early gastrulation. The neural plate is a transient structure which has antero-posterior and dorso-ventral polarity but is initially morphologically uniform.

The neural plate condenses to form the neural tube through the process of neurulation. In most vertebrates, the lateral edges of the neural plate thicken and form neural folds, which become raised above the surface of the rest of the neural plate. A U-shaped groove in the centre of the neural plate forms rostrally and spreads caudally (Schoenwolf and Smith, 1990). The combination of cell movement events which orchestrate the formation of the folds and groove eventually lead to the fusion of the neural folds, separation from adjacent non neural ectoderm and formation of a hollow neural tube. In the zebrafish, the process of neurulation occurs through cavitation of a thickened neural keel but the outcome is the same (Papan and Campos-Ortega, 1994).
<table>
<thead>
<tr>
<th>Structure</th>
<th>Subdivision</th>
<th>Components of subdivision</th>
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<tbody>
<tr>
<td>Pallium</td>
<td>limbic pallium</td>
<td>hippocampus, dentate gyrus, Ammon's horn</td>
<td>memory, emotion, learning</td>
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<td></td>
<td>dorsal pallium</td>
<td>neocortex</td>
<td>sensory processing, motor control</td>
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<tr>
<td></td>
<td>lateral pallium</td>
<td>olfactory (piriform) cortex</td>
<td>olfactory processing</td>
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<tr>
<td>?</td>
<td></td>
<td>amygdala, claustrum</td>
<td>limbic processing, including fear</td>
</tr>
<tr>
<td>Sub-pallium</td>
<td>striatum</td>
<td>striatum, pallidum</td>
<td>motor responses, planning, initiation and execution of movement</td>
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<tr>
<td></td>
<td>septum</td>
<td>septum</td>
<td>limbic processing</td>
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**Table1.1. Brief summary of structure and function of the rodent telencephalon**

The telencephalon can be broadly divided into pallium and sub-pallium structures, which correlate to dorsal and ventral divisions respectively. Pallium and sub-pallium structures can be further delineated into more specific areas, with specific functions. The amygdala and claustrum can not be clearly defined as pallial or sub-pallial structures, as the origins of these structures have not been clearly established.
After neurulation, the neural tube starts to become divided into morphologically distinct regions and the overlying neural crest migrates. The neural tube develops several ring like constrictions which demarcate the major divisions of the brain, the forebrain, midbrain and hindbrain. The forebrain is composed of the telencephalon and diencephalon. In most vertebrates, the telencephalon is further subdivided into the dorsal pallium and ventral sub-pallium. The rodent telencephalon is one of the most extensively described structures with the regions well defined by cytoarchitecture and function. The components of the telencephalon and their associated functions are illustrated in Table1.1.

1.1.2. Morphogenetic events during the development of the teleosts telencephalon.

In the zebrafish, the adult telencephalon is broadly subdivide into the area dorsalis and area ventralis (Wulliman et al., 1995). Comparative neuroanatomy between brains of different species from different vertebrate taxa has advanced our understanding of the overall phylogeny of vertebrates and helped assign homologies to telencephalic structures of different species. However such comparative neuroanatomy is hampered in zebrafish due to a significant difference in teleostan and mammalian telencephalic development.

In mammals and many other amniotes, the telencephalon undergoes a process called evagination. During evagination, the dorsal telencephalon expands exponentially and bulges outwards. The central lumen also enlarges to form the lateral telencephalic ventricles. The end result of evagination is that structures, which were originally positioned dorsally are placed in the medial most part of the telencephalon (Fig1.1A,B). Other displacements are also caused by evagination, for example the most ventral palliai area coming to lie most laterally to give rise to the olfactory pallium (Fig1.1A).

In zebrafish and other teleosts, the telencephalon undergoes the process of eversion. The roof of the telencephalon elongates and bends over the rest of the forebrain (Fig1.1B). Following eversion, structures originating from dorsal neural tube lie at the lateral most extent of the telencephalon. The intermediate part of the pallium comes to lie dorsally and ventral most pallial structure become positioned as the medial most structure (Butler and Hodos, 1996). The timing and processes involved in eversion are unknown in the zebrafish.

1.2. The role of segmentation in forebrain development
1.0 Introduction

**Fig 1.1. Different developmental processes in the teleost and tetrapod telencephalon**
(A) In most groups of vertebrates, the telencephalon develops through the process of evagination. During evagination, the dorsal telencephalon expands and the central lumen expands to form the lateral ventricles. Consequently dorsal most structures become medial and ventral most pallial structures become the lateral most telencephalic structures in the adult. (B) In teleosts, the telencephalon undergoes eversion during which the dorsal telencephalon thins and elongates. Thus the dorsal most structures in the neural tube lie at the lateral most extent of the adult brain. Additionally the lateral ventricle does not form. Subdivisions a-e refer to different positions along the dorso-ventral axis. After evagination, a becomes part of the limbic pallium, b, neocortex, c, the olfactory cortex, d, striatum and e, septum. In teleosts, a, b becomes the area dorsalis and d, e, the area ventralis. c contributes to parts of the area dorsalis and area ventralis.

Adapted from Butler and Hodos, 1996.
Evagination

Eversion
1.0 Introduction

The presence of constrictions, which delineate the major divisions indicate that the CNS is divided into segments from an early stage. The further segmentation of the hindbrain, as visualised by the series of bulges known as rhombomeres, suggests that segmentation may play an important role in the development of other regions of the CNS, such as the forebrain (Lumsden and Krumlauf, 1996). The study of forebrain segmentation has also led to the discussion and examination of the evolutionary links between vertebrate and invertebrate segmentation.

The advent of developmental genetics has allowed the study of many distinct processes at the molecular level. The importance of segmentation was underlined by mutagenesis screens in Drosophila, which revealed the hierarchy of transcription factors and signalling proteins involved in establishing the embryonic bauplan (Lawrence, 1990).

1.2.1. Establishment of segment identity in Drosophila embryos.

The body of adult Drosophila displays the characteristic segmental organisation of insects. These segments are specified by the action of a number of different classes of genes, which interpret the gradients of maternal genes, like bicoid (St Johnstone and Nüsslein-Volhard, 1992). Bicoid acts as a true morphogen, as activation of different gap genes depends on the amount of bicoid protein present in a domain. The zygotic gap genes, in turn, act to further regionalise the embryo by activating the pair rule genes. The gap genes also act upon each other to refine their expression domains and therefore the eventual embryonic segments.

At this point of Drosophila development, segmentation becomes apparent through the expression of genes. The gap genes act in combination to activate pair-rule gene expression in seven transverse stripes along the antero-posterior (AP) axis. The pair-rule genes, such as fushi-tarazu and even-skipped, divide the embryo into 14 parasegments. The parasegments are also visible through the appearance of transient grooves on the surface of the embryos after gastrulation. Regionalisation of the embryos, in the AP axis, continues through the activation of the segment polarity genes. The segment polarity genes are responsible for fixing parasegment boundaries and the final larval segment boundaries.

Although the Drosophila embryos is segmented, the same genes are expressed in each of the different segments along the AP axis. The expression of gap genes is transient and therefore no longer defines different AP values. Individual segment identity is established through the combinatorial action of homeotic selector (HOM) genes (Lawrence
1.0 Introduction

and Morata, 1994). The HOM genes dictate the future development of each segment by controlling the activation of other genes throughout development. Classically HOM genes are defined by their cluster like sequence organisation on chromosomes and by functional studies. Loss of parasegment identity is observed upon loss of a homeotic gene function and transformation of parasegment identity when the gene is misexpressed.

1.2.2. Segmental organisation and development of the Drosophila head.

The larval Drosophila head also shows segmental organisation at the extended germ band stage. The head can be divided into 6 different segments but the three more posterior segments are thought to be specialised trunk derivatives (Fig 1.2; Cohen and Jürgens, 1991). Three gap genes have been identified, which are under the control of maternal genes. The three gap genes, orthodenticle (otd), empty spiracles (ems) and buttonhead (btd), are expressed in overlapping domains in the larval head. Mutations in these genes leads to loss of groups of adjacent segmental primordia with the corresponding loss of segment polarity genes, such as engrailed and wingless (Cohen and Jürgens, 1990).

In Drosophila and Tribolium, segmental organisation of the head and trunk differ in one important aspect. In neither species have HOM genes been isolated, which are responsible for establishing head segment identity. However the expression domains and mutant phenotypes of cephalic gap gene mutants suggest they may initiate and maintain head segment identity.

In order to explain the differences in head and trunk segmentation, Cohen and Jürgens (1990) proposed the combinatorial model for head development in Drosophila. The model states that the specification of cephalic segment identity occurs through the combinatorial expression of the gap genes. Thus the cephalic gap genes would accomplish the functions of both trunk gap genes and homeotic selector genes. In support for this model, ems and otd are both homeobox containing transcription factors, like the homeotic selector genes. Although the loss of function studies support the combinatorial model, gain of function experiments with otd and btd do not achieve the transformation of cephalic segment identity predicted by the model (Wimmer et al., 1997; Gallitano-Mendel and Finkelstein, 1998).

1.2.3. Segmentation of the vertebrate forebrain.
Fig 1.2. Segmentation of the *Drosophila* head

(A) The *Drosophila* head is clearly segmented at the extended germ band stage. There are three head segments, the labral (LR), antennal (AN) and intercalary (IC). The three posterior segments, the mandibular (MD), maxillary (MX) and labial (LI), are thought to be specialised trunk segments. The black spots in the segments indicate the anlagen of sense organs. (B) A schematic representation of gene expression and head segment identity reveals that the cephalic gap genes are expressed in overlapping territories in the head segment primordia. The cephalic gap genes are *orthodenticle* (*otd*), *empty spiracles* (*ems*) and *buttonhead* (*btd*). The gap genes are directly under control of the maternal gene, *bicoid*. The acron and labral differ from the other head segments as they require *tor* activity for correct formation. The ? denotes the preantennal area (PC), which may be a cryptic cephalic segment. The expression of *hunchback*(*hb*) and subsequently homeotic genes indicate that the posterior segments are derived from special trunk segments.

Adapted from Cohens and Jurgens, 1991.
The importance of segmental organisation in vertebrate embryonic development is revealed by the formation of somites and rhombomeres, soon after gastrulation has been completed. In the CNS, rhombomeric subdivisions of the hindbrain display all the features commonly associated with segments (Guthrie, 1995; Papalopulu, 1995). The idea of compartmentalisation underlying embryonic development was investigated by anatomists in the last century. Recently, the criteria for segmentation has been revised to include the role of genes in development (Lumsden and Keynes, 1989) and are as follows. First, segments should have reiterative patterns of cellular or molecular differentiation. Second, each segment should have a pattern of proliferation, which would correspond to particular patterns of neurogenesis within the CNS. Third, segments should have cell lineage restrictions, such that cells from segments do not mix. Finally, restricted gene expression must occur within each segment.

Two general models for forebrain segmentation have been proposed and debated amongst several authors. The columnar model was proposed by His, Herrick and Kuhlenbeck, whilst the neuromeric model was developed by Rendahl, Bergquist, Kallen, Vaage and others (Vaage, 1969; Kuhlenbeck, 1973; Puelles, 1987).

1.2.4. The Columnar Model.

In the His-Herrick columnar model, the CNS is divided into longitudinal columns which are most obvious in caudal regions. In the hindbrain and spinal cord there 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. The alar plate is the sensory (afferent) zone and the basal plate the motor (efferent) zone of the CNS (Heijdra and Niewenhuys, 1994).

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 (Puelles, 1987) or only as far as the midbrain. The His-Herrick model suggests that the diencephalon is divided by three sulci into four longitudinal zones and the telencephalon subdivided by two sulci into three longitudinal zones (Kuhlenbeck, 1973). The uncertainty over the arrangement of longitudinal divisions has led to criticism of the columnar model.
A major criticism of the His-Herrick model stems from the fact that 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 rostro-caudal levels (Northcutt, 1995). The use of sulci to delineate divisions is also controversial, as sulci do not appear to be reliable markers for histogenic boundaries (Northcutt and Butler, 1993).

1.2.5. Neuromeric models.

The neuromeric model suggests that the brain is subdivided by transverse furrows along the rostrocaudal axis into segments called neuromeres. 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 and Keynes, 1989). These rhombomeres are transient developmental structures, which were previously thought to be artefacts of no functional importance. However, the identification of serially repeated brainstem neurons (Trevorrow et al., 1990) and of the rhombomeric origin of branchiomeric cranial nerves (Gilland and Baker, 1993) led to the recognition that the rhombomeres are important developmental structures (Lumsden and Krumlauf, 1996).

In fulfilment of the criteria for true segmental organisation, rhombomeres have been shown to be sites of organised cell proliferation (Guthrie et al., 1991; Wulliman and Puelles, 1999), to be sites of cell migration (Puelles, 1987; Figdor and Stern, 1993), have boundaries that restrict cell mixing (Fraser et al., 1990) and specific gene expression (Wilkinson et al., 1990). Rhombomeres may acquire their distinct characteristics through rhombomere specific cell adhesion, potentially mediated by Eph signalling (Cooke et al., 1999).

Segmental organisation of the forebrain has been mostly studied in rodents and most of the following data presented will relate to how the rodent forebrain is thought to be regionalised into neuromeres. Initially it was suggested that there were three prosomeres in the forebrain, corresponding to the telencephalon, diencephalon and pretectum. Subsequent models sub-divided the telencephalon (the secondary prosencephalon) into three prosomeres. The most recent prosomeric neuromeric model distinguishes six neuromeres, otherwise known as prosomeres, in the forebrain (Puelles and Rubenstein, 1993). The diencephalon is divided into six prosomeres (p1-6), of which the last three also extend into the telencephalon (Fig1.3A). Alternative neuromeric models of the forebrain have also been proposed (Figdor and Stern, 1993) but currently the prosomeric model championed by Puelles and Rubenstein is the most favoured forebrain segmentation model. In the following sections, I will analyse the prosomeric model using the segmental criteria described above.
Fig1.3. Different models for the segmental organisation of the forebrain

A schematic diagram illustrating the positions of the six prosomeres, as proposed by Puelles, Rubenstein and colleagues, along with the gene expression boundaries in a E10.5 mouse embryonic brain. The provisional transverse and longitudinal boundaries are drawn in thick black lines and the six genes shown are *Dlx2, Gbx2, Nkx2.1, Nkx2.2, Otx2* and *Shh*. The positions of the neuromeres in the midbrain and hindbrain are also shown. In comparison, Figdor and Stern describe the position of four neuromeres shown in (B). The neuromeres described were delineated using morphology, cell lineage restriction and gene expression. (C) In the zebrafish, neuromeres have been proposed to be organised in a radial, rather than transverse, fashion. The radial organisation is particularly striking in the telencephalon. Abbreviations: a, alar plate, b, basal plate, fp, floor plate, m, mesencephalon-midbrain, p, prosomere, r, rhombomere, rp, roof plate, os, optic stalk.

1.2.6. Morphological boundaries in the vertebrate forebrain.

The revised prosomeric model suggested by Puelles and Rubenstein differed from (A) previous models, including the columnar model, in that the bending of the longitudinal axis in the diencephalic territory is taken into account when subdivisions are designated. The classical view that the telencephalon is the anterior most structure in the CNS is challenged by recent observations regarding the flexure of the CNS (Ross et al., 1992; Puelles and Rubenstein, 1993; Fernandez et al., 1998). Correcting for the flexure results in the telencephalon becoming a dorsal structure and parts of the telencephalon and hypothalamus becoming the most anterior brain structures. In contrast to the use of sulci in the columnar model, the revised prosomere model uses constrictions in the wall of the neural tube. These constrictions are similar to those observed at rhombomere boundaries in the hindbrain, appear before sulci and are characterised by an intraventricular ridge and external furrow.

Comparison of the prosomere model to the adult brain indicates how the prosomeres relate to adult structures (Bulfone et al., 1993, Papalopulu, 1995). The telencephalon and hypothalamus would develop from p4-6, the ventral thalamus from p3-6, the dorsal thalamus and epithalamus from p2 and the pretectum would occupy p1.

The longitudinal subdivisions of the forebrain, in the new prosomeric model, also take into account the flexure of the brain. However the positions of the roof, floor, alar and basal plates can not be easily distinguished by using morphological markers and researchers are dependent on gene expression domains to distinguish these territories (see later). However the transverse boundaries of the designated prosomeres do coincide with constrictions in the neural tube (Bulfone et al., 1993, Puelles and Rubenstein, 1993). Perhaps the most prominent constriction lies at the p2/p3 boundary. This boundary coincides with the zona limitans intrathalamica (ZLI), a structure which has been identified as a boundary between segments in a number of models. The position and prominence of the ZLI suggests it may play an important role during development. Although constrictions have been noted for the telencephalic boundaries, the existence and positions of these constrictions remains uncertain (Bulfone et al., 1993, Puelles, pers. comm.).

1.2.7. Cell lineage restrictions and prosomeres.
Maintenance of segment identity would be expected to occur through restrictions on cell mixing between neighbouring units. Unrestrained cell mixing would complicate the mechanisms required for specifying segment identity and may even challenge the integrity of individual segments. Numerous cell lineage and migration studies have been performed to address the issues of cell commitment to segment identity.

Figdor and Stern (1993) studied cell mixing in the diencephalon through fluorescent labelling experiments. If cells were labelled before neuromeres were formed, fluorescent cells were often discovered in other neuromeres but neuromere boundaries were respected if cells were labelled after neuromere formation. Figdor and Stern (1993) further analysed the diencephalon using cell lineage, morphological and gene expression studies. From their results, they conclude that the chick diencephalon is divided into four neuromeres (Fig.1.3B).

One boundary that both Rubenstein and colleagues (1994) and Figdor and Stern observe is the ZLI, which separates the dorsal and ventral hypothalamus. The recognition of this structure as important across species and neuromere models further suggests that it forms an important developmental landmark (Puelles et al., 1987).

In the telencephalon, grafts and cell labelling experiments have been used to address the issue of cell lineage restrictions. By introducing cells into the cerebral vesicles, Fishell and colleagues have described how cell lineage restrictions depend mainly on the age of the embryo (Fishell, 1995; Campbell et al., 1995; Brüstle et al., 1995). In these experiments, researchers transplanted cells from one telencephalic territory to another and assayed lineage restriction through morphology, host specific gene expression and axonal projection. Lineage restrictions appear to depend on age of the transplanted tissue. The older the transplanted cells are, the less likely that they are to form host derivatives. However according to these studies lineage restrictions only apply after neuromeric boundaries have become difficult to distinguish. A recent study of early cell identity suggests that cells maybe restricted in the anteroposterior axis, but not the dorsoventral, from an early age (Na et al., 1998). Such a restriction would support the identity and position of the transverse neuromeres but casts further doubt on the identity of longitudinal telencephalic domains.

Cell labelling experiments have also highlighted apparent discrepancies in telencephalic neuromeric boundaries (O'Rourke et al., 1997; Anderson et al., 1997). Putative segment boundaries in the telencephalon would be expected to be populated by cells from a specific area and restrict migration within its boundaries. DiI labelling has described how post mitotic cells migrate tangentially in the cortical ventricular zone to
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populate all parts of the rat pallium (O'Rourke et al., 1997). Such migration would not be expected if longitudinal and transverse domains existed in the telencephalon. In addition to migration within the anteroposterior (AP) and dorsoventral (DV) pallium, Anderson and co-workers describe migration of GABA-ergic precursor cells from lateral ganglionic eminence to the neocortex (1997).

Although these studies would suggest that cell lineage restriction boundaries do not play a significant role in forebrain development, two explanations for the apparent failure of the neuromere model have been offered. One possibility is that migration of precursors between neuromeres follows a distinct differentiation route, which includes the tangential migration of post mitotic neurons to different forebrain regions (Anderson et al., 1997). In this way, the integrity of the neuromeres would not be significantly compromised. Other observers indicate that cell mixing occurs between the well described neuromeres of the hindbrain (Birgbauer and Fraser, 1994), suggesting that restrictions upon cell mixing does not form an integral part of segmental identity.

1.2.8. Proliferation and neurogenesis in the vertebrate forebrain.

Another essential feature of an individual segment is the localised pattern of proliferation. In the CNS, zones of proliferation are often associated with neurogenic domains with the early neurogenic pathway being activated in mitotic cells (Edlund and Jessell, 1999). Discontinuities in proliferation also underlie many of the distinct morphologies observed in the CNS (Puelles et al., 1987).

Curiously few studies have addressed how proliferative zones relate to the proposed forebrain prosomeres. In mice the prosomeres become more difficult to distinguish during late development (E12-17) and it is only at these late stages that distinct proliferative zones appear. Therefore comparative studies have been difficult to perform (Fishell, 1997). In a recent study of the proliferation in 5 days postfertilisation zebrafish larval, Wulliman and Puelles (1999) report a correlation between proliferation patterns and prosomeres. Zones of proliferation can be classed as belonging to either the basal or alar plates. However these classifications can only be assigned to diencephalic structures and telencephalic proliferative zones do not appear to significantly correlate to the proposed p4-p6 prosomeres.

Support for the prosomeric model does come from studies of zonal localisation of neurogenesis. In chick and stickle-back, examination of markers of neuronal cell types reveals patterns of expression which correlate with neuromeres (Figdor and Stern, 1993;
Ekström and Ohlin, 1995). Figdor and Stern (1993) drew different prosomere boundaries, after characterising cell lineage and acetylcholinesterase (AChE) expression in the chick embryo. Diencephalic AChE positive neurons show a periodic pattern in chicks, with strong expression detected in the prosomeres D1 and D3 and weaker expression observed in prosomeres D2 and D4. Although the equivalent analysis of AChE expression in the framework of the revised prosomere model of Puelles and colleagues has not been performed, the periodicity of expression favours a segmental organisation of the forebrain. Examination of GABA-ergic neurons in the stickle-back also corroborate the prosomere model, with clusters of GABA positive neurons present in the alar plates of the p1 to p3 prosomeres (Ekström and Ohlin, 1995). However studies of GABA expression in the telencephalon and hypothalamus do not allow a full investigation of the proposed rostral prosomeres.

The precise nature of branchiomeric projections from specific rhombomeres best illustrates the notion of segment identity and the functional importance of a segment (Trevarrow et al., 1990; Moens et al., 1996). An equivalent examination of axonal projections within the forebrain also reveal an underlying segmental organisation. Indeed Herrick (1936, 1948) investigated such projections in Amblystoma and Necturus forebrains and drew conclusions for segmental organisation of the brain. Re-evaluation of these projections, in the context of the revised prosomeric model, suggests that these projections closely adhere to the described longitudinal subdivisions. In addition when rostral brain commissures turn or branch, they often do so at right angles, which correlate with the transverse boundaries. In mice and zebrafish, analysis of early axonal projections also suggest the presence of neuromeres within the brain (Wilson et al., 1990; Mastick and Easter, 1996).

1.2.9. Gene expression studies define the vertebrate prosomeres.

Reassessment of forebrain segmentation, in light of recent gene expression studies, strongly supports the revised neuromeric model of Puelles, Rubenstein and others (1994). The restricted expression of vertebrate homologues of the Drosophila homeotic selector genes, Hox genes, further supported the rhombomeric organisation of the hindbrain (Wilkinson et al., 1990). The Hox genes are expressed in overlapping domains but differ in the rostral limits of expression, such that Hoxb-1 is expressed within rhombomere 4, whilst Hoxb-2 has a rostral limit at the rhombomere 2-3 boundary. Other genes, such as Krox-20 and members of the Eph family, are also expressed in a restricted domains within the hindbrain. Expression of many of these genes precedes the establishment of morphological
boundaries and this early expression is thought to be vital for conveying segment identity. For example, loss of *Krox-20* or *Kreisler/valentino* results in the loss of rhombomeres r3 and r5 (Sham *et al.*, 1993; Moens *et al.*, 1996).

In the anterior CNS, gene expression domains also appears to delineate individual segments (Puelles and Rubenstein, 1993; Bulfone *et al.*, 1995). Examination of over 30 genes has aided the identification of telencephalic and diencephalic prosomeres. Indeed the positions of some boundaries have been drawn using gene expression as the most important criteria.

1.2.9.1. The floor plate.

In the DV axis, the relative positions of the longitudinal domains have been resolved using gene expression (Puelles, 1995). No consensus has been reached upon the exact position of the floor plate using morphological landmarks but the expression of *Shh, HNF-3β* and *Bmp4* suggests that the floor plate, at early stages, may extend as far anterior as the tuberal region of the hypothalamus (Fig 1.3A). Examination of the same genes suggests the rostral extent of the floor plate would include and/or overlie the prechordal mesoderm.

1.2.9.2. The roof plate.

The rostral tip of the roof plate has been established through fate map experiments (Eagleson *et al.*, 1995) and supported by the expression of genes, such as *Fgf8* and *noggin* (Crossley and Martin, 1995; Shimamura *et al.*, 1995). *Fgf8* is expressed in the anterior margin of the neural plate and continues to be expressed in the anterior limit of the forebrain after neurulation. The expression of *Fgf8* and genes in the roof of the diencephalon, like *Wnt1*, appear to delimit the roof plate of the forebrain (Parr *et al.*, 1993).

1.2.9.3. The alar and basal plates.

The alar and basal plates form the main longitudinal subdivisions of the brain. However histological analysis does not reveal a distinct boundary between the two subdivisions and the sulci used by His have been discredited. Fate mapping studies have been utilised to approximate the position of the boundary (Eagleson *et al.*, 1995). Other useful tools for establishing the boundary have been to analyse the formation of early axonal scaffolds (Wilson *et al.*, 1990; Mastick and Easter, 1996). In particular, the postoptic commissure forms at the expected alar/basal boundary.
In mice and zebrafish, the expression of \textit{nk2.2} approximates the location of the alar/basal boundary (Price, 1993; Barth and Wilson, 1995). After neurulation, \textit{nk2.2} is expressed in the dorsal thalamus, ventral thalamus, optic stalks and pretectum, indicating that it is probably located in the forebrain alar plate. A marked inflexion is also observed at the ZLI (Fig.1.3A,C). Expression of \textit{nk2.2} is detected immediately dorsal to \textit{shh}, in the expected alar/ basal region. \textit{shh} is also expressed at the flexure of the ZLI and regulation of forebrain \textit{nk2.2} expression by \textit{shh} has been demonstrated (Barth and Wilson, 1995). Furthermore \textit{Wnt7a} and \textit{Wnt5a} are expressed with \textit{Shh} in the basal plate of the forebrain (Parr \textit{et al.}, 1993). Rostral to the ZLI, \textit{BF1} and \textit{BF2} are expressed in the alar plate (Tao and Lai, 1992; Hatini \textit{et al.}, 1994). The ventral extent of \textit{BF} and other alar plate genes, such as \textit{Pax3} and \textit{Pax6}, also coincide with the \textit{Nk2.2}-labelled alar/basal boundary (Puelles and Rubenstein, 1993).

1.2.9.4. Transverse prosomere domains.

In addition to longitudinal subdivisions of the forebrain, detailed examination of a number of genes has allowed researchers to identify transverse segments (Puelles and Rubenstein, 1993; Bulfone \textit{et al.}, 1993). In the diencephalon, the caudal prosomeres align with specific structures and can be relatively easily distinguished by gene expression. For example, p2 and p3 (which encompass the dorsal and ventral hypothalamus) specifically express Pax6. Although prosomeric specific gene expression is rare, the overlapping gene expression is a common feature of the prosomere model (Fig.1.3C). For example, the diencephalic prosomeres express a combination of genes, such as \textit{Shh}, \textit{Nkx2.1} and \textit{Nk2.2}. Gene expression studies also indicate that the ZLI is a significant neuromere boundary, with genes like \textit{Dlx}, \textit{Gbx}, \textit{Wnt3} and \textit{Otx}, showing sharp limits of expression at the ZLI (Bulfone \textit{et al.}, 1993).

The location of diencephalic transverse domains have also been established in other species, including chicks and zebrafish (Figdor and Stern, 1993; Bulfone \textit{et al.}, 1993; Hauptmann and Gerster, 1999). A variation in diencephalic prosomere numbers is observed across species, with the zebrafish diencephalon being segmented into 8 prosomeres and the chick reported to have between 4 and 6 prosomeres. The difference in prosomere number may underlie the differential development of the forebrain across species or may reflect differences in experimental interpretation.
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The positions of telencephalic prosomeres remains unresolved. Most observers suggest that the majority of telencephalon is part of the alar plate, with a thin roof plate highlighted by Fgf8 expression (Puelles, 1995). The expression of BFl and other genes in the telencephalon supports this assertion. The suggestion that the telencephalon forms the alar plate is consistent with Na and colleagues' (1998) observations that cell mixing is not restricted in the dorsoventral axis.

Members of Dlx, Emx, Pax and Otx gene family are expressed in restricted telencephalic domains (Simeone et al., 1992; Price, 1993a; Stoykova and Gruss, 1993). In particular, dix and emx expression domains sharply abut each other in apparently transverse domains (Simeone et al., 1992; Price, 1993a). The sharp limits of these genes enabled three transverse telencephalic prosomeres to be described (Puelles and Rubenstein, 1993). However there is little support for the position of these domains from morphological, proliferative and cell lineage studies.

In zebrafish and Xenopus, the segmental organisation of the forebrain has been described to follow radial domains of gene expression (Fig1.3C; Papalopulu and Kintner; 1993; Li et al., 1994; Macdonald et al., 1994). Radial expression of genes is observed in the neural plate in a number of species (Rubenstein et al., 1994; Papalopulu, 1995; Delaney, 1999). Following neurulation, the early radial expression of genes may be modified into a more complex pattern. Therefore neurulation may be responsible for separating tissue primordia that are induced by the same signal. The maintenance of radial organisation may reflect the more primitive state of the zebrafish and Xenopus forebrains.

1.2.9.5. Summary of the prosomere model.

Since the revised prosomere model was proposed, a number of studies have sought to investigate the validity of the proposed boundaries (Puelles and Rubenstein, 1993; Puelles, 1995; Rubenstein et al., 1998). Although the positions of the diencephalic longitudinal and transverse prosomeres have proved to be relatively robust, the telencephalon has proved harder to segment in a satisfactory manner (Rubenstein et al., 1994). The post-natal neocortex does display region specific gene expression and histological structure (Rakic, 1988) and although the embryonic telencephalon does display restricted gene expression, the other criteria of morphology, proliferative units and cell lineage restrictions are not fully fulfilled.
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Despite the shortcomings of the revised prosomere model, it has significantly aided our comprehension of forebrain development and evolution. Understanding the organisation of the forebrain also facilitates investigations into the mechanisms involved in patterning this complex structure. For example, the ZLI, identified as an important prosomere boundary, may act as a neuroepithelial organiser (Marin and Puelles, 1994). The prosomeric model also provides a framework for understanding the evolution of the vertebrate forebrain (Rubenstein et al., 1994). Neuromeres may originate from a common vertebrate ancestor. Identification of neuromeres and studies in alterations in their development would allow the forebrains of different species to be compared and homologous structures identified.

Using this principle of neuromeric identity and homology, Fernandez and colleagues (1998; Puelles et al., 1999) investigated telencephalic organisation in developing mice, chick, Xenopus and turtle embryos. Of the genes expressed in the telencephalon, members of the Emx and Dlx gene family have distinct transverse boundaries. Emx family members can be used to distinguish pallial structures and similarly, Dlx family members delineate subpallial structures. In the four organisms examined, the two genes distinguished these respective areas and an additional intermediate territory, which does not express either gene, was also observed. The fate of the intermediate area varies between species. In mice this territory develops into parts of the amygdala, medial septum and paleocortex, and in chicks, the intermediate territory will give rise to parts of the chick Dorso-Ventricular Ridge (DVR). In the future, neuromeric studies may reveal the origin of highly evolved structures, such as the mammalian neocortex.

1.3. Initial induction and patterning of the vertebrate forebrain

1.3.1. Induction of the neural plate.

Spemann and Mangold described how the dorsal blastopore lip of the amphibian embryo has the ability to induce a second axis when transplanted to the ventral side of the host embryo (Spemann and Mangold, 1924). The second axis was formed by the conversion of ventral ectoderm, which normally gives rise to epidermis, into neural tissue, which normally arises from dorsal ectoderm. The dorsal blastopore lip was christened the organiser as it has the ability to organise the second axis on the host embryo. Tissues equivalent to the amphibian organiser have been described in other species, such as the embryonic shield of fish, node in mice and the Hensen's node in chicks (Bouwmeester and Leyns, 1997). Recent advances in identifying the molecular mechanisms responsible for organiser activity came
after the realisation that the embryonic default state may be neural instead of epidermal (Kelly and Melton, 1995).

The key molecular event underlying neural plate formation appears to be the antagonism between non-neural and neural inducers. The epidermal inducing signal is thought to be predominantly mediated by Bone Morphogenetic Protein-4 (Bmp4) (Tanabe and Jessell, 1996). Bmp4 is expressed in the early ectoderm and gradually excluded from the neural plate during neural induction. The organiser induces neural fates by inhibiting the action of the Bmp signalling, which suppresses neural fates and promotes epidermal differentiation. A number of Bmp antagonists have been identified, including follistatin, noggin, chordin and cerberus (Smith and Harland, 1992; Sasai et al., 1994; Hansen et al., 1997; Piccolo et al., 1999; Streit and Stern, 1999). The overexpression of these proteins results in ectopic development of neural tissue and at least follistatin, noggin and chordin are known to inhibit Bmp signalling by directly binding to Bmps to prevent receptor activation (Hansen et al., 1997; Streit and Stern, 1999; Piccolo et al., 1999).

1.3.2. AP patterning of the neural plate.

In recent times, the two signal model proposed by Nuwekoop, Eyal-Giladi, Toivonen and Saxen (Doniach, 1995) has been the most widely accepted model of AP patterning of the neural plate. In this model, the organiser induces a neural plate with anterior character and subsequent signals would specify more caudal CNS regions. Molecular evidence for this model comes from studies of gene expression in the neural plate during gastrulation. Sive and colleagues (1989) were among the first to report that anterior neural plate markers were initially expressed posteriorly and gradually become more anterior. Subsequently studies of genes, such as Otx2, show that many anterior neural plate markers are expressed throughout the entire neural plate and are restricted anteriorly as gastrulation proceeds (Ang and Rossant, 1994).

The source of signals responsible for specifying posterior neural plate identity appears to be mesodermal (Muhr et al., 1997). Fibroblast growth factors (Fgf) and Retinoic acid (RA) have been suggested to act as posteriorising signals (Doniach, 1995; Papalopulu et al., 1996). Fgf is expressed in the posterior mesoderm and can induce posterior neural identities (Crossley and Martin, 1995, Mason, 1996, Storey, 1998). However posterior neural patterning can be established without Fgf signalling (Koshida et al., 1998). Additionally the induction of mesoderm by Fgf make it difficult to precisely describe the role Fgf has in posterior neural plate patterning (Doniach, 1995). RA has the ability to suppress
anteriorgene markers (Ang and Rossant, 1994; Pannese et al., 1995) and cause anterior
defects but the difficulty to localise RAactivity to specific embryonic regions has impeded
our understanding of the role of RA in neural plate patterning (Blumberget al., 1997).

1.3.3. Anterior extraembryonic endoderm can induce anterior neural plate identity.

In addition to the organiser, anterior extraembryonic endoderm (AEE) has been
shown to play an important role in anterior neural plate induction. The role of this tissue in
establishing the anterior neural plate was exposed by studies on the cerberus gene in
Xenopus. Cerberus is expressed in the non-involuting yolky endoderm, which will eventually
form the foregut and liver (Bouwmeester et al., 1996). In Xenopus, ectopic expression of
cerberus induces partial head duplication. The ectopic heads are cyclopic, which may result
from a lack of axial mesoderm. The downstream effectors of cerberus are not known but
may include xotx2, which also induces anterior identity in the overlying rostral neural plate.

The pathways involved in AEE signalling are not known but evidence for the
 suppression of the Wnt signalling pathway has been recently proposed. Wnt signalling has
previously been implicated in anterior patterning. Overexpressing wnt8 signalling in mouse
embryos leads to expansion of midbrain territories at the expense of the forebrain (Pöpperl
et al., 1997). Inhibition of wnt8 activity also severely affects head development (Hoppler
et al., 1996). Additional studies have demonstrated that simultaneous inhibition of Bmp and
Wnt signalling leads to head induction (Glinka et al., 1997). Inhibitors of Wnt activity which
have recently been discovered, are expressed in anterior tissues and may act directly as
antagonists. Frzb is expressed in the AEE and prechordal plate (PCP) and can increase head
size when ectopically expressed (Leyns et al., 1997). Like the Bmp antagonists, Frzb is
thought to bind Wnt proteins and inhibit receptor binding and activation. Dickkopf, another
wnt inhibitor, is expressed solely in the PCP and induce ectopic heads in Xenopus when
misexpressed (Glinka et al., 1998). Suppression of the Wnt pathway is clearly necessary for
head induction in Xenopus (Itoh and Sokol, 1999). Further evidence for the role of
extraembryonic tissue in head induction and patterning comes from experiments done in
other model organisms.

1.3.4. The anterior visceral endoderm functions as an anterior organiser.

The mouse node was identified as being the equivalent of the Xenopus organiser
through the expression of similar genes, similar embryonic cell fates and the ability to induce
secondary axes upon transplantation (Beddington and Smith, 1993; Beddington, 1994).
However the second axis induced by node transplantation does not have forebrain structures and morphological signs of AP pattern appear before the node develops, suggesting that other signalling centres with essential roles in forebrain patterning exist in mammalian embryos (Beddington, 1994; Viebahn et al., 1995; Pöpperl et al., 1997; Tam et al., 1997). Recently the extraembryonic anterior visceral endoderm (AVE) has been shown to have similar properties to the AEE in AP patterning of the neural plate (Beddington and Robertson, 1999).

The AVE derives from a small population of endodermal cells, which exclusively express Hex in E5.5 mouse embryos (Thomas et al., 1998). Hex expression is maintained in the AVE and 12hrs before the primitive streak is observed, the AVE is already becoming a finely patterned tissue expressing a number of genes, including Otx2, Lim1, goosecoid and cerberus-related 1 (Ang and Rossant, 1994; Acampora et al., 1995; Belo et al., 1997; Thomas et al., 1998). These genes are initially expressed in a medial territory underlying the epiblast. The AVE can ectopically induce anterior markers in mouse and chicks (Ding et al., 1998; Knotgen et al., 1999) and the ablation of the AVE affects forebrain patterning (Thomas and Beddington, 1996). The molecular mechanisms underlying this organiser like activity have been delineated through the study of transgenic and chimeric mice.

Mice lacking correct Otx2 or Lim1 have truncated heads, which apparently lack all forebrain structure (Shawlot and Berringer, 1995; Ang et al., 1996). As both these genes are expressed in the AVE and neural plate or PCP, previous studies have been unable to delineate where the gene needs to be active for head formation. However injection of ES cells into blastocysts results in chimeric embryos, where the ES cells do not contribute to extraembryonic tissue (Beddington and Robertson, 1989). Using this technique, the role of gene expression in the AVE has been analysed. For example, when wild-type cells are injected into Otx2^-^ blastocysts, rostral neural plate development fails in a phenotype similar to that observed in transgenic Otx2^-^ embryos (Rhinn et al., 1998). When the converse experiment is performed, head development is initiated but not maintained. These results suggest that Otx2 function is initially required in the AVE to induce forebrain structures but subsequently Otx2 is also required in the neural plate or PCP.

The chimeric mice approach has also been used to investigate the role of other genes in mediating AVE activity. Although nodal is expressed in the visceral endoderm, the failure of nodal single mutants to gastrulate properly means that potential roles in head patterning are difficult to interpret (Varlet et al., 1997). When wildtype ES cells are injected into nodal^-^- blastocysts, gastrulation occurs but anterior structures fail to form. In wildtype
embryos injected with \textit{nodal} \textsuperscript{-/-} cells, gastrulation and anterior development occur normally, suggesting that \textit{nodal} is also required in the visceral endoderm for correct head specification. Chimeric studies on genes, such as \textit{Gsc}, \textit{HNF3\textbeta}, \textit{Smad2} and \textit{Smad4}, have further described roles for these genes in correct AVE and hence anterior development (Filosa \textit{et al}., 1997; Waldrip \textit{et al}., 1998; Sirard \textit{et al}., 1998). Although the relationships between these molecules is still being resolved, the antagonism of Wnt and Bmp signalling pathways appears to have a significant role in the initial induction and patterning of the anterior neural plate.

1.3.5. \textit{The yolk syncitial layer (YSL) provides anterior information in the zebrafish.}

Studies performed on \textit{Xenopus} and mouse AEE differentiation have powerfully demonstrated the role of extraembryonic tissues in head induction. In the zebrafish, the YSL is thought to be the functional equivalent of \textit{Xenopus} AEE and mouse AVE. In addition to expressing molecules found in \textit{Xenopus} and mouse AEE, a number of new molecules have also been shown to be expressed in the zebrafish YSL (Mizuno \textit{et al}., 1996; Fekany \textit{et al}., 1999). The expression and mutagenesis of \textit{Boz} further implicates Wnts in forebrain patterning. \textit{Boz} is a putative target of \textbeta-catenin and mutation at the \textit{boz} locus results in reduced forebrain structures (Fekany \textit{et al}., 1999).

1.4. \textbf{Refinement of the AP pattern of the neural plate}

1.4.1. \textit{Planar signals within the neural plate.}

The actions of the AEE and organiser induce anterior neural plate identity and instigate patterning of this territory. After these inductive events, a number of distinct processes are utilised by the embryo to achieve the regionalisation of the forebrain described earlier in this chapter. Regionalisation of the neural plate is mediated by specialised cell populations called signalling centres, which influence neural patterning through the action of secreted or membrane bound proteins.

1.4.2. \textit{Signalling by the mid-hindbrain boundary (MHB) is required for correct AP patterning of the midbrain, cerebellum and anterior hindbrain.}

The MHB, also known as the isthmus, is a signalling centre implicated in refining the AP pattern of the neural tube (Lumsden and Krumlauf, 1996). The signalling activity of the MHB was demonstrated by transplant experiments (Martinez \textit{et al}., 1991; Bally-Cuif \textit{et al}., 1996).
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1992), in which transplantation of the MHB to the ZLI results in ectopic mid-hindbrain gene expression in the surrounding forebrain. The forebrain tissue expressing ectopic mid-hindbrain markers differentiate into organised midbrain structures but are inverted with respect to the host midbrain and cerebellum (Marin and Puelles, 1994). Furthermore, the MHB can induce a cerebellum in the dorsal spinal cord (Martinez et al., 1995).

Candidate molecules for mediators of MHB signalling include members of the Wnt and Fgf families of secreted proteins. \textit{Wnt1} is expressed in the presumptive MHB from early neural plate stages and has shown to be required for maintenance but not initiation of midbrain and cerebellum development (McMahon et al., 1992). The co-operation of \textit{wingless} and \textit{engrailed}, a transcription factor, in \textit{Drosophila} is also observed in the development of the MHB. \textit{Wnt1} is required for the maintenance of \textit{En1} expression in the MHB (Danielian and McMahon, 1996), as is the paired box gene \textit{Pax2.1} (Brand et al., 1996; Favor et al., 1996).

Ectopic induction of midbrain-like territories by the MHB can be mimicked using Fgf8/Fgf4 soaked beads (Crossley and Martin, 1996). Implantation of these beads at the ZLI upregulates the expression of \textit{Wnt1}, \textit{En2} and \textit{Fgf8}, demonstrating the importance of these genes in MHB development and signalling (Crossley and Martin, 1996). Zebrafish mutant fish, which lack Fgf8 signalling, lack cerebellar structures and have other, more subtle defects in midbrain and hindbrain development (Brand et al., 1996; Reifers et al., 1998; Picker et al., 1999). Detailed analysis of the Fgf8 mutant fish, \textit{acerebellar (ace)}, revealed that the MHB territory is induced but fails to develop, suggesting that Fgf8/Ace is required for the maintenance of gene expression at the MHB.

1.4.3. Planar signalling by the anterior neural plate margin patterns the forebrain territory.

Recent work on zebrafish and mice has elucidated a signalling centre in the anterior neural plate. In the early gastrulating zebrafish embryo, a row of 6-8 cells in the anterior most edge of the neural plate (row1) are required for patterning of the telencephalon (Houart et al., 1998). Ablation of these cells results in loss of global telencephalic patterning, whilst transplantation of the row1 cells to more caudal neural plate loci induces ectopic telencephalic gene expression.

Similar signalling activity for cells in the anterior neural plate has been demonstrated in mice (Shimamura and Rubenstein, 1997). Prior to neurulation, the lateral edges of the
neural plate condense to form thickened folds. In the anterior neural plate, this condensation results in the formation of the anterior neural ridge (ANR). Excision of the ANR results in loss of telencephalic markers, such as BF1, whilst transplantation can ectopically activate such markers. Fgf8 is expressed in the ANR and beads soaked in Fgf8 can reproduce the activity of the ANR in explant cultures. The importance of Fgf8 signalling in the forebrain was highlighted by recent explant culture experiments performed by Ye and workers (1998). In these experiments, exposure of the explants to ectopic Fgf8 resulted in an expansion of forebrain dopaminergic neurons and blocking Fgf signalling, through antibodies to Fgf receptors, decreased the number of dopaminergic neurons. Row1 cells' activity maybe partially mediated through Fgf8 signalling but the relatively late expression of fgf8 in the forebrain and loss of fgf8.1 in row1 ablated embryos suggest that other molecules play a more important role in mediating row1 signalling activity (Houart, unpubl.).

Random mutagenesis screens have also isolated zebrafish mutants with defects in AP patterning of the neural plate. In the masterblind (mbl) mutant, the loss of the telencephalon and eyes is coincident with an expansion of rostral diencephalic territories (Heisenberg et al., 1996). In contrast the zebrafish floating head (flh) mutant has defects in differentiation of the epiphysial neurons (Masai et al., 1997). A genetic interaction between mbl and flh is responsible for specifying the positions of the epiphysial neurons in the neural plate. Subtractive cDNA library techniques, which have yielded many novel molecules involved in a range of biological processes, will also help elucidate molecules involved in signalling from the ANR.

1.5. Dorso-ventral (DV) patterning of the neural plate

1.5.1. Ventral patterning: insights from more caudal tissues.

In the caudal neural tube, numerous studies have greatly enhanced our understanding of the structures and molecules involved in DV patterning. During early development, the caudal neural tube can be separated into ventral structures, such as the floor plate and motor neurons, and dorsal structures, such as sensory neurons. Intermediate positions are characterised by the presence of interneurons. Ventral patterning appears to be initially governed by the notochord, a mesodermal tube of cells underlying the neural plate from the ZLI to the caudal spinal cord (Placzek et al., 1990). The action of the notochord induces the formation of floor plate cells in the ventral neural tube and also can initiate the differentiation of motor neurons (Yamada et al., 1991). Subsequently the floor plate also develops the ability to induce motor neurons (Placzek et al., 1993).
1.5.2. *Shh as the ventralising signal in the caudal neural tube.*

The expression of the vertebrate homologue of the *Drosophila* segment polarity gene *hedgehog, sonic hedgehog*, in the notochord from early gastrulation and later in the floor plate suggested that it may mediate their inductive actions (Echelard et al., 1993; Riddle et al., 1993; Krauss et al., 1993). This was confirmed by ectopic expression of *shh* in mice, zebrafish and *Xenopus*, which results in induction of floor plate markers (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994). Blocking *Shh*, through an anti-Shh antibody, results in loss of floor plate induction and similarly *shh* null mutant mice lack floor plate cells (Chiang et al., 1996; Ericson et al., 1996). Although initial studies on zebrafish mutants, such as *flh* (Talbot et al., 1995) and *no tail* (*ntl*, Schulte-Merke et al., 1994), suggested that *shh* expression was required for induction of the floor plate, more recent studies on the *shh* mutant, *sonic you* (Schauerte et al., 1998) suggest that zebrafish floor plate induction may require molecules other than *shh*.

A series of loss of function and gain of function studies implicate long range *shh* signalling in induction of motor neurons and interneurons (Roelink et al., 1994; Ericson et al., 1996; Chiang et al., 1996). Retinoid signalling also plays an integral role in establishment of neuronal identity in the caudal neural tube (Sockanathan and Jessell, 1998) and retinoid signalling acts in a *shh* independent manner to induce interneurons (Pierani et al., 1999).

1.5.3. *Modification and diffusion of Shh.*

The Shh protein undergoes a number of modifications prior to secretion. Initially the protein undergoes an autocatalytic cleavage to give two separate peptides, the aminoterminal active (N-Shh) and the inactive carboxy-terminal (C-Shh) form (Porter et al., 1995). The N-Shh form mediates all actions of *Shh* and is well conserved in evolution (Porter et al., 1995; Roelink et al., 1995; Ekker et al., 1995). The C-Shh component of the protein appears to be required for auto-proteolysis and concurrent attachment of cholesterol to the carboxy terminus of N-Shh. Cholesterol addition is important for Shh function as defects in cholesterol biosynthesis result in a phenotype similar to the *shh* null mice (Chiang et al., 1996; Cooper et al., 1998).

Short range contact dependent and long range contact independent roles for *Shh* have been proposed to occur in the caudal neural tube. Cholesterol modification has been
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proposed to regulate the extent of diffusion of Shh molecules, as cholesterol modification may anchor Shh proteins to the surface of the expressing cell, preventing long range direct signalling (Rubenstein and Beachy, 1997). The induction of floor plate can occur in the absence of protein synthesis, suggesting that this Shh activity is mediated over a short range (Roelink et al., 1995). However studies of mice lacking Pax6 or Nkx2.2 suggest cells at different DV positions react to different concentrations of Shh (Ericson et al., 1997; Osumi et al., 1997; Briscoe et al., 1999). Other regulators of diffusion of Hh gene family members have also been identified. Tout-velu, a Drosophila homologue of the putative tumour suppressor EXT-1, has been shown to be required for the diffusion of Hedgehog proteins. A failure in diffusion of Indian Hedgehog may result in the human Multiple Exostoses Syndrome (Bellaiche et al., 1998).

1.5.4. Downstream targets of Shh.

In the Shh pathway, ligand reception is mediated by Patched (Ptc) and Smoothened (Smo; Alcedo et al., 1996; Chen and Struhl, 1996; Goodrich et al., 1997; van den Heuvel and Ingham, 1996). Binding of Shh to Ptc relieves the Ptc-mediated repression of Smo, allowing Smo to activate intracellular targets. Commonly Shh signalling regulates the activities of vertebrate homologues of Drosophila cubitus interruptus, called Gli proteins (Dahmane et al., 1997; Hynes et al., 1997; Lee et al., 1997; Ruiz i Altaba, 1998). Both Gli1 and Gli2 are induced by Shh and are able to induce motor neurons (Dahmane et al., 1997). The inhibition of Gli2 mediated motor neuron induction by Gli3 suggests a Gli combinatorial code may interpret Shh activities at different locations (Sasaki et al., 1997).

Protein Kinase-A (PKA) provides a cAMP dependent mechanism for Shh signal transduction (Fan et al., 1995). Although the exact relationship between PKA and Shh has yet to be established, inhibition of PKA in zebrafish results in a phenotype similar to the shh null mice mutant (Blader et al., 1997). Other vertebrate molecules involved in the common Shh signal transduction pathway include the membrane glycoprotein, Hip (Chuang and McMahon, 1999). Hip proteins, named for their ability to bind Shh (Hedgehog interacting protein), are expressed in cells adjacent to those expressing shh and may attenuate Hedgehog signalling. In addition a negative regulatory feedback loop may operate, similar to that observed with Ptc, to regulate Shh activity (Chen and Struhl, 1996; Chuang and McMahon, 1999).

1.5.5. Patterning of the dorsal neural tube.
Although long range signalling from the floor plate may induce interneurons, it is unlikely to influence dorsal patterning in the caudal neural tube (Ruiz i Altaba, 1994). Dorsal gene expression is normal in shh null mice (Chiang et al., 1996) and differentiation of dorsal cell types, such as neural crest, occurs in the absence of a notochord (Liem et al., 1995). The signals required to induce the dorsal cell types probably originate from the non-neural ectoderm overlying the neural tube.

Non-neural ectoderm can induce the expression of dorsal markers, such as pax3 and slug, upon culturing with ventral neural plate tissue (Dickinson et al., 1995; Liem et al., 1995). However ventral cell fates are detected around the notochord if it is not removed in these studies, suggesting that an antagonistic mechanism may pattern the DV axis of the caudal neural tube (Dickinson et al., 1995).

Members of the TGF-β superfamily, including BMP4, BMP7 and dorsalin-1 (dsll; Liem et al., 1995; Dickinson et al., 1995), are expressed in the correct spatio-temporal manner to act as effectors of this dorsalising signal. BMP4 and dsll are initially expressed in the non-neural ectoderm and subsequently in the dorsal neural tube (Basler et al., 1993; Hemmati-Brivanlou and Thomsen, 1995). The dorsalising activity of these proteins has been displayed in a number of different studies in different organisms. In chicks, COS cells expressing BMP4 and BMP7 can induce ectopic dorsal neural tube identities (Liem et al., 1995). In zebrafish, overexpression of Bmp4 leads to a reduction in ventral motor neurons and a loss of expression of ventral midline genes (Neave et al., 1997). The zebrafish swirl mutant has a mutation in bmp2b and a subsequent loss of neural crest cells (Nguyen et al., 1998). These and other studies have firmly established a role for BMPs in regulating dorsal neural tube identity.

As the notochord induces the floor plate, to maintain ventral signals, the non-neural ectoderm may also induce a similar signalling centre in the dorsal neural tube. Bmp4 expression is first detected in the non-neural ectoderm and subsequently in the dorsal neural tube, a host of other genes show similar spatiotemporal expression (Fan and Tessier-Lavigne, 1994). Thus patterning of the caudal neural plate appears to depend on opposing BMP and Shh signals, which lead to the induction of specific neurons along the DV axis (Roelink, 1996).

1.5.6. Pattern of the ventral forebrain.

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Although the notochord does not extend past the level of the ZLI, another mesodermal derivative, the prechordal plate (PCP), underlies the forebrain anlage (Adelmann, 1922). The PCP forms the rostral most part of the axial mesendoderm and is continuous with the notochord. The role of the PCP in forebrain patterning was first suggested by Adelmann (1922) and more recent studies have elaborated the nature of this patterning activity (Dale et al., 1997; Blader and Strähle, 1998).

Insights into the role of the PCP have come from analysis of zebrafish mutants. The absence of the PCP in zebrafish mutants, such as cyclops (cyc) and one-eyed pinhead (oep), leads to severe defects in the ventral forebrain (Hatta et al., 1991; Barth and Wilson, 1995; Schier et al., 1996a; Zhang et al., 1998). In mice and chicks, ablation of the PCP leads to loss of ventral forebrain specific genes (Dale et al., 1997; Shimamura and Rubenstein, 1997).

The notochord and PCP share similar gene expression, developmental origins and morphologies (Krauss et al., 1993; Roelink et al., 1994; Sulik et al., 1994; Shih and Fraser, 1996). Differences between the PCP and notochord may result in the distinct properties observed at different AP levels. For example, PCP expresses some genes that are not expressed in the notochord, such as gsc, otx2 and ntl (Pannese et al., 1995; Blitz and Cho, 1995; Thisse et al., 1994; Schulte-Merke, 1994; Talbot et al., 1995). In addition, the PCP and notochord have different inductive activities. PCP can not induce floor plate derivatives in caudal neural plate explants and conversely, notochord can not induce ventral identity in the rostral neural plate (Placzek et al., 1993).

Despite the apparent differences in the PCP and notochord, initial studies suggested that ventral patterning of the forebrain is mostly mediated through the action of Shh. Overexpression of shh leads to the expansion of ventral forebrain fates, as assayed by nkx2.1, nk2.2 and pax2, and a corresponding reduction of dorsal fates, as assayed by pax6 expression (Barth and Wilson, 1995; Macdonald et al., 1995; Dale et al., 1997; Shimamura and Rubenstein, 1997). In zebrafish another Hh family member, twiggy winkle hedgehog (twhh), can also mediate ventral forebrain patterning (Ekker et al., 1995). Blocking shh signalling, with an anti-SHH antibody or a dominant negative form of PKA, leads to a loss of ventral forebrain patterning (Dale et al., 1997; Blader et al., 1997). In mice, loss of shh activity severely affects the development of the ventral forebrain (Chiang et al., 1996). A similar loss of activity in the zebrafish syu/shh and yot/gli2 mutants also causes ventral forebrain patterning defects (Schauerte et al., 1998; Karlstrom et al., 1999; Barth and Wilson, unpubl.). Typically ventral patterning has been assayed using the Nkx2.1 gene, which is expressed in the hypothalamus and the ventral telencephalon (Sussel et al., 1999).
Recent studies have demonstrated that shh is required for patterning the ventral telencephalon and not just the diencephalic component of the forebrain (Chiang et al., 1996; Kohtz et al., 1998). The patterning of the telencephalon by shh may be mediated by late shh expression in the midline of the hypothalamus and telencephalon. One potential difference in the action of shh in the PCP and notochord may be mediated through differences in concentration at different AP levels (Dale et al., 1997). In explant cultures, higher shh concentrations were required for ventral forebrain specification than for more caudal regions.

Shh plays an important role in ventral neural plate patterning and differences between anterior and caudal neural plate identities are probably specified through the action of other molecules. In chicks the activity of shh has been proposed to be modulated by the action of Bmp7 (Dale et al., 1997). Bmp7 is also expressed in the PCP and can co-operate with shh to pattern the ventral forebrain. However Bmp7 and Shh are expressed in the rostral notochord underlying the hindbrain but do not activate forebrain specific genes in hindbrain region. One explanation for this observation may stem from the fact that ventral midline cells of the forebrain initially have caudal identity. Subsequent respecification of the midline cells to a rostral identity may be mediated by the action of chordin (Dale et al., 1999). chordin is expressed throughout the axial mesoderm but is excluded from rostral midline cells when anterior identity is assumed.

Several zebrafish mutants have been identified with defects in forebrain patterning (Heisenberg et al., 1996, Schier et al., 1996). Recently some of the genes perturbed in these mutants have been identified through a combination of positional and syntenic cloning (Zhang et al., 1998; Karlstrom et al., 1999). The zebrafish cyclops mutant has severe midline defects, including cyclopia and loss of floor plate cells. Positional cloning of the cyclops allele revealed it to be a nodal related gene, expressed in the dorsal mesendoderm and PCP (Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998). A loss of rostral shh expression in cyc mutants suggests that the two molecules may interact. oep mutants are also characterised by cyclopia, caused by the lack of PCP (Schier et al., 1996). Positional cloning revealed that the oep gene encodes a membrane bound EGF-related ligand, which interacts with nodal signals and is required for the continued development of the PCP (Zhang et al, 1998). Other genes, such as gsc, have also been implicated in formation of the PCP and thus ventral forebrain patterning (Thisse et al., 1994).

1.5.7. Patterning of the dorsal forebrain.
Patterning of the dorsal forebrain appears to depend on the same signals that pattern the dorsal parts of the caudal neural plate. In particular, the BMPs appear to play a conserved role in the differentiation of the dorsal neural tube (Rubenstein and Beachy, 1998).

BMPs are expressed by the non-neural ectoderm during gastrulation and subsequently in the dorsal forebrain (Furuta et al., 1997; Barth et al., 1999, Golden et al., 1999). A significant role in dorsal forebrain development is suggested by examination of the zebrafish bmp2b mutant, swirl, where telencephalic identity is lost (Barth et al., 1999). In mice and chicks expression of Bmp4 and Bmp7 is excluded from the anterior non-neural ectoderm during midgastrulation but recovers by late gastrulation (Muhr et al., 1997, Furuta et al., 1997). This spatio-temporal switch in BMP expression may explain why no neural crest cells are found around the telencephalon. Ectopic expression of BMP4 results in the induction of neural crest in more caudal levels of the neural plate and in contrast, ectopic expression of dorsal telencephalic genes in the rostral neural plate (Muhr et al., 1997; Furuta et al., 1997). In chicks overexpression studies in chicks suggest that BMP4/5 also regulate dorsal forebrain development (Furuta et al., 1997; Golden et al., 1999). A further role for BMPs in the patterning of the dorsal forebrain is suggested by the expression of the BMP antagonist, noggin, in the roof of the forebrain (Shimamura and Rubenstein, 1995; Knecht and Harland, 1997).

Further insights into the mechanisms responsible for the patterning of the DV forebrain axis come from studies on transcription factors. Expression of these transcription factors are regulated by the activities of the signalling centres described (Shimamura and Rubenstein, 1997; Houart et al., 1998). For example, row1 activity is required to activate the expression of the Emx genes, which are zebrafish homologues of the Drosophila empty spiracles genes (Houart et al., 1998). Emx genes are expressed in the dorsal telencephalon of many species (Gulisano et al., 1996; Morita et al., 1995; Pannese et al., 1998). In mice, mutations in these genes lead to severe defects in the dorsal telencephalon. Emx2 null mice have hypoplastic telencephalons, lack limbic cortex structures, such as the hippocampus and dentate gyrus, and have disrupted cortical layering (Pellegrini et al., 1996, Yoshida et al., 1997). Emx1 null mice exhibit less severe defects, such as the loss of corpus callosum. The loss of the corpus callosum, a fibrous connection between the two telencephalic hemispheres, is thought to be due to loss of glial cell differentiation (Yoshida et al., 1997).

The development of the dorsal telencephalon is also affected in mice with mutations in Pax6 (defective dorsal patterning and cortical lamination), Gli3 (abnormal lamination and
loss of choroid plexus) and Lhx5 (loss of hippocampus) (Stoykova et al., 1996; Frantz, 1994; Zhao et al., 1999).

Ventral telencephalic defects are observed in Bfl null mice, which display hypoplasia and loss of the basal telencephalon (Xuan et al., 1995). A further role in positioning neurogenesis in the anterior neural plate has been demonstrated for the Xenopus homologue of Bfl (Bourguignon et al., 1998). The loss of both Dlx1 and Dlx2 results in defects in neuronal differentiation in the striatum and olfactory bulb (Anderson et al., 1997a). Another homeobox containing gene, Nkx2.1, is required for the correct development of ventral telencephalic structures, such as the pallidum (Sussel et al., 1999). These transcription factors and others are likely to be downstream targets of members of the TGFB and Hh family.

1.5.8. Further patterning of the vertebrate forebrain.

In addition to the signalling centres, a number of other mechanisms are also involved in the regionalisation of the forebrain, most notably the effects of axonal connectivity. For instance, organisation of the neocortex has been proposed to occur through two distinct mechanisms involving the input of thalamic afferents (O'Leary, 1989) and the activity of intrinsic cues within the neocortical ventricular zone (Rakic, 1988). Within the olfactory system, pioneer olfactory axons are thought to instigate the differentiation and eventual evagination of the olfactory bulb (Gong and Shipley, 1995). A central role for the olfactory placode in patterning of the telencephalon has been confirmed by a series of ablation experiments (Graziadei and Graziadei, 1992).

1.6. Axon guidance within the forebrain

During development, axons extend over considerable distances to form precise and reliable connections. The problem of accomplishing the complex axonal connections observed in the adult is simplified in the developing embryos by pioneering axons. Pioneer axons travel through the embryos in a stereotyped manner to establish axonal tracts, over which subsequent axons will navigate. Although these pioneer axons frequently navigate in the absence of obvious morphological landmarks, it is increasingly becoming apparent that the axonal growth cones sense cues within the neuroepithelium (Goodman and Shatz, 1993).

The route of an axon offers many favourable targets but these are consistently bypassed to reach the correct terminus. Thus the pathway can be considered to be
fragmented into a series of choice points (Cook et al., 1998). Choice points represent specialised areas, which provide the navigating axon with cues that direct it to the next stage of the route. Perhaps the most extensively studied choice point is the CNS midline of both vertebrates and invertebrates. Studies from Drosophila and Caenorhabditis elegans (C. elegans) have helped elucidate a number of molecules involved in axon guidance but before we consider these molecules, I will briefly describe the mechanisms which may influence guidance at the midline.

1.6.1. Attraction and repulsion in axon guidance.

Four guidance forces are thought to control all aspects of axonal navigation. A host of embryological manipulations have described how axons will respond either favourably, by outgrowth, or unfavourably, typically through growth cone collapse and repulsion, to contact mediated or long range signalling cues. Long range chemoattraction was first postulated by Ramon Y Cajal (1892) to explain axon guidance over long distances. Chemorepulsion can also be mediated through the action of long range signalling molecules and both these features of axon guidance have been extensively described through in vitro experiments (Tessier-Lavigne et al., 1988; Guthrie and Pini, 1995). Axons are also influenced by contact mediated signalling and indeed require certain extracellular matrix molecules over which to extend. Similarly repulsion can be mediated by preventing axons entering a territory through the use of contact mediated chemorepulsion (Keynes and Cook, 1990). Thus the process of axon guidance can be viewed as the channelling of axons through intermediate territories by using attractive signals to maintain correct pathfinding and repulsive signals to inhibit ectopic outgrowth.

1.6.2. The role of boundaries in axon guidance.

Recent observations suggest that early axonal tracts are established at the boundaries of gene expression domains (Wilson et al., 1990; Macdonald et al., 1994, Marcus et al., 1999). A number of models could explain how these regulatory genes act to guide axons at these boundaries (Fig1.4; Wilson et al., 1993; Wilson et al., 1997). All of these models establish a complement of attractive and repulsive cues, which serve to push and pull the axons across a particular region. The simplest model involves the creation of a boundary between two groups of cells that have different cell surface properties (Fig1.4A). If these cell surface proteins both discourage exploration, then the axon is forced to extend at the
Fig1.4. Potential mechanisms by which axon navigate at boundaries of gene expression

(A) Two regions of cells may discourage axon growth within their respective territories, thus forcing the axon to navigate between the two domains. (B-D) In these models, one domain secretes a molecule which exerts influence over adjacent domains. (B) In this scenario, the secreted molecule serves to discourage axon outgrowth into the adjacent territory. Domain 2 continues to also exert a repulsive influence on the axon growth cone. In (C) the secreted molecule leads to domain 1 allowing growth of the axon over cells receiving the secreted signal. This may occur through the upregulation of favourable cell surface proteins. In (D) the secreted signal acts in a graded manner to draw axons towards the boundary territory. However domain 2 cells still do not favour axon extension and thus growth cones turn away from this area.

Adapted from Wilson et al., 1997
boundary between the two domains. Subsequent axons would be tightly fasciculated to form a tight bundle at the boundary.

Contact independent mechanisms can also be incorporated into boundary models, such that a signal from one domain may influence the attractive or repulsive properties of adjacent domains (Fig 1.4B-D). These signals may stem from one domain or may depend upon interactions between domains. With the introduction of long range signalling comes the potential that axons are able to react to a signal gradient, such gradients may be active at some distance away from their source in a particular gene expression domain (Fig 1.4D).

1.6.3. Axon guidance in the zebrafish forebrain.

A simple axonal scaffold is established relatively early during zebrafish development. In the forebrain, only a handful of axon bundles are observed in the prim 12 (28hpf) embryo (Fig 1.5; Chitnis and Kuwada, 1990; Wilson et al., 1990). The telencephalon is dominated by the anterior commissure (AC), whose axons project ventrally to cross the midline in the basal telencephalon. The supraoptic tract (SOT) is formed by reciprocal connections between the telencephalon and hypothalamus. Axons cross the rostral diencephalic midline to form the postoptic commissure (POC), the dorsal diencephalon to form the posterior commissure (PC) and the ventral tegmental commissure (VTC) crosses the tegmentum. In particular, the AC and POC form either side of the optic stalk territory. By prim 26 stage, retinal axons form the optic chiasm by exiting the eye to project to the contralateral tectum. The points where these axons cross the midline represent specialised choice points with the axons remaining tightly fasciculated at these locations.

The role of boundaries in axon guidance is suggested by the positions of neuronal differentiation in the forebrain (Macdonald et al., 1994). The boundaries of Pax6, Shh and Wnt1 are coincident or adjacent to the sites of neuronal differentiation in the telencephalon and rostral diencephalon. The relationship between the location where the AC and POC cross the midline and gene expression has been well characterised (Macdonald et al., 1994; Macdonald et al., 1997). As development proceeds, neurons appear to extend axons along the boundaries of gene expression (Fig 1.5B). The positions of the commissures exhibit a strong correlation with the boundaries of gene expression. Insights into the exact mechanisms which may regulate axon guidance have come from studies zebrafish mutants (Macdonald et al., 1997).

1.6.4. Noi/Pax2.1 demarcates a repulsive territory in the zebrafish forebrain.
Fig 1.5. Axon guidance and gene expression boundaries in the zebrafish forebrain

(A) A simple axon scaffold is established in the prim 10 zebrafish embryo, as viewed by N-acetylated tubulin antibody staining (a lateral view is shown). In the forebrain two commissures, the anterior and postoptic, and one tract, the supraoptic, are detected. (B) A schematic of a frontal view a prim 10 zebrafish embryo illustrating the positions of the two commissures, which coincide with the expression domains of several genes. The genes, which display restricted patterns, include transcription factors and genes with known axon guidance properties, such as netrins. (C) Frontal view of a prim 10 embryos stained with anti-Noi/Pax2.1 and anti N-acetylated tubulin antibody. Noi is expressed in the nuclei of cells in the ventral optic stalk, directly dorsal to the position of the POC. Loss of Noi activity leads to invasion of the ventral optic stalk territory by axons of the POC.

Abbreviations: ac, anterior commissure, dd, dorsal diencephalon, e, epiphysis, ey, eye, hy, hypothalamus, mlf, medial longitudinal fasciculus, os, optic stalk, poc, postoptic commissure, sot, supraoptic commissure, t, telencephalon, te, tectum, tpc, tract of the posterior commissure, tpoc, tract of the postoptic commissure, vtc, ventral tegmentum commissure.

Adapted from Wilson et al., 1997
The Pax family transcription factor *pax2.1* is expressed in the ventral optic stalk region, dorsal to the position the POC will form (Fig.1.5C). Defects in forebrain axon pathfinding were examined in the zebrafish *pax2.1* mutant, *no isthmus (noi)*. In *noi* mutants the POC is defasciculated and axons begin to invade the ventral optic stalk territory, which normally excludes axons. Axons of the POC do not send ectopic projections into the anterior hypothalamus, suggesting that patterning of this area is normal in the *noi* mutant. Further examination of the rostral forebrain suggests that *noi*-expressing cells do not differentiate properly in the *noi* mutant. These observations strongly suggest that the Noi protein regulates molecules which repulse axons of the tract of the POC from the ventral optic stalk region (Macdonald *et al*., 1997).

Examination of other zebrafish mutants have been less informative about forebrain axon guidance (Lauderdale *et al*., 1997; Halloran *et al*., 1999). Overexpression of *shh* and *twhh* leads to the upregulation of *noi/pax2.1* expression, thereby suggesting that these molecules are required to establish the axon free optic stalk territory (Ekker *et al*., 1995; Macdonald *et al*., 1995). As Shh is expressed adjacent to POC, it may prove to be an example of how a secreted signal can influence cell behaviour and axon guidance in adjacent territories.

Several molecules with known axon guidance properties are expressed in the zebrafish forebrain (Cooke *et al*., 1997; Stråhle *et al*., 1997; Halloran *et al*., 1999) and these are discussed in the following sections.

1.6.5. **Netrins regulate axon guidance.**

Netrins (net) were isolated as floor plate secreted proteins, that possess the ability to attract spinal cord commissural axons to the ventral midline (Serafini *et al*., 1994). Homologues of netrins have been isolated in both invertebrates and vertebrates. In *C. elegans* the homologue of netrin, UNC-6, is required for the circumferential growth of axons in the body wall (Hedgecock *et al*., 1990) and the two *Drosophila* homologues of netrin are required for correct motor neuron projection and commissure formation at the midline (Harris *et al*., 1996; Mitchell *et al*., 1996). Diffusion of netrins is probably regulated by the binding to extracellular matrix, as netrins are related to laminins.

In vertebrates netrins have been shown to have differential effects on different classes of axons. The wandering of spinal cord commissural axons in *net1* deficient mice support a
role for Netrin-1 in attracting these axons towards the floor plate (Serafini et al., 1996). A similar role has been described in the zebrafish. In zebrafish flh mutants netl expression is found in patches along the AP axis of the floor plate and axons abhorrently project towards those cells where netl expression is maintained (Lauderale et al., 1997). The patchy expression of netl in the flh mutant, corresponds to patchy shh expression also observed in the mutant embryos. Indeed a regulatory link between the two molecules is also observed when overexpression of shh results in the upregulation of netl (Strähle et al., 1997).

Netrins have been shown to have similar attractive effects in different tissues. netrin-1 is expressed in the optic disc and can attract retinal axons in vitro (Denier et al., 1997; Macdonald et al., 1997). In netrin-1 null mice, retinal axons fail to exit the eye indicating netrins are required for axon pathfinding within the eye (Serafini et al., 1996; de la Torres et al., 1997).

Netrins have also been described to influence axon guidance in the forebrain. Loss of netrinl activity leads to defects in the corpus callosum, hippocampal and anterior commissure (Serafini et al., 1996). Each commissure fails to cross the midline, suggesting that a lack of chemoattraction is the cause of the defect. Chemoattractive properties have also been described for netrins with regard to cortical projections (Métin et al., 1997).

Chemorepulsive properties have also been described for Netrin family members. The chemorepulsive properties of netrins are best described in relation to cell and axon migration in C. elegans. In the body wall of C. elegans, cells migrate either dorsally or ventrally. The migration of the cells appears to depend upon a response to the netrin homologue, UNC-6, which is expressed in the ventral midline (Chan et al., 1996). Interestingly the response to UNC-6 may depend on the type of netrin receptor expressed on the migrating cells. Cells which migrate ventrally towards the UNC-6 source express UNC-40, the C. elegans homologue of the vertebrate netrin receptor DCC (Keino-Masau et al., 1996). Dorsally migrating cells express the co-receptor UNC-5, along with UNC-40, and loss of UNC-5 leads to a reduction in dorsal migration. UNC-6 probably has a direct effect on these cells as UNC-5 is thought to bind UNC-6 (Leonardo et al., 1997). In support of this hypothesis, recent domain swapping experiments reveal that the cytoplasmic domain of the Drosophila netrin receptor, frazzled, dictates the response of the axon (Bashaw and Goodman, 1999). In vertebrates only trochlear motor axons are repelled by cells expressing netrins and the basis of this repulsion is not understood (Colamarino and Tessier-Lavigne, 1995).
In the zebrafish forebrain, two netrin homologues have been isolated with overlapping expression in the optic stalk territory (Strähle et al., 1996, Macdonald et al., 1997). The expression patterns of zebrafish netl and net2 would suggest that they attract axons towards the midline. In noi mutants, the axons of the tracts of the AC and POC are attracted towards the midline and correspondingly netrin gene expression is maintained (Macdonald et al., 1997).

1.6.6. Multiple roles for Semaphorins.

The semaphorin (sema) gene family consists of more than 20 secreted and membrane associated proteins. Members of the family are characterised by a 500 amino acid extracellular domain (termed the sema domain) and can be grouped into eight subclasses. Of the two classes of invertebrate semas, one is secreted and the other is transmembrane protein. The five vertebrate subclasses of semas are characterised according to their membrane association (secreted, transmembrane or glycosylphosphatidylinositol (GPI)-linked) and presence of Ig or thrombospondin type 1 repeat domain.

Semaphorins were initially characterised through their ability to cause growth cone collapse. Sema3A (previously known as collapsin-1 and H-Sema III) was purified from adult chick brain as a secreted glycoprotein, that could induce the collapse of dorsal root ganglion sensory axons (Luo et al., 1993). Sema3A is expressed throughout the ventral neural tube during development and probably delimits the site at which sensory afferents enter the spinal cord. The repulsive nature of Sema3A is confirmed by antibody blocking experiments, application of the antibodies, results in axons no longer being repelled in the presence of a Sema3A source (Shepherd et al., 1997). Different sensory afferents display temporal sensitivity towards Sema3A, as initially all sensory afferents do not invade the sema3A territory but later NT3-dependent afferents project into the sema3A territory (Messersmith et al., 1995).

Analysis of sema function in insects, such as Drosophila and grasshoppers, indicate that sema-1a (previously known as G-Sema 1 and D-Sema 1) may have a dual role in axon guidance (Kolodkin et al., 1993; Wong et al., 1997). In the grasshopper limb bud, Til pioneer neurons apparently regard a stripe of Sema-1a as a repulsive cue and project axon away from it. Later axons originating in the distal region of the limb will cross this stripe of sema-1a to fasciculate with the Til axons. In the second case, the stripe of sema-1a apparently acts as an attractive/permissive cue (Wong et al., 1997). Similar
attractive/repulsive functions for Semas have been demonstrated for different axonal pathways in vertebrates.

In the forebrain, semas also present repulsive and attractive cues to navigating cortical axons (Bagnard et al., 1998). Cortical neurons also express the putative Sema receptor, neuropilins. Application of neutralising antibodies to neuropilins block Sema3A mediated growth cone collapse and neuropilin deficient mice partially phenocopies the Sema3A null mice (He et al., 1997). Neuropilins can be grouped into two classes according to their binding to the different classes of Semas. In contrast to netrin/DCC, neuropilins do not appear to mediate the biological activity of Semas and other as yet unidentified molecules maybe responsible for imparting biological activity of Semas (Koppel et al., 1997). Plexin A is also a semaphorin receptor, which can mediate axon guidance responses (Windberg et al., 1998). In the zebrafish, two semas have been recently cloned (Halloran et al., 1999). The expression of sema3D between the AC and POC suggests that it may provide a repulsive cue to prevent axons invading the optic stalk territory.

In the developing embryo, Semas display dynamic expression patterns and recent reports have demonstrated roles for Semas in processes ranging from bone and cartilage formation to development of the immune system (Behar et al., 1996; Hall et al., 1996).

1.6.7. Ephrin ligands and Eph receptors as repulsive cues.

The Ephrin ligand family and their receptors have also been widely implicated in regulating axon guidance. As with the Semas, Eph ligands can be classed according to their membrane attachment, which is achieved either by GPI-linkage or through a transmembrane domain (Orioli and Klein, 1997). The receptors are characterised by an extracellular domain containing a cysteine-rich domain and two fibronectin type III repeats and an intracellular domain with tyrosine kinase activity. The discovery that signal transduction can occur in both the ligand and receptor expressing cells suggests that Ephrins do not act as conventional ligands (Holland et al., 1996).

Ephrin-A5 and Ephrin-A2 appear to be involved in topographic mapping in the retinotectal system. Both ephrins are expressed in a posterior to anterior gradient in the tectum, with ephrin-A5 expressed in a steeper gradient, whilst the ephrin-A2 gradient is shallower and extends further into the anterior tectum (Drescher et al., 1995; Cheng et al., 1995). The receptor (Eph3) for these ligands is also expressed in a gradient in the retina and growing axons of the retinal ganglion cells. Experiments have examined the specificity of the
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receptor-ligand pairs and the reaction of retinal axons to changes in ephrin gradients. From these experiments it appears that temporal axons, which express high amounts of Eph3, enter the tectum but are repelled from the posterior portion by the action of ephrin-A2 (Monschau et al., 1997; Brennan et al., 1997; Picker et al., 1999).

In general the ephrins and Eph receptors appear to mediate their actions on axons through repulsive mechanisms. Axon pathfinding through somites initially occurs through the anterior rather than posterior portion and indeed the posterior portion has been shown to contain growth cone collapsing properties when co-cultured with motor and sensory axons and early migrating neural crest (Keynes et al., 1996). Restricted ephrin-B1 expression in the posterior half of the somite suggested that these molecules may impart the repulsive activity of the somite (Wang and Anderson, 1997). Neural crest cells express the EphB2 receptor and avoid clustered ephrin ligands in stripe assays. A similar repulsion activity is also noted for the motor axons. In C. elegans, expression of the VAB-1/Eph receptor in the ventral midline is required to prevent incorrect pathfinding by axons projecting to the head nerve ring (Zallen et al., 1999).

The role of Eph receptors in brain commissural pathfinding has been studied in mice lacking EphB2 function (Henkemeyer et al., 1996). The anterior commissure does not fully form but rather axons, which would normally contribute to this commissure, ectopically project to the ventral forebrain. EphB2 is normally expressed in the ventral forebrain territory suggesting that EphB2 functions in wildtype embryos to repel axons away from the ventral forebrain and towards the midline. Similar failure of commissural formation is observed in EphB3 deficient mice (Orioli et al., 1996). Recently a chemoattractive role for the ephrins and Eph family has been proposed during artery and vein formation (Adams et al., 1999). Other roles for the Eph family have been described in the embryo, including somite formation (Durbin et al., 1998; Holder and Klein, 1999).

In the zebrafish forebrain two ephrins are expressed in the hypothalamus in cells bordering the tract of the POC (Brennan et al., 1997; Macdonald et al., 1997). The expression patterns of these two ligands would suggest that they prevent ectopic axonal projections into the hypothalamus. Indeed these ligands may serve to facilitate the fasciculation of the POC as the commissure is initially loosely organised and becomes tightly fasciculated as development proceeds (Wilson et al., 1990). In support of this hypothesis, the normal expression of the two ephrins in noi mutants correlates to the correct exclusion of axons from the anterior hypothalamus (Macdonald et al., 1997).
1.6.8. Co-ordinated robo, slit and comm function in the midline.

In early development, most midline cells prevent axons from navigating to the contralateral side of the embryo. The repellent nature of the midline appears to depend on members of the Sema and Eph gene families. A particularly striking feature of the midline is that once axons have crossed, rarely do they cross again suggesting that the character of the axon is transformed by contact with the midline. In the Drosophila roundabout (robo) mutant, axons fail to respect the midline boundary and repeatedly traverse the midline (Seeger et al., 1993). The robo gene encodes a receptor-like protein of the Ig superfamily, which is abundantly expressed on the growth cones of non-crossing axons, downregulated whilst the axons cross and then upregulated after they have crossed the midline (Kidd et al., 1998). The regulation of this protein by midline cells apparently dictates the ability of axons to cross the midline.

Despite successive rescreens, no Drosophila mutants were identified with the same commissural phenotype as robo. However mild overexpression of another midline determinant, commissureless (comm), leads to a robo like phenotype whilst high overexpression of comm lead to a phenotype similar to that observed when the Slit protein is mutated (Kidd et al., 1999). The discovery of another Robo protein, Robo2, suggested that Comm may regulate both robo genes and that Slit may function as a ligand for both receptors. Kidd and colleagues tested the possibility that Slit may be the ligand for Robo by creating a double mutant. Heterozygotes of either genes do not have significantly perturbed axon guidance at the midline but heterozygotes for both genes have dramatic midline crossing defects suggesting that the proteins function in the same molecular pathway. Similarly expression of Slit in muscle cells results in the growth cones failing to extend over these cells. Further conformation of a functional interaction between Robo and Slit came through biochemical binding studies (Brose et al., 1999).

In vertebrates, Slit was isolated through biochemical purification of molecules that promote elongation and branching (Wang et al., 1999) and shown to bind Robo receptors with dissociation constants in the physiological range (Li et al., 1999). Slit acts at many sites during vertebrate development. Its initial characterisation as a factor which promotes branching within the rat spinal cord contrasts to the chemorepulsive properties described in Drosophila (Wang et al., 1999). However in the forebrain, Slit is required to repel olfactory axons from another midline structure, the septum (Li et al., 1999). Slit1/2 are expressed in the septum and complementary expression of Robo is detected in cells of the olfactory bulb. Furthermore the repulsion of olfactory axons by the septum can be mimicked by cells
expressing *Slit* (Wang *et al.*, 1999; Li *et al.*, 1999). The expression patterns of *Slit* and *Robo* in the cortex and hippocampus respectively suggests these two molecules may also underlie the repulsion of hippocampal axons by cortical territories (Li *et al.*, 1999). In vertebrates, the nature of Slit and Robo interactions are not yet fully understood. For instance Slit proteins can undergo cleavage to give either membrane associated or secreted forms, the significance of this cleavage is not understood (Wang *et al.*, 1999). The expression of *Slit* and *Robo* in the same territories also add a further level of complexity in elucidation of potential interactions between the two proteins.

1.6.9. *Other molecules involved in axon guidance.*

The process of axon guidance is a multi-step process (Holt and Harris, 1998). The molecules described above provide environmental cues for navigating axons. Commissural axons must be able to sense their environment and react accordingly. In *Drosophila* intracellular signalling molecules and cell adhesion molecules have been shown to mediate the response of axons to their environment (Chien, 1998). Mutations in the cytoplasmic proteins, *abl, disabled (dab), enabled (ena)* and the cell adhesion molecule, *fasciclin I*, lead to defects in commissural formation. Recent reports suggest these molecules maybe required for co-ordinating axon outgrowth and consequently pathfinding (Wills *et al.*, 1999). Future studies will hopefully address the regulation of these proteins in response to axon guidance cues such as, netrins.

1.7. *Zebrafish as a developmental model*

1.7.1. *Features of the zebrafish.*

Zebrafish have a number of features that facilitate isolation and characterisation of genes essential for vertebrate development (Streisinger *et al.*, 1981; Kimmel, 1989, Eisen, 1996). Zebrafish are small hardy, freshwater fish which can live in high density in relatively small tanks without adverse effects on their development or reproduction. A single female can lay a clutch of 200-300 eggs per week, which undergo external development. External development of the embryos allows study of all stages of development with relative ease. The embryos are transparent during early stages of development which allows investigators to follow the cell movements that contribute to the development of internal structures. Additionally external development of the embryos means that transplantation and ablation experiments can be performed. Optical clarity of older embryos can be maintained by
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growing the embryos in 1-phenyl-2-thiourea (PTU) without affecting development. These features allow comprehensive mutagenesis screens of zebrafish embryos to be performed.

Mutagenesis screens in invertebrates, such as *Drosophila* and *C. elegans*, have facilitated the isolation of many novel genes involved in all stages of development (Nüsslein-Volhard and Wieschaus, 1980; Brenner, 1974). Regulatory genes involved in specific processes can be isolated as screens will only detect genes with unique or at least partially non-redundant functions. Extensive genetic screens have recently performed to isolate mutations in genes affecting the development of the zebrafish. (Driever *et al.*, 1996; Haffter *et al.*, 1996). Chemically induced points mutations were generated with high efficiency in these screens. The large number of transparent, synchronous stage embryos were screened for mutants with obvious morphological traits using a dissecting microscope. Over 300 single mutants were isolated and a concerted effort to identify the genes responsible for the mutations by mapping or positional cloning has begun.

Extensive mapping of the zebrafish genome has started to facilitate the identification of genes through the positional cloning or candidate gene approach. Mapping has already helped elucidate the genes mutated in a number of mutants, such as *noi* which is caused by a mutation to *pax2.1* (Brand *et al.*, 1996). Positional cloning has also been used to identify genes. For example, the *oep* mutant was discovered to be caused by a mutation in a cripto-like gene (Zhang *et al.*, 1998). A further approach, that of comparative synteny, has been used to show how the zebrafish *you-too* mutant lacks functional *gli2* activity (Karlstrom *et al.*, 1999). Comparison of zebrafish and other vertebrate chromosomes reveals conserved synteny (gene linkage), which facilitates the identification of candidate genes for mutations.

Insertional mutagenesis mediated by retroviral vectors (Schier *et al.*, 1996) or transposons (Weinberg, 1998) to generate mutants. This method of mutagenesis allows mutated genes to be identified much more quickly and easily than through positional cloning or mapping. Pilot studies have yielded encouraging results for these screens and novel developmental genes have been identified, including *dead eye* and *pescadillo* (Allende *et al.*, 1996). A large mutagenesis screen using retroviral vectors has been initiated in Nancy Hopkins' laboratory and the results are eagerly awaited.

One 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 (Amores *et al.*, 1998). Despite subsequent loss of genes, genetic redundancy will be expected to occur in...
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zebrafish. However, duplication of the genome is likely to have led to two genes separating the functions of the single gene in tetrapods (Blader and Strähle, 1998) and so zebrafish may provide a powerful tool to dissect the functions of vertebrate genes. The development of reverse genetics in zebrafish, using ribozymes (Xie et al., 1997) or double-strand injection (Fire et al., 1998; Kennerdell and Carthow, 1998) will allow null mutants to be generated of known genes.

1.7.2. Stages of zebrafish development.

The stages of zebrafish development are well characterised (Kimmel et al., 1995). There are seven defined stages of development - zygote, cleavage, blastula, segmentation, pharyngula and hatching periods. All these stages occur over the first three days after fertilisation (Fig 1.6).

1.7.3. Development of the zebrafish brain.

The dorsal epiblast thickens abruptly near the end of gastrulation, approximately around the bud stage (10hpf). At this stage the neural plate becomes visible morphologically (Kimmel et al., 1995). The neural plate is thicker in the head region and the prechordal plate, hypoblast and polster lie underneath it. The neural plate is formed from a monolayer of pseudostratified neuroepithelium which is composed of columnar cells and so is raised above the adjacent non-neural ectoderm (Papan and Campos-Ortega, 1994). Further condensation of the neural plate leads to the formation of the neural keel. The ventricular system is formed through the cavitation of the neural plate and consequently the neural tube is formed.

This study focuses on the development of the zebrafish forebrain during late segmentation through to post hatching stages. During this time, the telencephalon can be distinguished from the diencephalon by the forming telencephalic ventricle. The brain undergoes a series of morphogenetic movements as development proceeds and these will be discussed later in this thesis. The general stages of interest are outlined in Figure 1.6.
Fig1.6. Summary of zebrafish development

(A-J) Camera lucida drawings of embryos at selected stages. Panel (A) represents the fertilised embryos and subsequent panels (B,C) show the embryo undergoing gastrulation. Soon after tailbud stage (C), somitogenesis begins to give rise to the 14 somite embryo shown in panel (D). Late somitoegensis and pharyngula stages are shown in panels (E-G). Hatching begins around 48hpf (H) and continues during the hatching period til 72hpf (J). (K) A photograph of a 5 day postfertilisation embryo.
Adapted from Kimmel et al. (1995).
Chapter Two

Materials and methods

2.1. Maintenance of Zebrafish

A breeding colony of zebrafish (Danio Rerio) were maintained at 28.5°C on a 14 hour light/10 hour dark cycle (Westerfield, 1993). Embryos were collected by natural spawning and staged according to Kimmel et al. (1995). Non pigmented embryos were generated by raising embryos, older than prim 2 (24hpf), in 0.2mM 1-phenyl-2-thiourea (Vischer, 1989) at 28.5°C.

Wild type embryos were generated from the kcwt, uwt, *AB, Tübingen and Tup Longfin strains. The recessive lethal mutant acerebellar\textsuperscript{li282a} (ace) (Brand et al., 1996), acerebellar\textsuperscript{li282a} x sonic you\textsuperscript{tbx392} (ace:syu) (Haffter et al., 1996) were used.

2.2. Observation of Live Embryos

Embryos were observed in fish tank water, and manually dechorionated with watchmakers forceps. Where required, embryos were anaesthetised with 0.02% tricaine (3-amino benzoic acid ethylester) and mounted for viewing in 3% methyl cellulose in fish tank water.

2.3. Molecular Biology Techniques

Standard molecular biology techniques were carried out according to Sambrook et al., (1989). For large scale DNA preparations, plasmids were transformed into E.Coli XL1 Blue and cultured in 100ml LB medium and ampicillin (70µg/ml). DNA was purified using a Qiagen Midi prep column (Qiagen).

Restriction enzyme digests were carried out in 20-100µl using an appropriate 1x enzyme buffer and 2-5 units of enzyme (Promega) per 1µg DNA. Digests were carried out at 37°C and checked on 0.8-1.5% agarose TAE gel by electrophoresis. Purification of DNA was carried out by phenol extraction and was precipitated with either 0.1 volume 3M NaAc and 2.5 volumes 100% ethanol at -20°C for 30 minutes, 0.25 volume 10 M NH₄Ac and 1 volume 1000% iso-propanol at room temperature for 10 minutes. Precipitated DNA was
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microfuged for 10 minutes, the pellet was washed in 70% ethanol and resuspended in TE buffer (10mM Tris-HCL, pH8.0, 1mM EDTA) or distilled water.

2.4. Whole In Situ Hybridisation to detect mRNA transcripts

2.4.1. Synthesis of antisense RNA probes for in situ hybridisation.

Templates for synthesis of antisense RNA probes were generated by linearising the clone at the 5' end with an appropriate restriction enzyme. Following phenol:chloroform extraction and precipitation (section 2.3.) the DNA was resuspended in low TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). 1 μg of the linear DNA was used for the in vitro transcription reaction, in which the RNA probe was labelled with either digoxigenin-11-UTP or fluorescein-11-UTP. A 20 μl reaction was set up in 1x transcription buffer (200 mM Tris-HCl, pH 7.5, 30 mM MgCl₂, 10 mM spermidine, 50 mM NaCl), 10 mM DTT, NTP-digoxigenin/fluorescein mix (1 mM ATP, CTP, GTP, 0.65 mM UTP, 0.35 mM UTP-digoxigenin/fluorescein), 40 units RNasin, 10 units T3/T7/SP6 RNA polymerase. The transcription mix was incubated at 37°C for 2 hours before 1 unit of DNase was added to remove the template. The DNase reaction was stopped after 15 minutes at 37°C by the addition of 1 μl 0.5 M EDTA, pH 8.0. The RNA was precipitated with 0.5 volumes 7.5 M NH₄Ac, 2.5 volumes 100% ethanol at -20°C for 30 minutes, washed in 70% ethanol and air dried. The RNA pellet was resuspended in 20 μl water and made up to 100 μl with prehybridisation mix (50% formamide, 5x SSC, 50 μg/ml heparin, 500 μg/ml torula RNA, 9.2 mM citric acid, 0.1% Tween-20), and used initially at a dilution of 1 in 200.

2.4.2. Single whole-mount in situ hybridisation.

Single whole-mount in situ hybridisation protocol was based on that of Thisse and colleagues (1993). Embryos were fixed in 4% paraformaldehyde in PBS, pH 7.4 (4% PFA), overnight at 4°C or for 3 hours at room temperature. Embryos younger than 20 hours post fertilisation (hpf) were dechorionated following fixation, and older embryos were dechorionated prior to fixation. Embryos were rinsed in PBT (PBS, 0.1% Tween-20), 50% PBS:50% methanol and stored in 100% methanol at -20°C.

Embryos were rehydrated with 5 minute washes of 75% methanol:25% PBS, 50% methanol:50% PBS, 25% methanol:75% PBS, followed by 4 washes in PBT, before prehybridisation at 65°C for 2 hours in prehybridisation mix. Embryos older than 100% epiboly (10 hpf) were treated with 10 μg/ml proteinase K at 20°C (in general, embryos were
treated 20 minutes per day of development), rinsed twice in PBT and refixed in 4% PFA for 20 minutes at room temperature. After refixation embryos were washed 5x 5 minutes in PBT before prehybridisation. The RNA probe was added and hybridised overnight at 65°C.

Post hybridisation washes were carried out at 65°C. Embryos were rinsed in prehybridisation mix, and then 15 minute washes with 75% prehybridisation mix:25% 2x SSC, 50% prehybridisation mix:50% 2x SSC, 25% prehybridisation mix:75% 2x SSC, 100% 2x SSC. This was followed by 2x 30 minute washes in 0.2x SSC.

Embryos were washed into antibody block at room temperature. 5 minute washes in 75% 0.2x SSC:25% MAB (0.1 M Maleic Acid, 0.15 M NaCl, pH 7.4), 50% 0.2x SSC:50% MAB, 25% 0.2x SSC:75% MAB, and 100% MAB. Embryos were blocked in 2% Boehringer block (Boehringer Mannheim) in MAB for 2 hours. The embryos were incubated in the appropriate antibody, either anti-digoxigenin-alkaline phosphatase F_{ab} fragments (1 in 5000) or pre-absorbed anti-fluorescein-alkaline phosphatase F_{ab} fragments (1 in 2000) overnight at 4°C.

The antibody was washed off 8x 15 minutes in MAB at room temperature. The embryos were equilibrated 3x 5 minutes in developing buffer (0.1 M Tris, pH 9.5, 50 mM MgCl₂, 0.1 M NaCl, 0.1% Tween-20). The embryos were developed in BM Purple Substrate (Boehringer Mannheim) in the dark. The colour reaction was stopped by rinsing 3x PBT, and refixing. Embryos were stored and photographed in 70% glycerol in PBS.

2.4.3. Double whole mount in situ hybridisation.

Double whole-mount in situ hybridisation was carried out following the same methodology of Hauptman and Gerster (1994). The methodology was the same as the single whole-mount in situ hybridisation unless otherwise stated. Two RNA probes were used in the hybridisation mix, one labelled with fluorescein-11-UTP and the other with digoxigenin-11-UTP.

Anti-fluorescein-alkaline phosphatase F_{ab} fragments (1 in 2000) was incubated first, and washed as previously described. The embryos were equilibrated 3 x 5 minutes in the developing buffer (0.1M Tris-HCL, pH8.2, 0.1% Tween-20). The first RNA probe was stained with 1 Fast Red tablet (Boehringer Mannheim) per 2ml of developing buffer.
After the first colour reaction was complete, the embryos were washed 3 x in MAB before inactivating the primary antibody with 4 washes in 0.1M glycine-HCl, pH2.2, 0.1% Tween-20. The embryos were washed 5 x 5 minutes in MAB, blocked for at least 5 minutes before incubation in the anti-digoxigenin-alkaline phosphatase $F_{ab}$ fragments (1 in 5000). Embryos were washed and second colour (purple) was developed as for single whole mount in situ hybridisation. Embryos were washed, refixed and analysed in 70% glycerol.

The colour reactions were also carried out the other way around, i.e. the first antibody was detected using BMP purple substrate and the second with another red colour substrate, p-lodoneitrotetrazolium Violet (Sigma). The remainder of the protocol was performed as described above.

2.5. Whole mount antibody labelling

Standard procedures were used for antibody labelling embryos ranging from prim 5 to 5 days post fertilisation (dpf) (Wilson and Easter, 1990). Embryos were fixed in 4% PFA for 3 hours at room temperature or 4°C overnight; for the N-acetylated tubulin antibody, embryos older than prim 22 were fixed in 2% TCA for 3 hours at room temperature. Following fixation, the embryos were washed in 1 x IB (1 x PBS containing 0.7% TritonX-100 and 0.5% DMSO). Embryos older than prim 22 were treated with 10% trypsin for varying times prior to incubation with the primary antibody. After preincubation for 2 hours in IB containing 10% goat serum, incubation in the primary antibody was done in the presence of IB solution containing 1% goat serum overnight at 4°C. The embryos were washed for over 4 hours in several changes of IB and incubated with the HRP conjugated secondary antibody in IB containing 1% goat serum overnight at 4°C. Embryos were washed as before and the presence of the antigen was detected using diaminobenzidine (DAB) as the chromogen washed with IB and fixed overnight in 4% PFA. The embryos were analysed in 70% glycerol. Antibodies to N-acetylated tubulin (Sigma), HNK-1 (Sigma), zns2 (Zebrafish antibody stock centre), Pax6 (MacDonald et al., 1996) and anti phosphorylated histone H3 (Upstate Biotech) were used according to the above protocol.

For antibodies to islet-1 (Hybridoma bank), Tyrosine Hydroxylase (Sigma) and Green Fluorescent protein (Sigma), a modified version of the Vectastain protocol was deployed (Chandrasehkar et al., 1998). After incubation in a biotin conjugated secondary antibody for 6 hours at room temperature, the embryos were washed 3 x 30 minutes in IB and were incubated in Vectastain A + B solutions overnight at 4°C, washed several times in IB and detected as before using DAB.
2.0 Materials and methods

2.5.1. **Double whole mount in situ hybridisation and antibody labelling.**

For double labelling studies with RNA probes and antibodies, the standard in situ hybridisation protocol was followed but the primary antibody was incubated with the anti-Digoxigenin antibody. Following detection of the RNA probe, the embryos were washed 5 x 5 minute in 1 x PBT and incubated in the secondary antibody overnight at 4°C. Detection of the antibody was as described before.

2.6. **Detection of apoptotic cell death**

Detection of apoptotic cell death using Terminal deoxynucleotidyl transferase nick end labelling (TUNEL). The method of TUNEL used was a modification of the protocol suggested by the manufacturer (ApopTag In situ Apoptosis Detection Kit - Peroxidase; Oncor Inc.).

Embryos were manually dechorionated, fixed in 4% paraformaldehyde overnight, washed in PBS and stored in methanol at -20°C. After rehydration in PBT (PBS, 0.1% Tween 20), embryos were permeabilized by digestion with 10µg/ml proteinase K in PBS (20 mins for post 14 somite staged embryos). The embryos were then postfixed for 20 minutes in 4% paraformaldehyde in PBS and washed 5x 5 minutes in PBT. Next, embryos were postfixed with prechilled (-20°C) ethanol: acetic acid 2:1, washed 3x 5 minutes in PBT and incubated for 1 hour at room temperature in equilibration buffer (provided in the ApopTag In situ Apoptosis Detection Kit-peroxidase).

After incubation overnight at 37°C in working strength terminal deoxynucleotidyl transferase (TdT) enzyme, the DNA end labelling reaction using digoxigenin-labelled dUTP was stopped by washing in stop/wash buffer. Digoxigenin-tagged DNA was detected using sheep anti-digoxigenin alkaline phosphatase conjugated Fab fragments (Dig Nucleic Acid Detection Kit, Boehringer-Mannheim). For both cell death and mitosis studies, cells were counted in frontal views of whole embryos encompassing a territory bounded dorsally and ventrally by the anterior and postoptic commissures and laterally by the position at which the optic stalk separates from the forebrain neuroepithelium.

Cells were counted in frontal views of whole embryos encompassing a territory bounded dorsally and ventrally by the anterior and postoptic commissure and laterally by the position at which the optic stalk separates from the forebrain neuroepithelium.
2.7. Plastic embedded sectioning

Samples were rehydrated stepwise from MeOH or 70% glycerol into 100% EtOH, using 1 minute washes in the following series: 30% EtOH, 50% EtOH, 70% EtOH, 90% EtOH, 100% EtOH. Once in 100% EtOH, the samples were transferred to Activated A solution (Leica) using an intermediary of 50% EtOH, 50% Activated A solution and left for 30 minutes at room temperature. The samples were transferred to coffin moulds after incubation in activated A and solution B (mixed in the ratio 3:1). The coffin moulds were placed in a sealed box, air evacuated using N₂ and left overnight.

Once the blocks had solidified, they were cut and mounted on sectioning blocks using Riechert-Jung historessin. 5-10μM sections were produced using Jung 20555 Autocut, equipped with Leica tungsten carbide blades. The sections were placed in water drops on slides, dried and counterstained with toulene blue. The slides were fixed with DPX and analysed using microscopy.

2.8. Analysis of cell morphology using Bodipy Ceramide

Embryos were placed in embryo medium (Kimmel et al., 1995) containing 60 pg/μl Bodipy Ceramide at 31°C for 2 hours. The embryos were washed 3 x 5 minutes in embryo medium and anaesthetised using 0.02% tricane. The embryos were mounted in 1%agarose and analysed with a LEITZ DM IRB confocal microscope, equipped with a TCS 4D Argon/Krypton laser and SCANware 5 software.

2.9. Injection of mRNA into early stage embryos

2.9.1. Cloning of emx1 and emx2 constructs into expression vectors.

For synthesis of RNA for micro-injection emx1 and emx2 constructs were cloned into pUT-2, a derivative of pBluescript which flanks the cloned sequence with the untranslated regions of the β-globin gene taken from pSP64T. The emx1 and emx2 sequences with FLAG epitopes at the 3’ end were produced by PCR using specific primers, and subsequently cloned into the vector using unique restriction sites.

2.9.2. Other sequences used for RNA synthesis.
The other construct used for RNA synthesis was green fluorescent protein and dominant negative FgfR (Griffin et al., 1995).

2.9.3. *In vitro transcription of mRNA for injection.*

In vitro transcriptions were carried out using the Ambion Megascript Kit (Ambion). In a total reaction volume of 20 μl, 1 μg linear DNA template, 1x transcription buffer, 5 mM ATP, 5 mM CTP, 5 mM UTP, 1.5 mM GTP, 6 mM 7mG(5') ppp(5')G sodium salt (capGTP), and 2 μl SP6 or T3 RNA polymerase enzyme mix were used. Transcription reactions were incubated for 2 hours at 37°C. The DNA template was removed by a 15 minute incubation with DNase.

The reaction was stopped by the addition of 115 μl dH2O and 15 μl 3 M NaAc. The RNA was extracted twice with phenol:chloroform and then twice with chloroform. The RNA was precipitated by the addition of 375 μl 100% ethanol, and incubation for at least 30 minutes at -20°C. The RNA was microfuged for 15 minutes at 4°C, washed in 70% ethanol, and resuspended in 20 μl dH2O.

RNA was cleaned and concentrated using a microconcentrator (microcon 100, Amicon), microfuging at 4°C. RNA concentration was determined spectrophoto-metrically.

2.9.4. *Injection of mRNA into early stage embryos.*

Embryos, still in their chorions, were aligned in a plastic trough. RNA was injected in a volume of approximately 200 pl into one cell of a 1-4 cell stage embryo, using a glass capillary needle attached to a Picospritzer. The injected embryos were left to develop in fish tank water at 28.5°C, and unfertilised embryos were discarded during blastula stages. The number of remaining embryos were noted.

The amount of RNA injected was, for the emx constructs 10-100 ng/μl. The distribution of injected RNA in vivo was determined by co-injecting with RNA encoding GFP (at 130-150 ng/μl). The two species of RNA co-injected broadly segregate together during development (Griffin et al., 1995), and the distribution of GFP can subsequently be determined by whole mount antibody staining (section 2.5). Attempts to use antibodies to the 3' FLAG epitope were unsuccessful.

2.10. *Administering drugs to zebrafish*
Embryos were treated with a drug to block FgfR activity (Mohammadi et al., 1997). 60% epiboly stage embryos were incubated in SU5204 containing embryo medium at 30.5°C (Sigma). SU5204 was initially dissolved in DMSO and used at varying concentrations from 8µm to 20µm. Embryos were allowed to develop to prim 12 stage, fixed and analysed.
Chapter Three

Characterisation of morphology, gene expression, axon guidance and proliferation in the zebrafish telencephalon

3.1. Introduction

The striking conservation of midbrain and hindbrain components of the Central Nervous System during vertebrate evolution is apparent through their positions, cytoarchitecture, connectivity, neurochemistry and gene expression patterns. The rigorous description of the adult forebrain in many different species, together with comparative analysis between species, has also helped map out the functional organisation of various vertebrate forebrains and advanced our understanding of the overall phylogeny of vertebrates (Butler and Hodos, 1996). However this structural complexity and variability does not reveal a consensus, shared by researchers, regarding the homology of different forebrain structures.

The lack of obvious homology between adult telencephalic structures is particular striking. In general the adult telencephalon can be divided into a dorsal component, the pallium, and a ventral component, the subpallium. During the early stages of development, the neural plate undergoes neurulation to form the neural tube. In most vertebrates, subsequent stages of forebrain development are dominated by the process of evagination. During evagination, the dorsal telencephalon undergoes a disproportionate growth which results in dorsal structures becoming the most medial adult telencephalic structures and the ventral most pallium is displaced laterally. In vertebrates which undergo evagination, the pallium is divided into three domains; medial (limbic), dorsal pallium, and lateral pallium. In mammals these domains can be further subdivided, such that the medial pallium contains the components of the limbic system, such as the hippocampus, dentate gyrus and Ammon's Horn. The dorsal pallium is composed of the neocortex and the lateral pallium, the olfactory cortex (Fig3.1). The development of the layered neocortex is thought to be a recent evolutionary event specific to mammals (Karten, 1997).

The ventral sub-pallium also gives rise to a defined set of structures. Typically the sub-pallium is arranged into the striatum and the medially positioned septum. In mammals, the striatum have been extensively studied and can be further subdivided into striatal (also known as the lateral ganglionic eminence) and the pallidum structures. The dorsal pallidum
3.0 Characterisation of the developing zebrafish telencephalon

Fig 3.1. Representation of a transverse section through the rat forebrain
The telencephalon can be broadly divided into dorsal and ventral structures. The dorsal component of the rat telencephalon is composed of the limbic (red), dorsal (blue) and lateral (green) pallium. Extensive structural and functional studies have further delineated structures such as the hippocampus, Ammon's horn and dentate gyrus in the limbic pallium. The dorsal pallium is composed almost exclusively of the isocortex (also known as the neocortex). The lateral pallium is also known as the olfactory or piriform cortex. The ventral part of the telencephalon is composed of the striatum (also known as the basal ganglia) and the medially positioned septum. The striatum is further divided into the striatum and pallidum. A dorsal part of the striatum, called the caudate-putamen (purple), is depicted in this section. An intermediary domain, composed of the amygdala and claustrum (yellow), is positioned between the pallium and subpallium. Fibre tracts and diencephalic structures have been left uncoloured in this figure.
Adapted from Butler and Hodos, 1996
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is also referred to as the medial ganglionic eminence and will give rise to the adult globus pallidus (Smart and Sturrock, 1979). The dorsal part of the septum is connected to the limbic pallium. The claustrum and amygdala are composed of associated groups of nuclei, which are located at the boundary between the pallium and sub-pallium. In mammals, the origin and positions of two structures are disputed (Marin et al., 1997).

When the adult zebrafish is examined, further difficulty in identifying homologous telencephalic domains arise because the teleost telencephalon undergoes different morphogenetic event to the telencephalons of other vertebrates. The zebrafish dorsal telencephalon becomes everted during development, such that the parts lying dorso-medially in other vertebrates come to be positioned at the lateral flap-like extensions of the dorsal telencephalon. Analysis of the adult zebrafish reveals two broad domains, the area dorsalis and area ventralis (Wulliman et al., 1995) and the positions of specific groups of nuclei and axon tracts. However assignment of homology to specific areas has been hampered by the contrasting morphogenetic events underlying the development of the teleostean and evaginated vertebrates.

In search of homologous divisions of the forebrain, attention has increasingly focused on the genetic basis of development. Recent insights into possible brain homologies came from the analysis of vertebrate homologues of genes first identified in Drosophila. The expression domains of many these genes, notably the Hox genes, along the main body axes appears to be conserved throughout evolution. Further analysis of vertebrate homologues of genes involved in Drosophila head segmentation, such as orthodenticle (otd) and empty spiracles (ems), revealed zonal, overlapping expression patterns suggestive of a segmental organisation of the vertebrate brain (Simeone et al., 1992). Initial studies of these genes suggested that they may be used as a powerful tool to understand the underlying principles of vertebrate brain development and the mechanistic variations that have lead to the diversity of brain structures observed in the telencephalon of different vertebrates.

Analysis of gene expression domains in the forebrain has also lead to renewed interest in and support for neuromeric models of brain segmentation. By interpreting gene expression and morphological constrictions, Puelles and Rubenstein (1993) proposed a revised neuromeric model which divides the forebrain into 6 distinct prosomeric regions. Although the evidence for the diencephalic prosomeres continues to accumulate, recent evidence has cast doubt on the positions and organisation of the three telencephalic prosomeres (Figdor and Stern, 1993; Fishell, 1997). A neuromere/segment can only be considered unique if it fulfils a carefully defined set of criteria based upon morphology,
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lineage restriction, identity of neuronal elements, restricted gene expression and patterns of neurogenesis (Guthrie, 1995; Papalopulu, 1995). Numerous lineage tracing studies have concluded that tangential migration occurs through the proposed telencephalic neuromeres, the implications of these studies severely test the lineage restriction, morphological and neuronal element identity aspects of the neuromeric model (Anderson et al., 1997). The strongest evidence for longitudinal and transverse prosomeres has come from gene expression studies (Bulfone et al., 1993; Puelles and Rubenstein, 1993). However studies on zebrafish and Xenopus forebrain gene expression have suggested that the neuromeres are organised in radial territories rather than the transverse domains suggested for the mouse (Macdonald et al., 1994; Papalopulu, 1995).

Despite these concerns about neuromeric models, the comparative analysis of restricted gene expression, axonal connectivity and neurochemistry between species has proved invaluable in defining homologous structures (Karten, 1997). In this study, I have sought to characterise the development of the zebrafish telencephalon. Although the early development of the zebrafish forebrain has been extensively studied, relatively little research has addressed development past late somitogenesis stages. I have undertaken morphogenetic studies, primarily to characterise the events underlying late telencephalic development in the zebrafish. Differential zones of proliferation are responsible for many of the morphological features observed in the adult central nervous system. For example, the dorsal expansion or évagination of the mammalian telencephalon results from disproportionate proliferation in the cerebral cortex compared to other telencephalic structures (Butler and Hodos, 1996). Thus to appreciate how the zebrafish forebrain develops morphologically and genetically, we must understand where and how proliferation occurs. The eversion of the teleost brain has made comparative anatomical studies difficult in the adult and the study of the processes previously outlined during development will facilitate the identification of homologous telencephalic structure.

I have demonstrated that eversion occurs relatively late in zebrafish telencephalic development, but continued proliferation in this territory probably gives rise to the large dorsal area in the adult. Proliferation also becomes localised to the ventricles, as observed in other vertebrates, and later refinement leads to more specialised areas of proliferation. Gene expression analysis unveils the presence of three telencephalic domains, which have been described in other vertebrates (Fernandez et al., 1998, Puelles et al., 1999). Distinct neuronal populations can also be detected, using gene expression studies of genes involved in differentiation of neurons. Identification of these neuronal populations will aid studies of control of neurogenesis and axonal projections in the future.
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I would like to acknowledge the help of Sam Cooke and Susan Chapman, who performed some of the plastic sectioning used in this study.

3.2. Results

3.2.1. Telencephalic organisation can be visualised through gene expression but not morphology at the 18somite stage

3.2.1.1. Little discernible histological organisation of the 18 somite stage zebrafish telencephalon.

As relatively little is known about late morphogenetic events in zebrafish telencephalic development, an initial histological study was performed. By the 18 s stage, the neural tube has started to bend at the ventral flexure but is still relatively straight with regard to the axis of the caudal neural tube. The fluid filled ventricle system has started to develop throughout the central nervous system and the forming forebrain ventricle is used as a landmark to distinguish the telencephalon. In the dorso-ventral (DV) axis, the optic recess (OR) component of the ventricle separates the telencephalon from the diencephalon (Fig3.2A,B). In sagittal sections, the optic recess extends from the rostral optic stalk territory to the dorsal diencephalon. The layer of pale blue cells at the anterior end of the embryo are cells of the olfactory placode, which forms a large, uncondensed structure at this stage (Fig3.2A,B,K). Proliferation occurs throughout the body of the telencephalon and cellular organisation within the telencephalon is not apparent at this stage (Fig3.2A,B; Delaney, 1999).

3.2.1.2. dlx2 and emx1 divide the telencephalon into 3 molecularly distinct regions.

In addition to investigating telencephalic morphology using plastic sectioning, telencephalic gene expression was analysed. Gene expression can reveal additional morphological features, suggest functional interpretations, delineate structures and be used for comparative evolutionary analysis between species. I used a variety of genes to characterise late development of the zebrafish telencephalon. emx1 is a vertebrate homologue of the Drosophila homeodomain transcription factor, ems. A requirement for the vertebrate Emx genes in telencephalic development has been described and the wealth of data on emx expression allow good comparative studies to be performed between vertebrates (Gulisano et al., 1996; Yoshida et al., 1997; Pannese et al., 1998; Fernandez et
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**Fig 3.2. Telencephalic organisation can be visualised through gene expression at the 18 somite stage**

(A,B) Sagittal sections of embryos counterstained with toluidene blue. The levels of sections, which progress towards the midline, are indicated in panel (J). (A,B,K) Anterior cells constitute the olfactory placode and differences in telencephalic morphology can not be clearly distinguished. (C-E) Lateral views embryos stained with digoxygenin and fluorescein labelled RNA probes for (C) *emxl*, (D) *dlx2*, (E) *emxl* (blue) and *dlx2* (red). *emxl* and *dlx2* are expressed in dorsal and ventral telencephalic domains. A small group of cells do not express either *emxl* or *dlx2* and can be seen in (F) anterior views of the same embryos (see arrow in F). (G-I) Lateral and (J) dorsal views of embryos stained with digoxygenin labelled RNA probes for (G) *eome*, (H) *nk2.1*, (I) *ngn1* and (J) *neuroD*. (G, H) *eome* and *nk2.1* are expressed in dorsal telencephalic and hypothalamic regions of the embryo. (I,J) Neurogenesis can be visualised through the expression of proneural genes, like *ngn1*, and genes involved in neuronal differentiation, such as *neuroD*. Both genes are strongly expressed in the olfactory placodes and weaker expression is detected in the dorsal telencephalon. (K) A schematic diagram of the cellular organisation of the telencephalon at the 18 somite stage. (L,M) The complementary expression domains of *Dlxl/2* and *Emxl* exist in zebrafish (L) and mice (M). Both genes show restricted expression in the dorsal and ventral telencephalon and an intermediate domain (ID), which doe not express either gene, can be distinguished in both species. All embryos are at the 18 somite stage. Scale bars represent 40μm and 400μm in (M). Abbreviations: d, diencephalon, e, eye, hy, hypothalamus, id, intermediate domain, op, olfactory placode, or, optic recess, os, optic stalk, t, telencephalon, te, tectum, zli, zona limitans intrathalamica.
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emxl mRNA is first detected in the anterior neural plate around 100% epiboly. Expression is detected strongly in two lateral stripes and weakly at the tip of the anterior neural plate. As the neural plate closes, emxl is detected in cells in presumptive telencephalon (data not shown). A gradual refinement of expression occurs to a dorsal telencephalic territory, such that by the 18s expression is observed throughout a dorso-caudal territory (Fig3.2C,F).

Dlx2 is a vertebrate homologue of the Drosophila homeodomain transcription factor, distal-less, and is an important mediator of ventral telencephalic development (Anderson et al., 1997a). dlx2 mRNA is first expressed in a cluster of ventral forebrain cells at the 5 somite stage and soon afterwards expression in a more dorsal territory is detected (Akimenko et al., 1994 and data not shown). At the 18 s stage, dlx2 expression is detected in ventral telencephalic cells adjacent to the optic recess (Fig3.2D). dlx2 is weakly detected in medial telencephalic cells and more strongly in lateral cells. The dorsal limit of telencephalic dlx2 expression is adjacent to the ventral limit of emxl (Fig3.2E,F).

Examination of dlx2 and emxl in mice, chicks, Xenopus and turtles, at a stage when neuronal differentiation begins in the telencephalon, has revealed the conserved presence of a domain between dlx2 and emxl expressing territories (Fernandez et al., 1998). I performed double in situ hybridisation studies to discover whether a similar territory exists in zebrafish. In the rostral telencephalon, dlx2 and emx2 are expressed in separate longitudinal territories; emxl is expressed dorsally, dlx2 is expressed ventrally and the two domains are separated by a small group of cells, which do not express either gene (Fig3.2E,F; arrows indicate non dlx2/emxl expressing domain). Coronal sections reveals the position of the group of cells not expressing either emxl or dlx2. Like similar domains described in other vertebrates, the domain extends from the ventricular surface to the lateral limit of the telencephalon but is only 2-3 cells wide in the 18s zebrafish embryo (Fig3.2L,M; Fernandez et al., 1998). The group of cells which do not express either dlx2 or emxl will be referred to as the intermediate domain (ID). In the zebrafish telencephalon emxl expression appears to delineate the dorsal pallium as it does in other species, similarly dlx2 expression delineates the subpallium (Fig3.2L,M).

3.2.1.3. eome and nk2.1 are expressed in the dorsal telencephalon and rostral diencephalon respectively.

Gene expression analysis in other vertebrates has shown the forebrain can be broadly divided into prosomeres or segments by the examination of a number of genes. In addition
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to *emx1* and *dlx2*, the other two genes widely used for prosomeric analysis are the transcription factors, *tbrl* and *nkx2.1* (Price, 1993; Rubenstein *et al.*, 1994, Bulfone *et al.*, 1995; Puelles *et al.*, 1999). For this study, we have used the zebrafish homologues of *nkx2.1* and *eomesodermin* (*eome*). *Eome* was originally thought to be the homologue of mouse *tbrl* but further analysis revealed it was more homologous to mouse and *Xenopus eome*. However studies of *Tbrl* and *Eome* expression in the mouse forebrain have revealed that the two T-box transcription factors differ in their expression in mitotic and post-mitotic cells but are expressed in the same broad telencephalic domains (Ciruna and Rossant, 1999).

*eome* is first expressed in one or two cells in the dorsal telencephalon at 5 s. Expression increases in the dorsal telencephalic territory, such that at 18 s *eome* is still expressed in the dorsal telencephalon in a subset of *emx1* expressing cells (Fig 3.2G and data not shown). *nk2.1* is consistently expressed in the mouse hypothalamus and ventral pallidum throughout development. In zebrafish, *nk2.1* expression is detected during late gastrulation in the axial midline tissue. After neurulation occurs, *nk2.1* continues to be expressed in the axial midline tissue of the hypothalamus. At 18 s, *nk2.1* is expressed throughout the hypothalamus but is not excluded from a thin layer of cells lining the OR and cells in the presumptive posterior hypothalamus (Fig 3.2H and data not shown).

3.2.1.4. Neurogenesis within the developing telencephalon.

Although the genes described above probably have important roles in specifying neurons within their expression domains, they have not been shown to be directly involved in the general neurogenic pathway. Vertebrates have several different homologues of the *Drosophila* neurogenic genes, which are responsible for neuronal specification in a number of different regions. *ngn1*, a basic Helix-Loop-Helix transcription factor, was initially isolated in *Xenopus* but has been shown to have proneural gene activity in a number of different species (Ma *et al.*, 1996). *neuroD*, another bHLH gene, has shown to be required in the initial stages of neuronal differentiation (Lee *et al.*, 1995). Studying the expression pattern of these two genes will give us a valuable insight into where neuronal determination and differentiation occur in the zebrafish telencephalon.

*ngn1* is initially expressed in a subset of the *emx1* expressing cells in the anterior neural plate at the tailbud stage (data not shown). Upon neural tube formation, *ngn1* continues to be expressed weakly throughout the telencephalon. At 18 s, strong *ngn1* expression is detected in the olfactory placode and weak expression is detected in the dorsal half of the telencephalon (Fig 3.2I). *neuroD* is expressed later than *ngn1* around 3 somites in
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cells in the dorsal telencephalon (data not shown). By 18s \textit{neuroD} is strongly expressed in
the olfactory placode and more weakly in a small dorsal subset of \textit{ngn1} expressing
telencephalon (Fig3.2J).

3.2.1.5. Summary of the 18 s zebrafish telencephalon.

Although no cellular organisation of the telencephalon can be detected in the 18 s
embryo (Fig3.2K), gene expression studies reveal that the telencephalon is already well
patterned. Regulation of gene expression at this stage of development appears to be
generally conserved between mice and zebrafish. Restricted expression of \textit{emxl} and \textit{dlx2}
distinguish the pallium and sub pallium respectively (Fig3.2L,M). A third non-\textit{emxl/dlx2}
expressing domain is observed, which also correlates to similar domains observed in other
species (Fernandez \textit{et al.}, 1998). Furthermore zebrafish \textit{eome} is initially expressed in a
subset of \textit{emxl} expressing cells (Ciruna and Rossant, 1999). Slight variations in gene
expression do exist between species as unlike mice, zebrafish \textit{nk2.1} is not expressed in the
telencephalon but is restricted to the hypothalamus. The neurogenesis pathway, which
utilises \textit{ngn1} and \textit{neuroD}, appears to be restricted to the dorsal telencephalon. Numerous
other proneural genes can be utilised by embryos to generate neurons and genes, such as the
zebrafish homologues of \textit{Drosophila acheate-scute} genes. Different regions of the
telencephalon probably utilise different proneural genes and indeed other proneural genes
are expressed in different domains to \textit{ngn1} (Allende and Weinberg, 1994).

3.2.2. Histological organisation of the telencephalon is apparent at prim 5 (24hpf)
stage

3.2.2.1. Cellular and proliferative organisation of the telencephalon.

By prim 5, the forebrain has undergone further bending at the level of the zona
limitans intrathalamica (ZLI) such that the forebrain lies at a $90^\circ$ downward angle to the rest
of the neural tube. The consequence of this flexure is that anterior forebrain structures have
become more ventral and dorsal structures more anterior. The ventricular system has
become enlarged, and the telencephalic ventricle clearly separates the two hemispheres.
Commissural pathways can also be detected using a monoclonal antibody against the
neuronal acetylated form of tubulin. Although the term, 'tract of the commissure' and
'commissure' will here refer to the same continuous bundle of axons, I will use the term,
'commissure' to refer to the bundle as it crosses the midline. The term, 'tract of the
Fig3.3. Cellular organisation, axon guidance and proliferation in the prim 5 stage embryo

(A-D) Sagittal sections of embryos counterstained with toulidene blue. The level of sections, which again start laterally and proceed to the midline, are indicated in panel (G). (A) More lateral sections are characterised by anterior position olfactory placode cell. (B-C) As sections are made medially, a primitive layering organisation can be detected. Cells lining the optic recess are darkly stained and form a thin layer. More dorsally, post mitotic, pale cells which contribute to the anterior commissure are detected form the differentiation zone (DZ). (D) Sections, at or near the midline, are characterised by dense, darkly stained cells. (E, F) Coronal sections of embryos, also counterstained with toulidene blue. At the midline, cells are quite darkly stained and densely packed. Within the central body of the telencephalon, cells are more loosely packed. Lines in (H) indicate the level of the sections. (G) Anterior view of an embryo stained with an anti-N-acetylated tubulin antibody to reveal the positions of the nascent anterior commissure (AC) and the postoptic commissure (POC). (H) Dorsal view of an embryo stained with a marker for mitotic cells, an antibody for phosphorylated histone H3 (PH3). Cells, in a proliferative state, are localised to the ventricles. PH3 positive cells in a group of anterior cells (marked by asterix) may signify a special population of cells. (I, J) Schematic diagrams of morphological organisation and proliferation in the prim 5 embryo. Proliferative cells are restricted to the ventricles with more differentiating cells away from ventricle. All embryos are at the prim 5 stage. Scale bars represent 40μm.

Abbreviations: ac, anterior commissure, dz, differentiation zone, d, diencephalon, e, eye, hy, hypothalamus, op, olfactory placode, or, optic recess, poc, postoptic commissure, pz, proliferative zone, t, telencephalon, tv, telencephalic ventricle.

nac, nuclei of the anterior commissure, tpc, tract of the posterior commissure, dvdt, dorso-ventra diencephalic tract.
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The nascent AC can be seen in sagittal sections as a ventral, thin, pale cell free area (Fig3.3B,C,I). Cells surrounding the AC are pale with relatively large nuclei and probably are differentiating neurons that contribute to the AC. I will refer to the territory containing the AC and differentiating neurons as the differentiation zone (DZ). The DZ is bordered dorsally and ventrally side by darkly stained small cells (Fig3.3B,C,I). These cells line the ventricles and fill the entire telencephalon if sections are taken at the midline (Fig3.3D). To verify that these cells are indeed mitotic, embryos were labelled with an anti-phosphorylated Histone H3 (PH3) antibody, which detects cells in G2/M phase of mitosis (Wei et al., 1998). Expression of PH3 is restricted to cells lining the ventricle and is coincident with the positions of the darkly stained cells in the sections (Fig3.3H,J and data not shown). Coronal sections reinforce the view that mitotic cells are restricted to the ventricular surface, whilst differentiating neurons are present in the more lateral aspects of the telencephalon as cells lining the ventricles are darkly stained whilst cells in the body of the telencephalon are much paler (Fig3.3E,F).

The coronal sections indicate the position of the olfactory placode (Fig3.3E,F), which is also detected as the most anterior structure in lateral sagittal sections (Fig3.3A). PH3 labelling reveals mitosis is restricted to the basal region of the olfactory placodes (Fig3.3H,J).

In summary, cellular organisation of the telencephalon is apparent by prim 5 (Fig3.3I). The restricted of proliferation to cells at the midline is probably responsible for this primitive organisation as post mitotic cells must migrate away from the proliferative zone (Fig3.3I,J). Once these post mitotic cells have migrated into the main part of the telencephalon, they start to differentiate and contribute axons to the tract of the AC and the SOT.
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3.2.2.2. *dlx2* and *emx1* maintain dorsal and ventral telencephalic gene expression territories.

To further assess the changes in telencephalic development, I investigated the expression of the genes examined at earlier stages. At prim 5 stage *dlx2* expression, in the telencephalon, remains restricted to a ventral territory, representing the zebrafish sub-pallium (Fig 3.4A). However *dlx2* expression is weak in a 1-2 cell layer adjacent to the telencephalic midline (Fig 3.4B,C,G). Comparison with the tubulin stained embryos reveals that the AC axons will navigate through the *dlx2* positive telencephalic territory (compare Fig 3.3G and 3.4B).

In the diencephalon, *dlx2* expression is still expressed in a broad dorsal territory along the optic recess (Fig 3.4A). The expression of *dlx2* in post mitotic cells is consistent with expression observed in mice, where *dlx2* is initially expressed in both mitotic and post mitotic cells of the sub-pallial ventricular and sub-ventricular zone but becomes restricted to post mitotic cells as development proceed (Bulfone et al., 1993). *emxl* is also still expressed in a broad dorso-caudal territory in the telencephalon but expression is weak in the most medial cells (Fig 3.4D,E).

The effects of 6 hrs of development on the three telencephalic zones was assessed by performing double *in situ* hybridisation using probes to *dlx2* and *emx1*. The pallial, subpallial and ID regions are again revealed by the expression domains of *dlx2* and *emx1* (Fig 3.4F,G,O,P). The ID continues to stretch from the ventricle to the lateral walls of the rostral telencephalon. The *dlx2* and *emx1* expression domains may overlap slightly in the caudal telencephalon (Fig 3.4F). The expression domains of *emx1* and *dlx1* also overlap in the caudal telencephalon of mice and chick (Fernandez et al., 1998).

3.2.2.3. Refinements to the *eome* and *nk2.1* expression domains.

*eome* mRNA is detected in the dorso-caudal telencephalon, in a similar domain to *emx1* (Fig 3.4J). Expression of *eome* has expanded since 18 s, when *eome* was expressed within the *emx1* domain. Weak *eome* expression is detected in cells lining the ventricle and higher levels of *eome* mRNA are found in more lateral cells.

By prim 5, high levels of *nk2.1* expression can be detected in the rostral diencephalon. Expression is excluded from two domains within the hypothalamus, cells lining the optic recess and in cells of the posterior hypothalamus (Fig 3.4H,I-see arrows).
Fig 3.4. Zonal expression of genes at the prim 5 (24hpf) stage

(A-C) Embryos stained with digoxigenin labelled probes for *dlx2*. (A) In a lateral view, the two forebrain expression domains of *dlx2* can be identified. (B-C) Anterior and dorsal views show that *dlx2* is weakly expressed by midline cells and more strongly by postmitotic cells in the DZ. (D) Lateral and (E) dorsal views of embryos similarly stained for *emx1*. *Emx1* expression is restricted to the dorsal region, in cells close to the midline. (F) Lateral and anterior view of embryos double labelled for *emx1* (blue-using a fluorescein labelled probe) and *dlx2* (red-using a digoxigenin labelled probe). The regions of *emx1* and *dlx2* expression are similar to their domains at the 18 somite stage, the cells not expressing are also visible in the lateral and anterior views. A schematic representation of *dlx2* and *emx1* expression domains is show in panel (O). (H) Lateral and (I) anterior views of embryos labelled with digoxigenin RNA probes against *nk2.1* indicated that it is expressed in the hypothalamus but is excluded from some posterior hypothalamic cells (see arrow). (J-K) *eome* is expressed in a broadly similar territory to *emx1* as shown by the (J) lateral and (K) dorsal views of the embryos. (L) Lateral view of an embryo stained using a digoxigenin RNA probe for *ngn1* shows expression is restricted to the cells lining the optic recess and an dorso-rostral group of cells. (M,N) Dorsal view of embryos stained using digoxigenin RNA probes for *ngn1* and *neuroD*. *ngn1* expression is limited to cell at or near the ventricle, whilst *neuroD* is expressed predominantly in cells away from the midline. All embryos are at the prim 5 stage of development and the eyes have been removed before photography. Scale bars represent 50μm. Abbreviations: ac, anterior commissure, e, eye, hy, hypothalamus, id, intermediate domain, op, olfactory placode, or, optic recess, poc, postoptic commissure, t, telencephalon, tm, telencephalic midline, tv, telencephalic ventricle.
The non-expressing cell layer along the optic recess has expanded slightly since 18 s, probably due to the addition of new cells, rather than a downregulation of *nk2.1* expression. *nk2.1* continues to be expressed throughout the medio-lateral axis, with expression detected at the midline ventricle to the lateral wall of the hypothalamus (Fig3.4I). Within the rostral diencephalic expression domain of *nk2.1*, groups of cells with higher levels of expression are detected. Surprisingly *nk2.1* is not expressed in the ventral telencephalon, as it is in other species (Rubenstein *et al.*, 1994).

3.2.2.4. *Neurogenic gene expression reveals the presence of at least two zones of neuronal precursors.*

By prim 5, *ngn1* expression is refined from one broad telencephalic domain into two separate regions. A broad dorso-ventral domain of *ngn1* expression is found along the optic recess (Fig3.4L,M). This *ngn1* domain starts from the roof of the telencephalon and traverses the optic recess to a ventral limit which overlaps, with the telencephalic *dlx2* territory. Dorsal views reveal the restriction of *ngn1* expression to the caudal telencephalic ventricle surface, where PH3 positive cells are detected. *ngn1* is also weakly detected in cells in a dorso-rostral territory (Fig3.4L, marked by asterix). This *ngn1* expression domain is also located medially within the dorsal telencephalon and would be predicted to give rise to more anterior neurons (data not shown). High expression of *ngn1* is also detected in the olfactory placodes (Fig3.4M).

Analysis of *neuroD* expression in prim 5 wildtype embryos revealed an dorso-caudal group of cells expressing high levels of *neuroD* mRNA. Cells expressing high levels of *neuroD* are detected in the lateral telencephalon (Fig3.3N). The *neuroD* positive cells may overlap slightly with the *ngn1* expressing cells, but are no longer contained within the *ngn1* domain (compare Fig3.4M and Fig3.4N). High levels of *neuroD* mRNA are also expressed in the olfactory placode.

3.2.2.5. *Summary of gene expression studies in prim 5 stage embryos.*

Despite the apparent organisation of the brain into a ventricular and differentiation zone, gene expression domains remain relatively the same in the prim 5 stage when compared to the 18 s stage. The expression domains of *dlx2* and *emx1* continue to define pallial, subpallial and 1D regions (Fig3.4O,P). These three territories have expanded along with the rest of the forebrain. The expression domain of *eome* has expanded and the significance of this is difficult to determine. A similar situation occurs in mice, where
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*tbr1/eome* expression expands whilst the *emx1* expressing territory remains the same (Bulfone et al., 1995). Eventually *tbr1/eome* expression expands into the ID of mice and is used as a marker for the ID. Further double *in situ* hybridisation need to be performed before similar conclusions can be drawn in zebrafish. Additionally in mice, the switch from *eome* to *tbr1* expression may correlate with a cell cycle transition from being in a mitotic state to a post mitotic one (Ciruna and Rossant, 1999).

Mitotic cells lining the ventricles express *ngn1*, as would be predicted by comparison of *ngn* expression in other organisms (Ma et al., 1996). Cells competent to form neurons express *ngn1*, these cells subsequently express *neuroD* once they start differentiating. Similar expression of *ngn1* and *neuroD*, in mitotic and post mitotic cell respectively, has been observed in mice and *Xenopus* (Lee et al., 1995).

3.2.3. **An increase in forebrain size does not significantly affect organisation at prim 20 (33hpf)**

3.2.3.1. **Zonal organisation of morphology can be clearly seen in the prim 20 stage telencephalon.**

By prim 20, the forebrain has increased in size. A disproportionate increase in the size of the hypothalamus and tectum may force the forebrain to bend further at the ZLI. Due to the increased bending at the ventral flexure, structures originally rostrally positioned have shifted to a more ventral position and similarly, dorsal structures at prim 5 stage take on a more rostral appearance but in general the telencephalon remains a dorsal structure. In the telencephalon the AC and SOT have fully formed and become thicker but no other tracts or commissures can be identified (Fig3.5G).

The level of cellular organisation observed in prim 5 stage embryos has been expanded upon by prim 20. Proliferative zones lining the OR appear to form a pseudostratified layer of cells commonly seen in the telencephalic ventricular zones of other species (Fig3.5A-C). The DZ is characterised by the cell free AC and pale neurons, which probably contribute towards the AC and SOT. Another cell dense proliferative zone can be distinguished in the rostral telencephalon (Fig3.5A-C). This second proliferative area will be called, proliferative zone B (PZB), to distinguish it from proliferative cells lining the OR, which will be referred to as proliferative zone A (PZA). Each proliferative zone is distinctive but continuous with each other. Sections taken near the midline are characterised by having many proliferative cells throughout the dorso-ventral extent of the telencephalon (Fig3.5C).
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Fig3.5. An increase in telencephalic size does not significantly affect organisation at prim 20 (33hpf) stage

(A-C) Sagittal sections of embryos counterstained with toluidene blue. The level of sections, which again start laterally and proceed to the midline, are indicated in panel (E). A similar cellular organisation at prim 20 stage (33hpf) is seen as observed at prim 5 stage. Two distinct proliferative zones can be distinguished by the appearance of small darkly stained cells (PZA and PZB; A,B). The anterior commissure can be seen as cell free, pale ventral area in the DZ (A,B). Sections at the midline reveal many proliferative cells adjacent to the ventricle(C). (D-F) Horizontal sections of embryo counterstained with toluidene blue. The level of sections are indicated in panel (C). The cellular organisation seen in the sagittal section is reiterated in these sections. (G) Antibody labelling for N-acetylated tubulin showing the positions of the AC, POC and SOT. (H,I) Embryos stained with anti-PH3 antibody to mark mitotic cells. (H) A dorsal view shows the presence of mitotic cells at the ventricular midline. (I) Mitotic cells lining the optic recess are observed in anterior views. (J) A summary of the organisation of the telencephalon at this stage, with two clear proliferative areas and a differentiation zone. The diagram is drawn in sagittal section. (K) An anterior view diagram to illustrate the localisation of mitotic cells to the ventricular surface. All embryos are at the prim 20 stage of development. Scale bars represent 50μm. Abbreviations: ac, anterior commissure, dz, differentiation zone, e, eye, hy, hypothalamus, op, olfactory placode, or, optic recess, os, poc, postoptic commissure, pza, proliferative zone A, pzb, proliferative zone b, sot, supraoptic tract, t, telencephalon, tv, telencephalic ventricle.

Blue dots in (J) show distribution of putative proliferative cells.
The fact that these domains contain mitotic cells is confirmed by labelling embryos with anti-PH3 antibody (Fig3.5H,I,K).

The position of PZB is further confirmed by horizontal sections of embryos stained with anti N-acetylated tubulin antibody (Fig3.5E,F). The PZB is seen at the most rostral extent of the telencephalon. Mitotic cells of PZA are seen lining the ventricles of the telencephalon (Fig3.5D-F). Tiny processes from neurons are labelled with the anti-N-acetylated tubulin antibody and are seen to extend towards the AC (Fig3.5E).

In summary, the telencephalon is organised into a putative three layered structure, with a neuron rich area (DZ) sandwiched by two proliferative areas (PZA and PZB; Fig3.5J). The neurons contribute to the AC and SOT.

3.2.3.2. Overlapping expression of dlx2, emx1 and eome in the prim 20 telencephalon.

By prim 20, dlx2 expression in the telencephalon has expanded dorsally to fill a greater portion of each hemisphere but is still excluded from proliferative cells lining the optic recess and midline of the telencephalon (Fig3.6A,B). A faint stream of dlx2 expressing cells appears to migrate from the diencephalic band into the posterior hypothalamus (Fig3.6A,E). By prim 20 stage, emx1 positive cells are still detected in the dorsal telencephalon but expression spreads more rostrally. This expansion of the emx1 domain does not merely represent a change in the morphology of the forebrain but represents a genuine expansion of expression (see later results).

eome expression is similarly expressed in a broad dorsal telencephalic domain. However anterior expression has noticeably expanded, when compared to the expression at prim 5 stage (Fig3.6G). eome is excluded from most midline cells but may be expressed by cells in PZB. Weak eome expression is detected in cells of the dorsal diencephalon lining the ventricle (Fig3.6H).

In sagittal views of embryos stained with both dlx2 and emx1 RNA probes, the area not expressing either gene is difficult to identify due to the respective rostral and dorsal expansions of emx1 and dlx2 expression domains. However a small territory which has weak expression of both genes can be identified, this domain is more clearly seen in rostro-ventral views of the prim 20 forebrain (Fig3.6E,F,S,T).
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Fig 3.6. Overlapping expression of *dlx2* and *emx1* expression and zones of neuronal differentiation in the prim 20 stage embryo

(A,B) Embryos stained using digoxygenin labelled RNA probes for *dlx2*. (A) Lateral views show the ventral telencephalic domain is expanded in prim 20 stage but the diencephalic domain becomes more patchy. (B) Anterior views show *dlx2* expression is expressed away from the midline. (C,D) Lateral and dorsal views of embryos labelled with digoxygenin RNA probes for *emx1*. *emx1* continues to be expressed in the dorsal telencephalon in cells near the midline, as well as in cells in more lateral positions. (E,F) Lateral and anterior views of embryos double stained for *dlx2* (red, digoxygenin probe) and *emx1* (blue, fluorescein probe) show that the two domains considerably overlap and cells not expressing either gene are difficult to distinguish. (G,H) Lateral and dorsal views of *eome* mRNA expression shows expression is limited to the dorsal telencephalon in a similar domain to *emx1*. (I,J) Lateral and anterior views of embryos stained for *nk2.1* transcripts. *nk2.1* expressed in the hypothalamus but are excluded in the cells around the optic recess and posterior hypothalamus. (K-Q) Lateral views of embryos stained using digoxygenin RNA probes for (K) *ngn1*, (L) *neuroD*, (N) *isl-1*, (O) *lim1*, (P) *lim5*, (Q) *lim6* or (M) an anti-*pax6* antibody. Arrows indicate positions of putative neuronal groups. Expression of *ngn1* reveal neuronal competence of cells lining the optic recess and a group of dorso-rostral cells. *neuroD* is expressed in a subset of *ngn1* positive cells in the dorsal telencephalon. The telencephalon can be subdivided into different putative neuronal groups at the prim 20 stage. (O-R) Anterior cells can be characterised by the expression of *lim1* and *lim6* (marked G2 in the summary figure (R)). (M,P) Dorsal telencephalic cells express *lim1*, *lim5* and *pax6* (marked G3 in (R)). (N) Ventral neurons are demarcated by the expression of *isl-1* (N; G1 in (R)). The position of the ID is illustrated in (S). ID continues to occupy a similar position as seen in mice (T). All embryos are at the prim 20 stage of development and the eyes have been removed before photography. Scale bars represent 40μm (A-S) and 550μm in (T). Abbreviations: ac, anterior commissure, e, eye, emt, eminence thalamica, hy, hypothalamus, id, intermediate domain, op, olfactory placode, or, optic recess, os, poc, postoptic commissure, t, telencephalon, te, tectum.
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nk2.1 is expressed in a similar pattern to that observed in the prim 5 embryo (Fig3.6I,J).

3.2.3.4. Neurogenesis within the prim 20 telencephalon.

Neuronal precursors, identified by ngn1 expression, are situated in two broad domains, akin to the observed territories in prim 5 embryos (Fig3.6K). The two domains of ngn1 expression are coincident with PZA and PZB, further implying that ngn1 is expressed in mitotic cells. No ventral expression of ngn1 mRNA is detected. Although ngn1 expression is refined into two telencephalic domains, neuroD expression is detected only in a broad dorso-caudal domain (Fig3.6L). This neuroD territory forms a subset of the eome and emxl expressing cells.

3.2.3.6. Expression of Lim homeodomain genes may delineate different populations of neurons.

The combinatorial expression of Lim homeodomain transcription factors in neurons is thought to impart specific identity and orchestrate axonal pathfinding (Pfaff and Kintner, 1998). Although extensive studies of combinatorial expression of Lim genes have been performed in the spinal cord and hindbrain of several species, relatively little work has been performed in the forebrain. I have analysed neuronal differentiation in the telencephalon at the prim 20 stage using Lim genes and Pax6, a paired box transcription factor.

Of the different Lim genes, isl1 was one of the first to be identified and has been extensively described as a marker for primary motor neurons (Korzh et al., 1993; Pfaff et al., 1996). In zebrafish embryos, isl1 is first detected in the telencephalon around the 14 somite stage in a small population of ventral neurons (data not shown). As development proceeds, this population expands but remains restricted to the ventral telencephalon (Fig3.5N,R-marked G1 on summary figure). Of the other Lim genes identified in zebrafish, none are thought to be expressed in a similar ventral population.

The combinatorial expression of other Lim genes appears to delineate specific groups of neurons within the telencephalon. A group of neurons in the rostral telencephalon probably express lim1, lim5 and lim6 (Fig3.6O-R; marked G2 on summary figure). More dorsally, a group of neurons express lim1 and lim5 (Fig3.6O,P,R-marked G3 on summary figure). It is unclear whether lim5 expressing cells line the OR or are expressed in post
mitotic cells away from the OR (Fig3.6P). If \textit{lim5} is indeed expressed in post mitotic cells away from the OR, these cells will also express Pax6 (Fig3.6M,R). \textit{Pax6}, a paired box transcription factor, has been shown to have important roles in forebrain development and proved valuable in understanding forebrain segmentation (Macdonald \textit{et al.}, 1997a). In the diencephalon, \textit{Pax6} expression is widespread throughout the thalamus and is particularly strong in the roof of the diencephalon. The definitions of these groups of neurons (Fig3.6R-labelled G1-G3) is an initial attempt to describe populations of neurons in the developing zebrafish telencephalon and further work will be required for a full description of their positions and projections.

3.2.4. A complex telencephalic organisation is observed at 48hpf

3.2.4.1. A defined olfactory bulb has started to evaginate from the telencephalon.

By 48hpf, further morphogenetic movements position the telencephalon as the most rostral brain structure. Thus rostral structures at prim 20 stage, such as PZB region, are located more ventrally in the 48hpf embryo (Fig3.7B-D,Q). Structures which were originally dorsal have become rostral in the 48hpf embryo. The OR now is perpendicular to the AP axis of the embryo. Consequently the rostral hypothalamus is thrust underneath the tegmentum and the thalamus occupies a more rostral position. Additionally the whole brain has contracted along the rostro-caudal axis. The AC and SOT have become thicker and are the only detectable axon bundles in the 48hpf telencephalon.

An additional proliferative zone can be distinguished in the rostral 48hpf telencephalon (Fig3.7A-D, labelled proliferative zone C (PZC)). PZC maybe continuous with the original proliferative zone, PZA, at some levels. The appearance of PZC suggests that the process of eversion has begun and PZC may directly contribute cells to the everted part of the telencephalon. The olfactory bulb can be detected as a separate entity in 48hpf embryos (Fig3.7A-D). Within the olfactory bulb, neuropil areas correlate to nascent glomeruli (Fig3.7A-D,Q). The DZ has enlarged but is still dominated by the AC, which is surrounded by presumptive neurons (Fig3.7A-C).

Horizontal sections of embryos, labelled with anti-N-acetylated tubulin antibody, display the same level of medio-lateral organisation observed in the prim 20 stage embryos, with cells of PZA lining the telencephalic ventricle (Fig3.5E-H). More dorsal sections are characterised by cell dense areas, which may correspond to everted dorsal tissue (Fig3.7E). In ventral sections the PZB area can be detected in the rostral telencephalon (Fig3.7H).
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Fig3.7. Complex telencephalic organisation is observed at 48hpf

(A-D) Sagittal sections, progressing towards the midline, counterstained with toluidene blue. The level of the sections is indicated on panel (F). By 48hpf, the primitive layered morphological organisation has evolved into a more complex morphology. The rostral brain has further rotated to position the telencephalon as the most anterior CNS segment. (A,B,I) The AC has become a thick commissure projecting ventrally in the telencephalon. The olfactory bulb starts to be easily distinguished from the body of the telencephalon and little plaques indicate the positions of the forming glomeruli (shown by asterix and g). Another proliferative zone (PZC) can be distinguished in the roof of the telencephalon. (E-H) Horizontal sections of embryos, previously labelled with an anti-N-acetylated tubulin antibody and then counterstained with tolene blue. The levels of the sections is indicated in panel (D). (E) Dorsal most sections are characterised by proliferative cells in the everting roof of the telencephalon. (F, G) More ventrally, cells lining ventricles appear similar to those observed before and more lateral cells are, again, less darkly stained and more loosely packed. The brown staining reveals projections from these cells contributing to the AC and SOT. (H) The SOT is detected as a tract between the telencephalon and diencephalon. (I) An embryo labelled with an anti-N-acetylated tubulin antibody reveals that there are no additional forebrain commissure to the AC and POC, but both commissures have become considerably thicker. (J-P) Lateral views of embryos labelled with digoxigenin and fluorescein RNA probes to (J) dlx2, (K) emx1, (L) dlx2(red) and emx1 (blue), (M) nk2.1, (N) eome, (O) ngnl and (P) neuroD. (J) Expression of dlx2 in the diencephalon has significantly decreased but expression can still be detected in certain diencephalic areas, such as the posterior hypothalamus. (K) emx1 is strongly expressed in the rostral telencephalon, approximately to the evagination olfactory bulb. In more caudal telencephalic territories, emx1 expression is coincident with the roof of the telencephalon. (L) Double staining for emx1 (blue) and dlx2 (red) show that expression overlaps slightly in the dorsal telencephalon. The PZB domain does not appear to express either dlx2 nor emx1. (M) nk2.1 continues to be expressed in the hypothalamus. (N) eome is expressed in a similar territory to emx1 but is detected in a broader territory. (O) Zones of competence for neurogenesis, as specified by expression of ngnl, appear to be restricted to three areas in the telencephalon, coincident with the proliferative zone. (P) Neuronal differentiation of a subset of dorsal telencephalic cells can be observed through the expression of neuroD. (Q,R) Morphological organisation and gene expression domains are summarised in schematic diagrams. All embryos are at the 48hpf stage of development and the eyes have been removed before photography. Scale bars represent 60μm (A-I,L) and 90μm (J,K,M-R). Abbreviations: ac, anterior commissure, cb, cerebellum, dz, differentiation zone, hb, hindbrain, hy,
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hypothalamus, ob, olfactory bulb, or, optic recess, pc, posterior commissure, poc, postoptic commissure, pza, proliferative zone A, pzb, proliferative zone b, pzc, proliferative zone C, sot, supraoptic tract, t, telencephalon, te, tectum.

Red-dlx2, Blue-emx1, Purple-eome, Green, nk2.1.
supraoptic tract is also seen, projecting from the telencephalon to the diencephalon, in the ventral sections (Fig3.7G,H).

3.2.4.2. Overlapping expression of \textit{dlx2}, \textit{emxl} and \textit{eome} in the 48hpf telencephalon.

\textit{dlx2} mRNA is transcribed by many cells in the 48hpf telencephalon (Fig3.7J). In the caudal telencephalon \textit{dlx2} expression is detected in cells adjacent to cells lining the optic recess and rostral expression overlaps with \textit{emxl} expressing cells (Fig3.7L,R). In the diencephalon, \textit{dlx2} is expressed only in small groups of cells caudal to the optic recess. Patchy \textit{dlx2} expression is also detected in the hypothalamus (Fig3.7J,L). In the dorsal telencephalon, only a thin band of cells express \textit{emxl}. However a broad domain of \textit{emxl} expression is detected in cells of the evaginating olfactory bulb (Fig3.7K,L). It is unclear whether \textit{emxl} expression in the olfactory bulb arises from induction or migration of earlier expressing telencephalic cells. A small number of cells in the bulb express \textit{dlx2} and \textit{emxl} (Fig3.7L,R). Cells of the PZB area do not appear express either gene (compare Fig3.7C to Fig3.7L).

\textit{eome} is more strongly expressed in the telencephalon and olfactory bulb than \textit{emxl} (Fig3.7M). In the dorsal telencephalon, \textit{eome} expression occupies a domain approximately twice the size of the \textit{emxl} band (Fig3.7R). In the bulb, \textit{eome} is also expressed in more cells than \textit{emxl}, and so would be expected to have a greater overlap with \textit{dlx2} expression. \textit{nk2.1} is expressed in the hypothalamus (Fig3.7N).

3.2.4.3. \textit{ngnl} expression correlates with mitotic areas in the 48hpf telencephalon.

\textit{ngnl} is expressed in three areas in the 48hpf telencephalon, all of which correlate with the proliferative zones described above (Fig3.7O). Strong expression of \textit{ngnl} is detected in cells corresponding to PZC and weaker expression is detected in PZA and PZB. Again \textit{neuroD} is expressed only within a subset of the \textit{ngnl} expressing cells, in the dorsal telencephalon (Fig3.7P).

3.2.4.4. \textit{Summary of cellular organisation and gene expression} 48hpf zebrafish telencephalon.

By 48hpf, the zebrafish telencephalon can be subdivided into several different territories (Fig3.7Q). The olfactory bulb has started to evaginate from the dorsal telencephalon and nascent glomeruli can be detected as neurophil areas. The telencephalon
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can be subdivided into three distinct proliferative zones and a main differentiation zone. The third PZ, PZC, is located in the dorsal roof of the telencephalon and may correspond to everted tissue (Fig3.7Q, Wulliman and Puelles, 1999). The DZ is characterised by the tract of the AC, SOT and numerous neurons, which contribute to these tracts. The SOT also contains axons from thalamic neurons, which project into the telencephalon (Wilson et al., 1990).

The morphogenetic movements that give rise to the cellular organisation of the 48hpf embryo also greatly influences changes in gene expression (Fig3.7R). \textit{dlx2} expression has expanded into the presumptive olfactory bulb, as have \textit{emxl} and \textit{eome} expression (see discussion). \textit{dlx2} expression expands in the telencephalon, whilst \textit{emxl} expression appears to have receded.

3.2.5. Zonal organisation of the 72hpf telencephalon

3.2.5.1. Sections reveal continued thickening of commissures and an expansion of all telencephalic territories.

After 48hpf, the forebrain undergoes an apparent compression in the DV plane and extension in the AP axis, such that the olfactory bulb becomes the most rostral extent of the forebrain. All the subdivisions of the brain are larger due to significant growth over the past 24hrs but the overall organisation of the telencephalon is conserved from 48hpf. The AC and SOT have thickened considerably and form a major part of the 72hpf telencephalon (Fig3.8M). In sagittal sections, the anterior commissure is detected as a thick cell free area occupying a large central portion of the telencephalon (Fig3.8A-C,M). The supraoptic tract, projecting into the diencephalon can also be detected in sagittal section (Fig3.8B,C,I). The olfactory bulb has budded off from the telencephalon and forms a distinct structure at the rostral extent of the embryo (Fig3.8A,M). The large olfactory bulb is positioned rostral to the telencephalon (Fig3.8A). Within the bulb, the glomeruli can be detected as punctate cell free areas due to the mass of dendrites and axons associated with them. Sagittal sections also reveal the olfactory tract projecting from the bulb into the telencephalon (Fig3.8C,M).

The proliferative zones detected at 48hpf are also observed in the 72hpf embryo. In coronal sections, PZB is clearly observed in the basal telencephalon (Fig3.8D,E,M). Coronal sections also reveal everted tissue in the roof of the telencephalon. (Fig3.8E-G). The PZC region probably forms part of the everted tissue. The coronal sections stained with an antibody to tyrosine hydroxylase (TH), an enzyme expressed in dopaminergic neurons, show
3.0 Characterisation of the developing zebrafish telencephalon

**Fig3.8. Cellular organisation, gene expression and connectivity in the 72hpf telencephalon**

(A-C) Sagittal sections of 72hpf embryos, counterstained with toluidene blue. The levels of the sections are indicated in panel (G). The organisation of the telencephalon at 48hpf is still observed at 72hpf, with three proliferative areas and one large, broad differentiation domain detected. The AC and SOP can be detected as large cell free areas. (D-G) Coronal sections of embryos, labelled with anti-tyrosine hydroxylase antibody. Sections progress towards the optic recess and the levels of the sections are shown in panel (B). (D-E) Cells of the olfactory bulb can be detected in the rostral most sections. Many tyrosine hydroxylase positive neurons are detected in the olfactory bulb. (F,G) Fewer tyrosine hydroxylase neurons are detected in the telencephalon at this stage. The roof of the telencephalon has started the process of eversion and proliferative cells are observed in the everted tissue (as shown by arrows). In more caudal sections, the thick AC is seen to dominate the developing telencephalon. (H) A lateral view of an embryo stained with an antibody to N-acetylated tubulin reveals that the olfactory bulb has started to project to the telencephalon. The AC still is the only detectable commissure in the telencephalon. (I-L) Lateral views of embryos stained using digoxigenin RNA probes for (J) dlx2, (J) emxl, (K) nk2.1 and (L) eome. (J) Punctate expression dlx2 is detected in the olfactory bulb, suggestive of specific expression in or around the glomeruli. In the telencephalon, dlx2 is expressed extensively but weaker, more restricted expression is detected in the diencephalon. (K,M) emxl is expressed in a subset of eome cells in the telencephalon and olfactory bulb. Within the bulb, both emxl and eome are expressed in a punctate manner. (L) nk2.1 is expressed in the hypothalamus at this stage. (M,N) Morphological organisation and gene expression domains are summarised in schematic diagrams. All embryos are at the 72hpf stage of development and the eyes have been removed before photography. Scale bars represent 60μm. Abbreviations: ac, anterior commissure, dz, differentiation zone, g, glomeruli, hy, hypothalamus, ob, olfactory bulb, or, optic recess, pc, posterior commissure, poc, postoptic commissure, pza, proliferative zone A, pzb, proliferative zone b, pzc, proliferative zone C, sot, supra-optic tract, t, telencephalon, tv, telencephalic ventricle.

Red-dlx2, Blue-emxl, Purple-eome, Green, nk2.1.

(H) Lateral view of an embryo labelled with N-acetylated tubulin to reveal the positions of axonal pathways.
that many TH positive neurons are detected in the olfactory bulb. Only a handful of TH positive neurons are found in the telencephalon at this stage (Fig 3.8D-G).

3.2.5.2. *dlx2, emxl and eome are expressed in a punctate manner in the olfactory bulb.*

By 72hpf, *dlx2* expression in the ventral telencephalon and diencephalon has diminished but strong staining in the olfactory bulb is still observed. The staining in the olfactory bulb is punctate, suggesting that *dlx2* mRNA is localised to cells within the glomeruli (Fig 3.8I). *emxl* mRNA is detected in the dorsal olfactory bulb, displaying a punctate staining similar to *dlx2* (Fig 3.1, N). Weak expression is also detected in the dorso-caudal telencephalon. *eome* also shows prominent punctate staining in the dorsal olfactory bulb (Fig 3.8L, N). In the dorsal telencephalon, *eome* is expressed at higher levels and in a broader territory than *emxl*. It is not possible to determine whether *dlx2, emxl* and *eome* are expressed in the same cells in the olfactory bulb using my results. *nk2.1* continues to be expressed in the hypothalamus (Fig 3.8K).

3.2.6. Stable morphological composition, gene expression and axon guidance is observed in the 5 day telencephalon

3.2.6.1. *Organisational changes occur from 72hpf to 5days in the telencephalon.*

By 5 days post-fertilisation, the olfactory bulb is now fully rostral to the telencephalon and the telencephalon rostral to the diencephalon and tectum. The olfactory tract contains projections from individual glomeruli to the telencephalon. The thickening of the AC and SOT have continued to give large axon bundles (Fig 3.9D,E). The separation of the bulb can be seen in sagittal sections, where the rostrally placed olfactory bulb contains many distinct glomeruli and a cell dense caudal boundary (Fig 3.9A-C). In Fig 3.9B, the olfactory tract is seen to project from the bulb to the main telencephalon through the caudal boundary. By 5 days, the PZB region is displace more caudally. The PZA is detected in the part of the everted roof of the 5 day telencephalon (Fig 3.9A-C, J). Although the body of the telencephalon is dominated by the AC, foci of axonal or dendrite condensations can be observed in the telencephalon (Fig 3.9A-C, marked by asterix).

3.2.6.2. *dlx2, emxl and eome expression is maintained in the 5 day old telencephalon.*

*dlx2, emxl* and *eome* are expressed in similar domains in the day 5 olfactory bulb as in the 72hpf embryo (Fig 3.9F,G,I). Although the cellular expression in the olfactory bulb can
Fig3.9. Morphological composition, gene expression and connectivity in the 5 day postfertilisation (dpf) telencephalon

(A-C) Sagittal sections of 5 dpf embryos, progressing towards the midline, counterstained with toulidine blue. The levels of the sections are indicated in panel (E). (A-C) The rostral brain has fully rotated with the olfactory bulb positioned most rostrally and the telencephalon sitting caudally behind it. The darkly stained cells in the roof represent everted portion of the telencephalon. Proliferative and differentiation domains are also detected in the 5 dpf telencephalon. (C) The glomeruli in the olfactory bulb can be seen as punctate cell free areas. (A-C) The AC and SOT can also be identified as thick, large cell free areas. (B) The olfactory tract, projecting from the olfactory bulb to the telencephalon can also be detected in some sections. (D, E) Staining of 5 dpf embryos with an anti-N-acetylated tubulin antibody shows a large AC, SOT and POC. (F-I) Lateral views of embryos labelled with digoxygenin RNA probes for (F) dlx2, (G) emx1, (H) nk2.1 and (I) eome. (F) dlx2 is expressed widely in the telencephalon and olfactory bulb. (G, I) As in 72hpf embryos, emx1 is expressed in a subset of eome expressing cells in the telencephalon and olfactory bulb. eome is expressed in the midbrain. (H) nk2.1 is still expressed in the hypothalamus and not in the telencephalon. All embryos are at the 5 dpf stage of development and the brain has been dissected from the rest of the embryo before photography. (J,K) Morphological organisation and gene expression domains are summarised in schematic diagrams. Scale bars represent 60µm (A-C) and 100µm (D-K). Abbreviations: ac, anterior commissure, dz, differentiation zone, g, glomeruli, hy, hypothalamus, mb, midbrain, ob, olfactory bulb, ot, olfactory tract, pc, posterior commissure, poc, postoptic commissure, pza, proliferative zone A, pzb, proliferative zone b, pzc, proliferative zone C, sot, supra-optic tract, t, telencephalon, te, tectum.

Red-dlx2, Blue-emx1, Purple-eome, Green, nk2.1.

Blue dots in (J) show distribution of putative proliferative cells.
not be resolved, it appears the genes are expressed in all the glomeruli and show no zonal bulb expression. Expression of *dlx2* is detected in the ventral telencephalon (Fig3.9F). In the diencephalon, *dlx2* is expressed in a small group of cells caudal to the optic recess and situated close to the position of the supraoptic tract. *emxl* and *eome* are expressed in the dorsal telencephalon, with *emxl* being weakly expressed within a domain of *eome* expressing cells (Fig3.9G,I,K).

3.3 Discussion

Although histological studies of the adult zebrafish telencephalon have delineated dorsal and ventral areas, interpretation of the relative homologies of these telencephalic structures has been greatly limited by the lack of understanding of the developmental processes underlying the formation of these structures. Comparative analysis between adult telencephalons from different species, has facilitated the classification of homologous structures, however the teleostean process of eversion has hindered such studies. By investigating morphogenesis, gene expression, connectivity and proliferation in the developing zebrafish telencephalon, we can begin to understand how the adult structures arise and start to assign relative homology to teleostean telencephalic subdivisions.

3.3.1. Morphogenesis, proliferation and connectivity in the developing zebrafish telencephalon.

The adult teleostean telencephalon can be subdivided into two broad domains, the area ventralis and dorsalis telencephali. Within these two domains, the positions of defined nuclei have also been characterised. Whilst my studies do not allow us to identify the origin of specific nuclei, we can gain a greater understanding of the development of the area dorsalis and ventralis telencephali.

No apparent morphological organisation of the telencephalon can be detect at a stage when the first neurons are starting to differentiate (Macdonald *et al.*, 1994). Before this stage, the telencephalon is composed of a single sheet of pseudo-stratified cells, all of which are mitotic (Kimmel *et al.*, 1993). A few hours after neuronal differentiation has begun, a primitive layering of the telencephalon can be detected. In the prim 5 stage telencephalon, a ventricular proliferative layer, a layer of post mitotic cells interspersed with axons of the tract of the AC and at the most dorso-rostral extent, a smaller group of proliferative cells are
observed. This basic layering pattern is also observed at prim 20 stage and built upon during later development.

Eversion begins between prim 20 and 48hpf, as a thin dorsal, cell dense area covered by a thin choroidal tela is detected. This area continues to expand in all the stages examined and its morphological appearance, combined with studies performed by Wulliman and Puelles (1999), suggest that it continues to proliferate throughout larval stages to give rise to the large, adult area dorsalis telencephali. Although the area ventralis telencephali appears to be established earlier in development, it too has well defined proliferative domains until 5 days postfertilisation. In mice, proliferation in the ventral telencephalon occurs at two adjacent areas, the ventricular and subventricular zone. Ventricular zone cells are characterised by their attachment to the pial or ventricular surface, whilst overlying subventricular zone cells are detected by their nonepithelial appearance. Although proliferation at the zebrafish ventricle is established in these studies, it is unclear whether telencephalic cells also retain a proliferative capacity away from the ventricular surface. Wulliman and Puelles (1999) reported that isolated groups of mitotic cells in the 5 day diencephalon, but not in the telencephalon.

In the adult telencephalon, the AC is split into a dorsal and ventral component. I do not observe such a split, and studies have shown that the separation of the AC occurs after 6 days post fertilisation (Cooke et al., 1999). Of the other tracts observed in the adult, the medial and lateral olfactory tracts can be seen in 4 day postfertilisation embryos. Comparative analysis of neuronal connectivity can greatly benefit the identification of homologous structures. Although several studies have described certain connections in other vertebrates, for example the reciprocal connections from the thalamus to the telencephalons, further detailed analysis is required in the zebrafish before homology can be confidently described. The innovation of new techniques, like transgenic fish expressing GFP, will greatly assist our understanding of axonal projections, cell migration and mitosis within the zebrafish telencephalon.

3.5.2. Emx1 and Dlx2 distinguish pallial, subpallial and intermediate territories in the zebrafish telencephalon.

The use of Emx1 and Dlx2 expression to delineate pallial and subpallial territories of the telencephalon is widely established in a number of different species (Fernandez et al., 1998). Comparison of these genes reveals the position of similar domains in the zebrafish telencephalon. As in other species, emx1 is expressed in a dorsal telencephalic territory, in
proliferating and differentiating cells (Briata et al., 1996; Gulisano et al., 1996, Pannese et al., 1998). Later expression of *emx1* in zebrafish telencephalon correlates with the position of the everted tissue. Although my studies do not follow the development of the area dorsalis after 5 days postfertilisation, it appears that everted tissue probably forms the major component of the area dorsalis (Wulliman and Puelles, 1999). *emx1* expression in everted tissue suggests that a major part of the adult area dorsalis telencephali forms the zebrafish pallium.

In mice, *dlx2* is expressed in ventral telencephalic structures; the striatum, pallidum and septum (Bulfone et al., 1993). However *dlx2* is not expressed at the ventricular surface but rather in the subventricular and sub-mantle zones. *dlx2* is not expressed at the ventricular surface in zebrafish embryos but is expressed in post mitotic, differentiating cells; suggesting a conservation of a role in the progression from proliferation to differentiation (Akimenko et al., 1994; Porteus et al., 1994). Although no morphological constrictions are observed in the zebrafish telencephalon to help delineate specific ventral structures, analysis of *dlx2* expression does allow us to define the subpallium. By 48hpf, *dlx2* is expression is detected in a large ventral area of the telencephalon, suggesting that the sub-pallium forms a large part of the telencephalon at this stage. This observation is supported by the relatively late expansion of the prospective pallium.

### 3.3.3. Development of the intermediate domain in the zebrafish telencephalon.

In addition to dividing the telencephalon into pallial and subpallial regions a third ID, which expresses neither *emx1* or *dlx2*, has also been described in mice, chicks, frogs and turtles (Fernandez et al., 1998). It is detected when neurons first differentiate in the telencephalon. Although no specific gene expression has been detected in the fated ID territory, the domain can be further characterised as *tbr1* and *pax6* expression extends into this non-*dlx2/emx1* expression region. In mice, the intermediary domain will develop into parts of the amygdala, medial septum and paleocortex, whereas in chicks and turtles, the intermediary territory will form components of the chick Dorso-Ventricular Ridge (DVR) (Fernandez et al., 1998).

In the 18s zebrafish, an intermediate domain can be detected as a thin band of cells not expressing either *emx1* and *dlx2*. Although *tbr1* and *pax6* expression in this intermediate domain have not been examined in this study, *eome* expression in this territory appears likely from my results. As I have previously described *tbr1* and *eome* are expressed in same dorso-ventral domains. The fate of these cells remains unclear, however, as this region does not
expand greatly from 18s to prim 20 stage. At present we can not establish a firm connection between the early and late non-expressing territories, nor can we assign a fate to the intermediary domain in the zebrafish. The establishment of pallial and subpallial domain, by gene expression, in zebrafish will greatly assist the understanding of adult structures and evolutionary variations in the teleostean telencephalon. In addition, the localisation of the intermediate domain will greatly advance our understanding of the adult teleost structures that relate to the chick DVR and mouse amygdala. Examination of the expression of Nitric Oxide will also facilitate this understanding, as this molecule has been shown to be a reliable marker for the amygdala (Marin et al., 1997).

* Nkx2.1, in the basal telencephalon, is expressed in the mouse pallidum from E11.5 through to adulthood. Loss of *nkx2.1* in mice leads to a ventral to dorsal transformation, suggesting that *nkx2.1* is vital for specifying the pallidum. By analysing *nk2.1*, I hoped to locate the zebrafish pallidum but find that *nk2.1* is not expressed in the telencephalon at any stage examined. The existence of multiple copies of zebrafish genes, due to a whole genome duplication, explains the absence of *nk2.1* telencephalic expression. A newly isolated zebrafish *nk2.1* is expressed in the basal telencephalon from early somitic stages (Rohr, K. and Wilson, S.W., unpubl.). Further analysis of this *nk2.1* homologue will facilitate our understanding of basal telencephalic divisions in the zebrafish.

Other forebrain structures can be delineated using expression of a number of genes. For example, the position of the eminencia thalamica in the dorsal diencephalon can be identified as a *tbrl*, *pax6* positive but *dlx2* negative area (Puelles, pers. comm.). Similarly, the Lim homeobox gene, *Lhx5*, is required for hippocampal specification in mice and expression of *Lhx5/Lim5* genes in other species may delineate structures homologous to the hippocampus (Zhao et al., 1999).

### 3.3.4. Development of the olfactory bulb in the zebrafish.

The paired olfactory bulbs evaginate from the telencephalon during development. In mice, the main olfactory bulb has a laminar structure, with concentric layers of outer nerve layer, glomerular layer, external plexiform layer, mitral layer and granule cell layer. Similarly the adult zebrafish olfactory bulbs are paired evaginated structures, which are organised into concentric layers. However the development of the zebrafish olfactory bulb is poorly characterised.
3.0 Characterisation of the developing zebrafish forebrain

The location of the olfactory bulb primordia in the telencephalon have not been well characterised. Olfactory axons invade the dorsal telencephalon at prim 12 stage (Wilson et al., 1990), implying that the bulb is initially a dorsal structure, which undergoes a morphogenetic movement to position it at the rostral tip of the embryos. In mice and chicks, it has been proposed that the lateral portion of the intermediate telencephalic domain, not expressing either *emx1* or *dlx2*, will give rise to the olfactory cortex (Fernandez et al., 1990). In zebrafish, the intermediate region is located more ventrally than the entry point of olfactory axons (Wilson et al., 1990). However the rostral expression domains of *liml* and *lim6* may coincide with the rostral tip of the intermediate domain. If we follow this *liml*/*lim6* expression domain through development, we find that it is localised in the 72hpf bulb (data not shown). Although gene expressions are not reliable markers for cell migration studies, the expression of *liml*/*lim6* in the bulb supports the hypothesis that in the zebrafish, part of the intermediate domain may give rise to the zebrafish olfactory bulb.

In the olfactory bulb, interneurons and projection neurons can be distinguished through their expression of certain genes. The GABAergic interneurons express *dlx2*, indeed *dlx2* appears to be required for the generation of all GABAergic interneurons (Anderson et al., 1997a). The projection neurons express *emx1* and *tbr1* (Bulfone et al., 1999). We observe punctate expression of *dlx2*, *emx1* and *eome* in the olfactory bulb, suggesting that specification of these neuron is conserved in the telencephalon. Thus the overall development of the olfactory bulbs, through morphological and molecular events, are similar in teleosts and other species.
Chapter Four

Ace/Fgf8.1 is required for forebrain commissure formation and patterning of the telencephalon

4.1. Introduction

Cells throughout the vertebrate brain must acquire appropriate regional and cell-type identities during early development. Although the organiser produces signals that antagonise BMP signalling and induce neural tissue, it remains much less clear how initial broad neural plate domains are established (Beddington and Robertson, 1999). A gradual process of pattern refinement within these broad domains, involving the action of signalling centres, eventually gives rise to the complexity of neuronal cell type and connectivity observed in the adult.

Signalling centres are specialised groups of cells which impose neural identity in the rostrocaudal and dorsoventral axes through the action of secreted or membrane bound molecules. Recent studies have described the activity of several such signalling centres involved in patterning the forebrain anlage. Anterior extraembryonic endoderm has shown to be the source of signals, which induce and pattern head structures (Beddington and Robertson, 1999; Fekany et al., 1999). Planar and vertical signals originating from the axial mesoderm and have been shown to pattern the ventral forebrain and its derivatives (Rubenstein and Beachy, 1996; Blader and Strähle, 1998). The position of another signalling centre in the anterior neural plate has elucidated through a series of ablation and transplant experiments in the zebrafish. Ablation of a single row of anterior neural plate cells in early gastrulating embryos leads to a complete loss of telencephalic patterning and a marked reduction of patterning in the rostral diencephalon. Transplantation of this single row of cells to more caudal brain territories results in the ectopic induction of telencephalic genes, demonstrating an inherent signalling activity within these cells (Houart et al., 1998). Similar signalling activity from the anterior neural ridge (ANR) in mice has been demonstrated by Shimamura and Rubenstein (1997).

Relatively few candidate molecules, which may be responsible for the signalling activity of the anterior neural ridge, have been described. Of the candidate signalling molecules, research has increasingly focused on the members of the BMP and FGF gene families. BMP signalling is required for the induction of forebrain identity, as zebrafish embryos lacking BMP2/4 signaling do not express telencephalic markers (Barth et al., 114
4.0 Ace/Fgf8.1 is required for zebrafish forebrain development

In the caudal neural tube functional antagonism between members of the BMP gene family and Shh is required for correct dorsoventral patterning (Tanabe and Jessell, 1996). Experiments in mice and chick similarly implicate the BMP family in a patterning the dorsal forebrain. Ectopic expression of chick Bmp4 and 5 leads to a loss of ventral forebrain fates but dorsal gene expression is maintained (Golden et al., 1999). In mice, Bmp2 is expressed before neural plate closure in the dorsal surface ectoderm adjacent to the anterior neuroectoderm, whilst Bmp5 and 7 are expressed in the ANR. In vitro Bmp2/4 can induce dorsal telencephalic identity from tissue fated to form ventral telencephalon (Furuta et al., 1997). Differentiation of dorsal tissue, in response to Bmp2/4, is coincident with a downregulation of Bfl, a gene required for maintaining ventral telencephalic proliferation (Xuan et al., 1995).

Another candidate gene for signalling from the anterior neural margin is Fgf8. Fgf8 is a member of the large Fibroblast Growth Factor family, of which 9 mammalian ligands and 4 different receptors have been isolated (Johnson and Williams, 1993). The FGFs have been shown to function as mitogens, motogens, trophic factors and differentiation factors in oncogenic and developmental processes. Fgf8, in particular, has been shown to play an important role during development. For example, Fgf8 is thought to be vital for limb bud initiation and outgrowth, left-right asymmetry in the mouse and chick, patterning and proliferation in the anterior pituitary and is thought to partially mediate the patterning activity of the isthmus region (Crossley et al., 1996a; Ericson et al.; Reifers et al., 1998; Meyers and Martin, 1999; Picker et al., 1999). The conservation of signalling molecules in different tissues is well described and molecules utilised by the isthmus may be used by other signalling centres, such as the ANR.

The initial expression of Fgf8 in the ANR, and subsequent expression in the dorsal telencephalic midline and optic stalks, suggests a role in forebrain patterning. A dual role for Fgf8 in regionalisation and differentiation of the forebrain has been demonstrated in vitro. In mice, the ANR is required for induction of BF1. Removal of the ANR results in a loss of BF1 expression but Fgf8 soaked beads can induce BF1 in forebrain explants denuded of the ANR (Shimamura and Rubenstein, 1997). Gain of function and loss of function experiments using rat explant cultures suggests a role for Fgf8 in the specification of forebrain and midbrain dopaminergic neurons (Ye et al., 1998). Further evidence for a role for Fgf8 in controlling forebrain development comes from analysis of a zebrafish mutant, aussicht (aus), which has elevated levels of fgf8.1 transcription (Heisenberg et al., 1999). In the aus mutant, upregulation of fgf8.1 transcription correlates with an enlargement of the optic stalk territory.
4.0 Ace/Fgf8.1 is required for zebrafish forebrain development

Although previous studies have suggested roles for Fgf8 in forebrain development, no specific in vivo role has been demonstrated. I have utilised zebrafish genetics to study the role of Fgf8.1 in zebrafish forebrain development. In zebrafish two homologues of Fgf8 have been isolated. Fgf8.1 is expressed in the ANR and at other sites of Fgf8 expression described in mice. Fgf8.2 is expressed later than Fgf8.1 and only in a subset of the Fgf8.1 expression domains, including the optic stalk and dorsal diencephalon (S.S., S.W., unpubl.). The acerebellar (ace) mutant was isolated in the Tübingen mutagenesis screen and shown to have a G to A point mutation in the splice site after the second exon of the fgf8.1 gene. An incorrect splicing event occurs, due to the point mutation, which gives a truncated, non-functional protein (Brand et al., 1996; Reifers et al., 1998).

Analysis of the ace mutant reveals the loss of Ace in zebrafish embryos leads to incorrect patterning of the optic stalk. The optic stalk territory plays an important role in guiding forebrain commissures across the midline and ace embryos display major defects in forebrain axon pathfinding. Ace is also required for the differentiation of an anterior group of neurons in the telencephalon and for the correct specification of the olfactory system.

4.2. Results

4.2.1. ace expression in the forebrain suggests a role in midline development

4.2.1.1. ace is expressed in the anterior neural plate.

ace is first expressed in the prospective forebrain at 95% epiboly stage in cells around the rostral margin of the neural plate (Fig4.1A). The homeobox containing gene emx1 is expressed in a similar band of marginal cells and double labelling experiments show that the ace is expressed within a subset of emx1 expressing cells (Fig4.1A,B). Fate mapping experiments (Varga, pers. comm.) confirm comparative in situ hybridisation analyses which suggest that these marginal neural plate cells give rise to the telencephalon.

By 10 somites, the rostral neural plate has condensed to form the forebrain and ace expression is detected in dorsal midline cells throughout the telencephalon (Fig4.1B). Over time, ace expression is lost from the dorsal telencephalon and becomes localised to a patch of cells at the midline of basal telencephalon, just dorsal to the anterior commissure (Fig4.1C,D).
Ace/FgF8.1 is required for zebrafish forebrain development

Fig 4.1. *ace* expression in the forebrain suggests a role in midline development

Animal pole (A), lateral (B,D-G) and anterior (C) views of (A-F) wildtype and (G) *ace*~*−* embryos labelled with digoxigenin and fluorescein RNA probes for *ace* (blue), *emxl* (red) and anti-N-acetylated tubulin antibody (brown). (A) tailbud (tb) stage (B) 10 somites. *ace* initially expressed in the anterior neural plate in a subset of *emxl* expressing cells. As neurulation proceeds, *ace* expression becomes restricted to the dorsal forebrain midline but is upregulated in the optic stalk territory. (C) Prim 10 stage (D) Prim 18 stage *ace* expression becomes refined to a small group of midline cells dorsal to the AC, whilst *ace* expression is maintained throughout the ventral optic stalk territory dorsal to the POC. (C-E) Other areas of *ace* expression in the forebrain include the pituitary, dorsal diencephalon, posterior tuberculum and ventral nasal placode. (F,G) Upregulation of *ace* transcripts is observed in the telencephalon, pituitary and dorsal diencephalon of *ace*~*−* embryos, suggesting a negative feedback loop operates in these regions. Scale bars represent 100μm (A,B) and 50μm (C-G). Abbreviations: ac, anterior commissure, dd, dorsal diencephalon, e, eye, fb, forebrain, hy, hypothalamus, mhb, mid-hindbrain boundary, op, olfactory placode, os, optic stalk, p, pituitary, poc, posterior commissure, s, somites, t, telencephalon, tb, tailbud.
4.0 Ace/Fgf8.1 is required for zebrafish forebrain development

4.2.1.2. ace expression in the optic stalk territory.

*ace* is expressed within the developing optic stalks. This site of expression may initially be continuous with the telencephalic expression as the optic vesicles evaginate. However, by 22 somites stage, a clear gap between the basal telencephalic and optic stalk expression domains is visible. This gap corresponds to a narrow domain of cells just ventral to the anterior commissure. At the midline, *ace* is expressed similar to the Pax protein *noi/pax2.1* (Macdonald et al., 1997) in cells just dorsal to the postoptic commissure (Fig 4.1C). More laterally within the optic stalks, *ace* is expressed diffusely and, unlike *noi*, only weakly within the choroid fissure.

4.2.1.3. ace expression in other forebrain structures.

Separate to the optic stalk expression, *ace* is expressed within the neural retina from approximately 22 somites and from prim 16 stage in the anterior pituitary and posterior tuberculum (Fig 4.1D and Reifers et al., 1998). *ace* is also expressed in cells of the dorsal diencephalon that may contribute to the habenular nuclei and in cells in the ventral nasal placode from prim 18 stage (Fig 4.1D,E).

4.2.1.4. ace mRNA transcripts are upregulated in *ace*~ embryos.

*ace* mutants synthesise a truncated, non-functional *fgf8.1* protein and can be identified by the loss of the cerebellum. Within the forebrain, *ace* expression was upregulated in *ace*~ embryos. This upregulation is variable and most noticeable within the dorsal diencephalon, basal telencephalon and pituitary (Fig 4.1F,G). This result indicates that *ace* normally negatively regulates its own expression within the forebrain.

4.2.1.4. Fgf8.2 does not compensate for the loss of Ace in the forebrain.

Another zebrafish FGF8 has been isolated by Schulte-Merke and colleagues (unpubl.). In wildtype embryos, *Fgf8.2* are weakly expressed in the optic stalk territory at prim 5. *Fgf8.2* is normally expressed in *ace*~ embryos and no upregulation of transcription is observed (data not shown).

4.2.2. ace is required for patterning the anterior and postoptic commissures
4.2.2.1. The AC and POC invade the midline optic stalk territory in ace~ embryos.

Functional interaction between Noi and Ace patterns midbrain and anterior hindbrain tissue. As Noi and Ace also expressed in similar domains in the forebrain, we investigated whether forebrain axon pathfinding defects occurred in ace mutants, as they do in noi mutants.

ace~ embryos showed defects of variable severity in the establishment of the AC and POC at the rostral tip of the developing forebrain (Fig4.2). In all mutants axons wandered inappropriately between the two commissures around the base of the optic stalks, a territory which normally excludes axons (Fig4.2A-F). In contrast to noi~ embryos, axons frequently misprojected rostrally and caudally quite lateral to the midline (Fig4.2A-D). In more severely affected embryos, one or both commissures sometimes failed to form altogether (Fig4.2B,D) and axons gave the impression of losing all sense of direction as they approached the midline (Fig4.2B-D). By prim 18 stage of development, the failure of both commissures to cross the midline and instead project towards each other was the most common phenotype observed (Fig4.2B). Although axons showed severe pathfinding defects between the two commissures, they did not show comparable navigational errors either dorsal to the AC or ventral to the POC.

4.2.2.2. The morphology of the midline territory is altered in ace~ embryos.

The severity of the midline axon guidance phenotype in ace~ embryos suggested that the midline tissue itself may be disrupted in mutant embryos. We therefore examined the morphology of the midline cells located between the two commissures in ace mutants. In wildtype embryos, there are about 9-10 cells between the AC and POC, the majority of which have a highly characteristic cuboidal morphology (Fig4.2G). Some of these cells express noi and are likely to differentiate as primitive glial cells (Macdonald et al., 1997). In ace~ embryos, cuboidal midline cells were absent and the expansion of the optic recess was occluded (Fig4.2H). This result indicates that midline tissue between the two commissures fails to form properly in the absence of Ace function.

4.2.3. ace~ embryos exhibit severe defects in formation of the optic chiasm

4.2.3.1. Retinal axons exhibit severe pathfinding defects.
4.0 Ace/Fgf8.1 is required for zebrafish forebrain development

**Fig4.2. ace is required for patterning the anterior and posterior commissures**

Anterior (A-D), lateral (E,F) views of prim-20 wildtype (A,E,G) and ace− (B-D,F,H) embryos labelled with anti-N-acetylated tubulin antibody. In the most severe phenotype, neither commissure crosses the midline and instead project into the optic stalk (B). In other instances, both commissures form but ectopic projections into the optic stalk still occur or the POC fails to cross and projects into the optic stalk (C,D,F). Parasagittal sections of (G) wildtype and (H) ace− embryos labelled with anti-N-acetylated tubulin reveal a disrupted optic stalk territory, distinct cuboidal cells no longer line the optic recess in ace− embryos (G,H-arrows indicate cuboidal cells lining the optic recess). Additionally the optic recess is occluded. Scale bars represent 25μm (A-F) and 10μm(G,H). Abbreviations: ac, anterior commissure, e, eye, hy, hypothalamus, os, optic stalk, pc, posterior commissure, poc, postoptic commissure, t, telencephalon, tpoc, tract of the postoptic commissure.
About 12 hours after the POC is established, the first retinal axons cross the midline establishing the optic chiasm, directly adjacent and rostral to the POC (Burrill and Easter, 1994). To determine if Ace is also required for establishment of the optic chiasm, we examined retinal projections in ace~ embryos.

In ace~ embryos, retinal axons frequently misprojected ipsilaterally and rostrally to incorrect targets (Fig4.3A,D,F,G,I). Axons showed no obvious defects exiting the eye and appeared to be normally fasciculated as they approached the midline. However, they frequently exited from the optic stalk/nerve inappropriately between the AC and POC (Fig4.3B,C). Although the earlier commissural axons made pathfinding errors in this same region, retinal axons formed ectopic projections that were quite separate from the other commissural axons (Fig4.3B,C). Indeed, despite the severe axonal disorganisation at the midline, retinal axons remained tightly fasciculated with other retinal axons, even when they formed inappropriate projections (Fig4.3D-I).

Ipsilaterally arranged axons turned soon after they exited the eye, quite distant to the midline. The variable ace~ phenotype was usually bilateral symmetrical; again suggesting the fault lies at the midline tissue (Fig4.3D,F,G).

4.2.3.2. ace~ embryos display two phenotypes in AC and POC pathfinding by 72hpf.

Defects in the AC and POC pathways have refined themselves to two phenotypes by 72hpf. The two axons either fuse together (Fig4.3D) or an ectopic tightly fasciculated projection is established between the two commissures (Fig4.3B).

4.2.3.3. Retinal axons frequently project to the telencephalon in ace~ embryos.

Although optic axons often navigated normally to the tectum in ace~ embryos, in some cases retinal axons sometimes formed ectopic rostral projections into the telencephalon (Fig4.3F,G,I and Picker et al., 1999). Once within the telencephalon, retinal axons remained tightly fasciculated and either corrected their trajectory back towards the midbrain tectum (Fig4.3F,H) or established an ectopic pathway to the dorsal telencephalon (Fig4.3G,I). These ectopic axons appeared to form axonal terminals in the dorsal telencephalon at later stages (see Fig4.4) suggesting that they may form ectopic termini.
**Fig4.3. ace<sup>-</sup> embryos exhibit severe defects in formation of the optic chiasm**

Ventral (A-G) and lateral (H,I) views of 48-72hpf wildtype (A,E) and ace<sup>-</sup> (B-D,F-I) embryos in which the retinotectal projection is visualised with anti-N-acetylated tubulin antibody (A-D) or through anterograde labelling with Dil (E-I). In wildtype embryos, the optic nerves form an entirely contralateral projection crossing the midline in the optic chiasm directly rostral to the POC. In ace<sup>-</sup> embryos, the retinal axons often approach the midline further rostrally between the AC and POC (C,D), sometimes project ipsilaterally (D,G) and to the telencephalon rather than the midbrain (F,H,I). Axons projecting towards the telencephalon sometimes correct their trajectories back towards the midbrain (arrow, H, arrowheads I). At these stages, the POC and AC are either fused (D) or are connected by an ectopic midline tract (C). Scale bars represent 25μm. Abbreviations: ac, anterior commissure, d, diencephalon, e, eye, mb, midbrain, oc, optic chiasm, poc, postoptic commissure, r, retina, t, telencephalon.
4.2.4. The retinal misprojections in ace mutants are due to a requirement for Ace in the brain, not the eye.

4.2.4.1. Layering and differentiation of the eye appears normal in ace− embryos.

As ace is expressed both in the eye and in the brain, the defects in retinotectal pathfinding in ace mutants could be due to a requirement for Ace in either or both locations. To address this issue, we examined the patterning and differentiation of the retina in ace mutants and assessed pathfinding following transplantations of wildtype and ace− eyes to wildtype and ace− host brains.

In ace− embryos, retinal differentiation superficially proceeds as in wildtype such that by two days, all layers of the retina are visible and photoreceptors, as marked by anti-Fret43 antibody staining, have started to differentiate in both wildtype and ace− embryos (Fig4.4A,B). Furthermore, most retinal ganglion cells acquire appropriate positional identity as assayed by their termination patterns within the midbrain (Picker et al., 1999). There may be minor disruption to nasotemporal patterning in dorsal regions of ace− eyes as gene expression characteristic of temporal retina appears to be expanded at the expense of nasal gene expression (Reifers et al., 1998).

4.2.4.2. Eye transplant experiments indicate the main patterning defect is within the ace optic chiasm territory.

Wildtype and ace− eyes formed normal chiasms when transplanted onto wildtype host brains (Fig4.4C,D). Conversely, both wildtype and ace− eyes formed ectopic ipsilateral and contralateral projections when transplanted onto host ace− brains (Fig4.4E,F). As noted above, the ectopic telencephalic projections appeared to form ectopic axon terminals in the rostral telencephalon.

4.2.5. Midline gene expression is altered in ace mutants

4.2.5.1. Altered gene expression in early ace− embryos.

The defects described above suggest that patterning of midline tissue in the commissure forming region of the forebrain is disrupted in ace− embryos. To begin to
Fig 4.4. Retinal misprojections in ace− embryos represent a requirement for Ace in the brain

Coronal sections of 48hpf (A) wildtype and (B) ace− embryos stained with an anti-Fret43 antibody. No obvious reduction in retinal layering or photoreceptor differentiation (arrowheads) is observed in ace− embryos. (C,F) Chimaeric embryos in which wildtype eyes were transplanted into wildtype (C) or ace− (F) host embryos or ace− eyes were transplanted into wildtype (D) or ace− (E) hosts embryos and retinal pathfinding observed with Dil and DiO labelling. When ace− or wildtype eyes are transplanted into wildtype host, normal retinal pathfinding occurs to the contralateral tectum. However when wildtype or ace− eyes are transplanted into ace− host embryos, ectopic pathfinding to the ipsilateral tectum and telencephalon are observed. Anterior is to the right and the phenotype of the transplanted eye is represented in the top right of the figure whilst the host phenotype is shown in the bottom right of the panel. Scale bars represent 20μm. Abbreviations: cmz, ciliary marginal zone, gcl, ganglionic cell layer, l, lens, pl, pigment layer, rnl, retinal nerve layer.
4.0 Ace/Fgf8.1 is required for zebrafish forebrain development

elucidate the alterations in gene activity that might underlie the patterning defects, we
examined the expression of various genes that are expressed in the affected territories.

Shortly after ace is first expressed in cells at the anterior margin of the neural plate,
we detected altered gene expression in prospective midline tissue. Similar to ace, enxl is
expressed in marginal neural plate cells. In about 25% of embryos from a cross between two
heterozygous ace carriers, enxl expression was reduced (Fig4.5A,B); most noticeably at the
rostral midline of the neural plate. Expression of the homeobox gene, anf, was similarly
reduced in putative ace- embryos (Fig4.5C,D). These results suggest that Ace activity is
required for patterning the rostral margin of the neural plate. As the neural keel condensed
and marginal neural plate cells fused dorsally to form the telencephalon, it became more
difficult to assess if these markers were still altered in ace- embryos. However, the rostral tip
of the neural plate is believed to give rise to the basal telencephalon and commissural region
(Eagleson and Harris, 1990 and data not shown) and later analysis confirmed that gene
expression alterations persisted in this region of ace mutants (see below).

4.2.5.2. Gene expression in the midline optic stalk territory is altered in ace- embryos.

Analysis of several markers in prim 10 to prim 20 stage ace- embryos suggests that
midline tissue just dorsal to the POC is either absent or is mis-specified in ace- embryos. noi
is expressed throughout the optic stalk and in a narrow band of cells at the midline just
dorsal to the POC (Macdonald et al., 1997). In ace- embryos, midline expression of noi was
reduced or absent although more lateral expression was not obviously affected (Fig4.6C,D).
Six3 is a homeobox containing gene expressed in the same optic stalk domain as noi and
additionally in cells ventral to the POC. In ace- embryos, six3 expression in the cells
adjacent to the optic recess was reduced or absent (Fig4.6E,F) confirming that the territory
dorsal to the POC was disrupted. Net1 and sema3D are genes involved in axon guidance
that are also expressed in the diencephalic optic stalk territory (Fig4.6G,H,K,L). Examination of these genes in ace- embryos revealed their expression is reduced or absent.
net1 expression is also reduced in the basal telencephalon (Fig4.6G,H). net2 and sema3D
dexpression, in the anterobasal telencephalon, is also reduced in ace- embryos (Fig4.6K,L and
data not shown). These genes may play more direct roles in the pathfinding defect observed
in ace- embryos.

In addition to noi, six3 and net1, we also examined several other markers expressed
in similar regions of the forebrain. Diencephalic shh expression was relatively normal though
its rostral limit of expression extended further dorsally to the optic recess (Fig4.6A,B). shh
4.0 Ace/Fgf8.1 is required for zebrafish forebrain development

**Fig 4.5. Early gene expression is perturbed in ace− embryos**

Animal pole views of (A,C) wildtype and (B,D) ace− tailbud embryos labelled with RNA probes against (A,B) emxl or (C,D) anf. Both emxl and anf are expressed in the anterior neural plate in a characteristic horse shoe shape, thought to demarcate the telencephalic anlage and optic stalk territory. A reduction in emxl and anf expression is observed in the most rostral tip of the anterior neural plate. This is unlikely to be due to a general developmental delay in the mutants as anf is actually expressed in broader territories at earlier stages (Kazansky *et al.*, 1997). Scale bars represent 50 μm.
4.0 Ace/Fgf8.1 is required for zebrafish forebrain development

is also expressed in the basal telencephalon, just dorsal to the AC, and this domain of expression was reduced or absent in ace− embryos. Unlike shh, expression of twhh, a second hedgehog gene, was reduced in diencephalic cells ventral to the POC in ace− embryos (Fig4.6I,J). Ephrin-A-L4 is a GPI linked proteins with known growth cone collapse properties that is expressed in cells ventral to the POC and in the basal telencephalon in similar domains to shh. As with shh, expression in the basal telencephalon of ephrin-A-L4 is reduced or lost and within the diencephalon, expression of ephrin-A-L4 expands slightly towards the optic recess (Fig4.6K,L). nk2.1, a putative target gene of the Hedgehog pathway, showed no major changes in expression in ace− embryos (Fig4.6M,N).

4.2.6. The preoptic area and dorsal diencephalon are affected in ace− embryos

4.2.6.1. Reduction of the preoptic area in older ace− embryos.

The most prominent defect in late somitogenesis ace− embryos is in the patterning of midline cells between the two commissures at the base of the optic stalks. The fate of this region of the brain has not been followed in detail but is presumed to give rise to preoptic regions at the interface between telencephalon and diencephalon. Although the defect in this region in ace− was initially modest and there was no overall change in brain shape, the severity of the phenotype increased over time, particularly in embryos, that exhibited fusion of the commissures. By three days, a gross distortion in brain shape was visible around the commissures (Fig4.7A-C). Caudal to this region, hypothalamic tissue appeared superficially normal (Fig4.7A,B) and there was no obvious alteration in hypothalamic TH neurons (data not shown). Rostral and dorsal to the preoptic region, the telencephalon was smaller in ace− embryos, but there was usually good separation of the olfactory bulb from other telencephalic structures (Fig4.7A-C).

To determine if localised cell death or cell proliferation might underlie the abnormal patterning and subsequent loss of the midline territory of ace mutants, we compared cell counts of an apoptosis marker, TUNEL, and a marker for mitotic cells, phosphorylated histone H3 (PH3), of wildtype and ace− embryos. Cells positive for PH3 were counted in cells lining or around the optic recess. Similarly cells positive for TUNEL staining were counted in the optic stalk territory. In prim 5 stage embryos we observed no difference between wt and mutant embryos for TUNEL staining but noted a marked reduction in proliferation in ace− embryos (Table4.1). TUNEL staining was not quantified at prim 22, as virtually no positive cells could be detected in either the wildtype or ace− embryos (data not shown).
Fig4.6. Midline gene expression changes, late morphological abnormalities and dorsal diencephalic pathfinding defects in ace- embryos

Lateral views of (A) prim 14 stage wildtype and (B) ace- embryos hybridised with digoxygenin-labelled probes to shh. shh expression in the diencephalon expands slightly towards the optic recess (shown by asterix), ventral telencephalic expression is reduced or absent in ace- embryos.

Anterior views of 18 somite (C) wildtype and (D) ace- embryos labelled with RNA probes against noi mRNA. noi expression is reduced at the forebrain midline but is less affected in the lateral optic stalks and eyes.

Anterior views of prim 10 stage (E,G) wildtype and (F,H) ace- embryos double labelled with a N-acetylated tubulin antibody (brown) to reveal axon pathways and RNA probes to (E,F) six3 and (G,H) netrin1. six3 (E,F) is normally expressed throughout rostral diencephalic cells, upto the optic recess. In ace- embryos, six3 expression is lost in cells adjacent to the optic recess and axons start to invade the optic stalk territory (arrows indicate the position of axons of the tract of the POC and optic recess). netl (G,H) is expressed in cells ventral to the optic recess and dorsal to the POC. This expression is reduced in the ace- embryos.

Lateral views of (I,K,M,O) prim 10 to 20 stage wildtype and (J,L,N,P) ace- embryos hybridised with labelled digoxygenin RNA probes to (I,J) twhh, (K,L) sema3D, (M,N) ephrin-A-L4 and (O,P) nk2.1. (I,J) twhh is normally expressed in a rostral diencephalic subset of shh expressing cells, which lie adjacent to the position where POC axons will cross the midline. In ace- embryos, twhh expression is reduced in this territory but unaffected in other domains of expression. (K,L) sema3D is expressed in the ventral optic stalk, in a similar domain to ace and noi, and is reduced in ace- embryos. Expression is also reduced in the ventral telencephalon. (M,N) ephrin-A-L4 is expressed in the ventral hypothalamus underneath the point the POC crosses the midline and weakly in the basal telencephalon. In ace- embryos, ephrin-A-L4 expression in the basal telencephalon is reduced and hypothalamic expression is expanded slightly towards the optic recess. (O,P) nk2.1 is also expressed in a hypothalamic domain, away from the POC and is relatively unaffected in ace- embryos. Scale bars represent 25 µm (A,B,E-H) and 50 µm (C,D,I-P). Abbreviations: ac, anterior commissure, e, eye, hy, hypothalamus, ob, olfactory bulb, or, optic recess, os, optic stalk, pc, posterior commissure, poc, postoptic commissure, r, retina, t, telencephalon, te, tectum.
4.0 Ace/Fgf8.1 is required for zebrafish forebrain development

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<th>Stage</th>
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<td>prim 5</td>
<td>5.3</td>
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Table 4.1 Cell proliferation and apoptosis in ace⁻ embryos

To assess whether cell proliferation was responsible for the loss of the preoptic territory, cells in a proliferative state, as marked by anti-phosphorylated histone H3 antibody, were counted in the optic stalk territory. Similarly to assess apoptotic cells, we used TUNEL staining. ace⁻ embryos display a reduction in the mean no. of mitotic cells compared to wildtype, this reduction is greater with time.
4.0 Ace/Fgf8.1 is required for zebrafish forebrain development

4.2.6.2. Defects in habenular commissure pathfinding in ace~ embryos.

The dorsal diencephalic expression of ace at prim 18 stage and later is coincident with a territory thought to give rise to the habenular nuclei. The habenular commissure crosses the dorsal midline territory over the prospective habenular nuclei and is reduced or absent in ace~ embryos (Fig 4.7D,E).

4.2.7. Neurogenesis in the telencephalon is affected in ace~ embryos

4.2.7.1. Regionalisation of the telencephalon is unaffected but neuronal differentiation is perturbed in ace~ embryos.

At early stages, the telencephalon in ace~ embryos appears to be morphologically normal. To assess whether there were more subtle defects in the telencephalon at these stages, we examined a variety of markers expressed either regionally within the telencephalon, or within specific groups of putative telencephalic neurons.

Whereas regional patterning of the telencephalon appeared to be relatively normal in ace~ mutants, some patterns neuronal differentiation were disrupted. Markers of various broad telencephalic domains (dlx2, rtk7 arxl, unc4, eome) showed no obvious changes in their patterns of expression in wt and ace~ embryos (Fig 4.8A,B and data not shown). In contrast markers of neuronal differentiation are disrupted in ace~ embryos. lim1 and lim6 appear to be expressed in complementary domains of the rostral telencephalon, and while the identity of these cells is unknown in fish, by analogy with other regions of the CNS, one might predict that the Lim genes define different populations of neurons. In ace~ embryos, expression of both lim1 and lim6 in the telencephalon was reduced or absent (Fig 4.8E-H). In contrast, the Lim gene isl1, was expressed in neurons of the basal telencephalon in both wildtype and ace mutants (Fig 4.8C,D). Similarly lim5, which in mice demarcates hippocampal neurons, is also expressed normally in wildtype and ace~ embryos (data not shown).

4.2.8. Disruption of olfactory axon pathfinding in ace~ embryos

4.2.8.1. Olfactory axons enter the bulb but fail to make correct connections.

In wildtype embryos, olfactory neurons project axons into the bulb and form terminations that appear to prefigure the olfactory glomeruli of the mature olfactory bulb
Fig4.7. General brain morphology is disturbed in older ace mutants
Lateral (A-C) and dorsal (D,E) views of 72hpf (A,D) wildtype and (B,C,E) ace− embryos stained with anti-N-acetylated tubulin antibody. (A-C) Lateral views reveal the preoptic area (marked by an asterix), bordered by the AC and POC in wildtype embryos is reduced or absent in ace− embryos. The arrows in (C) indicate the retinal nerve ectopically projecting to the ipsilateral telencephalon.

ace is expressed in the dorsal diencephalon, an area through which the habenular commissure will navigate and cross the dorsal midline. In ace− (E) embryos, the habenular commissure is reduced and does not cross the dorsal midline correctly. Scale bars represent 30μm. Abbreviations: ac, anterior commissure, e, eye, hy, hypothalamus, ob, olfactory bulb, or, optic recess, os, optic stalk, pc, posterior commissure, poc, postoptic commissure, r, retina, t, telencephalon, te, tectum.
Fig 4.8. Telencephalic neurogenesis is affected in the ace embryos

(A-H) Lateral views of prim 14 stage wildtype and ace brains labelled with various probes.
(A,B) \textit{dlx2}, a marker of regional telencephalic patterning, is expressed in similar domains in wildtype and ace embryos.
(C,D) Islet1/2 is expressed in several clusters of neurons in the rostral forebrain (arrowheads). All of these clusters are present in the ace embryo.
(E,F) \textit{lim1} is expressed in two clusters of cells dorsally and anteriorly near the midline of the telencephalon of the wildtype embryo. The ventral domain of expression is absent and the dorsal domain reduced (arrowhead) in the ace embryo.
(G,H) um6 is expressed in an anterior group of cells in the wildtype embryo. This site of expression is severely reduced in the ace embryos. Scale bars represent 40μm.

Abbreviations: d, diencephalon, e, epiphysis, t, telencephalon.
4.0 Ace/Fgf8.1 is required for zebrafish forebrain development

(Fig4.9A,C; Whitlock and Westerfield, 1998). In ace− embryos, there were fewer axonal condensations and the condensations which did form were more typically seen in the medial bulb (Fig4.9A-D). As with the retinotectal projection, the olfactory axon phenotype of ace− embryos was bilaterally symmetrical, such that both halves of the embryo were affected equally.

4.2.8.2. The olfactory bulb is disorganised in ace− embryos.

Detailed examination revealed that the olfactory bulb was smaller and glomeruli were less well developed in ace− embryos compared to wildtype (Fig4.9E,F). Confocal imaging of bodipy ceramide labelled embryos revealed the cellular organisation of the ace− bulb to be disorganised with fewer, poorly formed glomeruli (Fig4.9G,H).

To further characterise the defect within the olfactory bulb, we looked at the expression of cell specific markers in ace− embryos. dlx2, a marker for interneurons in mice, is relatively unaffected in ace− embryos but markers for projection neurons, such as emx1 and eome, show severe perturbation (Fig4.9I-L and data not shown). By 72hpf, emx1 shows focal patches of expression in the olfactory bulb, which correlate with the positions of the glomeruli. In ace mutants, expression is reduced and there is less localisation to the glomeruli (Fig4.9K,L).

4.2.8.3. Olfactory placodal development is relatively normal but Ace is required for the differentiation of odorant receptor neurons.

ace is expressed in the olfactory placode as well as the telencephalon and we do not know which site of activity is important for correct development of the olfactory bulb. There were no obvious defects in the development of the nose as assessed by histological sectioning (Fig4.9M,N). Also patterning genes, such as eyab, and early neurogenic genes, such as ngn1 and neuroD, are not obviously altered in ace− embryos (data not shown). As odorant receptors are thought to be involved in olfactory axon pathfinding, I examined the expression of three odorant receptor genes, OR2.0, 2.4 and 13.0, in ace− embryos. The expression of OR2.0 was reduced or absent in mutant embryos while OR2.4 and 13.0 appear unaffected (Fig4.9O,P).
4.0 Ace/Fgfr1 is required for zebrafish forebrain development

**Fig4.9. Disruption of olfactory axon pathfinding, olfactory bulb and odorant receptor differentiation in ace<sup>-</sup> embryos**

(A-F) Frontal views of wildtype and ace<sup>-</sup> embryos labelled with antibodies to (A-D) anti-zns2 (A-D) and acetylated tubulin (E,F). The zns2 antibody detects a transient group of pioneer olfactory axons as they navigate into the olfactory bulb and form termini. In ace<sup>-</sup> embryos, fewer termini are detected and those that do form are detected around the medial olfactory bulb. Olfactory bulbs were dissected from whole 96hpf embryos stained with anti-N-acetylated tubulin. The olfactory bulb in the ace<sup>-</sup> embryo is typically smaller than the wildtype siblings, and has fewer projections and foci.

(G,H) The organisation of the bulb was examined using bodipy ceramide labelling of the cell membranes. The ace<sup>-</sup> bulb is more disorganised with fewer glomeruli.

(I-L) Lateral (I,J) and frontal (K,L) views of wildtype and ace<sup>-</sup> embryos hybridised with digoxigenin labelled RNA probes to *dlx2* and *emxl*. (I,J) *dlx2* expression is similar in the wildtype and the ace<sup>-</sup> embryos. (K,L) In the wildtype embryos, *emxl* is expressed in projections neurons of the glomeruli and expression is reduced and disorganised in the ace<sup>-</sup> embryo. (M,N) Horizontal sections of 48hpf wildtype and ace<sup>-</sup> embryos reveal that although the ace<sup>-</sup> placode is slightly smaller, its structure is relatively normal.

(O,P) Ventral views of 48hpf wildtype and ace<sup>-</sup> embryos hybridised with digoxigenin labelled RNA probes to *Odorant Receptor 2.0* (OR2.0). OR2.0 expression is reduced throughout the olfactory placode in the ace<sup>-</sup> embryos. Scale bars represent 25μm (A-F,K,L), 10μm (G,H), 50μm (I,J) and 15μm (M-P). Abbreviations: ob, olfactory bulb, op, olfactory placode, t, telencephalon.
4.2.9. Perturbation of FGF signalling leads to dramatic defects in midline patterning

Fgfs are a large family of proteins which are often expressed in overlapping domains in the embryo. The complexity of Fgf signals during embryonic development means that there may be some degree of functional redundancy such that loss of any one ligand may in part be compensated by other family members.

One way that is commonly used to broadly inhibit Fgf function is to overexpress a truncated receptor that dimerises with endogenous receptors and inhibits their function. While such experiments have proved useful, within the embryo, the specificity of the truncated receptors is unknown. Nevertheless, given this caveat, we overexpressed the XFD truncated receptor and compared the phenotype to ace mutants (Amaya et al., 1991).

As has previously been described, widespread overexpression of XFD gives embryos that lack a trunk and tail but possess a relatively normal looking head (Griffin et al., 1995). However, more detailed analysis of the brain in such embryos showed that the embryos exhibited phenotypes comparable to those observed in ace mutants. Most commonly, we observed that commissures were either fused or that one or other of the commissures was absent (Fig 4.10A,B).

The drug SU5204 has been described as a potent inhibitor of Fgfr1 function (Mohammadi et al., 1997). Fgf8 is thought to bind and activate Fgfr2 and Fgfr3 but the drug blocks Fgfr1 activity by binding to the hinge region, which is identical in Fgfr1 and Fgfr2. Therefore application of the drug may block Fgf8 signalling. I observed a dose dependent effect on SU5204 treated wildtype embryos (Fig 4.10C,D). At low concentrations, the embryos exhibit defects similar to ace− embryo, with one or both commissures failing to cross the midline and invading the optic stalk territory. At higher concentrations, neither commissure crossed the midline and again axons project towards each other across the optic stalk territory. In addition, the head and eyes are smaller than the wildtype or ace− embryos.

4.3. Discussion

Although the FGF family has been extensively studied in a multitude of developmental processes, relatively little work has addressed their role in patterning the vertebrate forebrain. Previously expression pattern and explant studies suggest that FGFs
Fig 4.10. Perturbation of FGF signalling leads to dramatic defects in midline patterning

Frontal views of prim-20 stage wildtype, (A), dominant negative FgfR (XFD) injected (B) and SU5204 treated embryos, labelled with anti-N-acetylated tubulin antibody. (B) Reducing FgfR activity through widespread overexpression of XFD leads to a phenotype similar to that observed in ace~ embryos with the AC and POC reduced and ectopic axonal projections between the two commissures. (C,D) Lower concentrations (8µM) of the drug, SU5204 (C), lead to similar commissural defects as observed in ace~ and XFD treated embryos. At higher concentrations (20µM, D) a more severe phenotype is observed, in which neither commissure crosses the midline and axons project ectopically lateral to the midline. The head and eyes of these embryos are also much smaller than their wildtype siblings. Scale bars represent 25µm. Abbreviations: ac, anterior commissure, os, optic stalk, poc, postoptic commissure.
may play an inductive role in forebrain development (Shimamura and Rubenstein, 1997; Ye et al., 1998). Further reports suggest reducing Fgf8 activity in mice reduces the size of the forebrain (Meyers et al., 1998). Here I have demonstrated specific in vivo roles for Fgf8 in specification of the optic stalk territory, guidance of forebrain commissural axons, differentiation of telencephalic neurons, olfactory bulb organisation and neuronal specification in the olfactory placode.

4.3.1 Ace and midline axon guidance through the POC and AC

In contrast to the caudal CNS, there are very few locations in the forebrain at which axons cross the midline. Those commissures that do form are established at invariant locations by tightly fasciculated bundles of axons (Chitnis and Kuwada, 1990; Wilson and Easter, 1990). At these locations, axons must be both attracted to the midline yet also inhibited from decussating at inappropriate locations, suggesting that a combination of both attractive and inhibitory guidance cues are required. These specialised positions represent choice points, an intermediate position which provides important navigational cues along the axon pathway, and coincide with, or are adjacent to, boundaries of regulatory gene expression domains within the neuroepithelium (Macdonald et al., 1994; Barth and Wilson, 1995; Cook et al., 1998). It has been previously suggested that axons may be funneled across the midline at the interface between two domains of cells, both of which discourage growth cone exploration (Macdonald et al., 1997). In support of this possibility, POC growth cones become less exploratory and axons more tightly fasciculated as they approach the midline.

The disruption of axon guidance at the forebrain midline is particularly severe in ace- embryos. Fgfs have previously been shown to have chemoattractive properties with regard to retinal nerves and one possibility is that ace acts to attract AC and POC axons towards the midline (McFarlane et al., 1995). If ace was acting purely as a chemoattractant, at least two criteria must be fulfilled. First, the AC and POC axons would be expected to express FgfRs. Only FgfR4 expression has been detailed in zebrafish, and although it is expressed in territories through which the AC and POC will navigate, it is unclear whether it is actually expressed by the navigating axons (Thisse et al., 1995). Secondly, the expression domain of ace must coincide with the area the axons are navigating towards. In the telencephalon, ace is expressed in the dorsal midline at the time when AC axon projections start. As the dorsal midline normally excludes axons, it is unlikely that ace does act as a chemoattractant in the telencephalon. Expression of ace, in the ventral optic stalk territory, does indeed coincide
with the intermediate target for POC axons. However other observations in ace− embryos lead us to favour a patterning role for ace in the forebrain.

In support of the hypothesis that ace patterns the optic stalk territory and does not act simply to attract axons to the midline, we detect a number of early and late morphological and gene expression changes in the forebrain. Gene expression changes, in ace− embryos, are detected soon after ace expression is initiated in the anterior neural plate. Both emxl and anf expression is missing at the anterior tip of the neural plate and fate maps indicate this region will form the basal telencephalon and commissural plate (Varga, personal communication). These early defects within the presumptive commissural plate territory probably influence later pathfinding defects by AC and POC axons. In mice, loss of emxl activity results in failure of the callosal commissure axons to cross the midline, thought to result from a loss of glial cell differentiation (Yoshida et al., 1997).

The reduction or loss of noi expression at the midline of ace− embryos suggests that at least some of the commissural defects of ace− embryos might be attributed to loss of Noi activity (Macdonald et al., 1997). However, the ace phenotype is much more severe than complete loss of function noi alleles and so Ace must also regulate midline patterning through Noi independent pathways. Indeed, in noi− embryos, the morphogenesis of midline tissue initially appears relatively normal whereas in ace− embryos, the cuboidal cells characteristic of midline tissue between the commissures fail to properly differentiate.

For successful commissure formation, the axons must be attracted to the midline but also repulsed from crossing the midline at ectopic locations. To achieve this ace and/or noi in the ventral optic stalk may regulate guidance factors; such as sema3D, netl and ephrinAL4. Semaphorins are secreted and transmembrane bound proteins which have been implicated in axon growth cone repulsion. Sema3D, an orthologue of chick collapsins, probably functions in the ventral optic stalk to exclude POC axons from this territory. In ace− embryos, reduction of sema3D expression may allow POC axons to enter the optic stalk territory (Chen et al., 1998; Halloran et al., 1999). A recent report has suggested Semaphorins may act as an attractive cue in forebrain projections (Bagnard et al., 1998) and a similar role for sema3D in POC guidance can not ruled out until further analysis involving overexpression and receptor analysis has been performed.

Ephrin-A-L4 is a GPI-linked membrane protein of the Eph ligand family, which can repel advancing axons (Brennan et al., 1997; Orioli and Klein, 1997). In the hypothalamus, expression of this ligand may prevent axons wandering inappropriately into that territory.
The basal telencephalic expression of ephrin-A-L4, and its loss in ace~ embryos, may suggest a chemoattractive role for the Eph family. Although no previous role for Eph family members as chemoattractants for axons has been reported, such a role has been described during the formation of blood vessels (Adams et al., 1999). Alternatively, ephrin-A-L4 expression in the basal telencephalon may convey identity towards a group of differentiating neurons and loss of expression may reflect a role for ace in neurogenesis, quite separate from an axon guidance role (Monschau et al., 1997, Chandrasekhar et al., 1999 and see later).

Although Netrin1 was initially isolated as a chemoattractant, it has been shown to have repulsive properties towards certain axons (Tessier-Lavigne and Goodman, 1996). Analysis of net1 expression in noi~ embryos suggests a chemoattractive role towards POC axons, as successful crossing of the midline by POC axons correlates with normal net1 expression (Macdonald et al., 1997). Further evidence for net1 having a chemoattractive role in the forebrain comes from investigations into the expression and loss of function analysis of net1 in mice (Serafini et al., 1996). In the mice forebrain, net1 is expressed in the commissural plate and forebrain commissures do not form correctly in the knockout mouse, suggesting that net1 is required to attract axons across the midline. Similarly the expression of zebrafish netl in the optic stalk, loss of netl in ace~ embryos and subsequent failure to form correct commissures may correlate with a chemoattractive role for netl in the zebrafish. However an alternative explanation for netl function in the forebrain may be to repulse axons away from the optic stalk territory. In this model, the loss of netl expression in ace~ embryos would allow axons to invade the optic stalk territory. The combination of failure to form correct commissures and invasion of the optic stalk in ace~ embryos does not allow us to assign a specific role for netl in the forebrain without undertaking more specific experiments.

Analysis of Hh pathway mutations indicates that this signalling pathway is required for patterning of midline tissue around the forebrain commissures (Schaurte et al., 1998; Karlstrom et al., 1999; KAB, SS and SW, unpublished data). Indeed noi expression is widely expressed in response to ectopic Hh expression (Ekker et al., 1995; Macdonald et al., 1995) and mice lacking Shh activity lack pax2 expression (Chiang et al., 1996) suggesting that noi/pax2 expression is normally regulated by Hh signalling. As noi expression and midline development is also affected in ace~ embryos, this raises the possibility that the Fgf and Hh pathways may cooperate to pattern rostral midline tissue in the forebrain. There are alterations to Hh gene expression in the midline of ace mutants with twhh expression reduced and shh expression slightly expanded dorsally. However, noi
expression is reduced despite the normal or expanded *shh* expression suggesting either that *twhh* may be more important in regulating noi expression or that *Ace* influences noi expression downstream or parallel to *Hh* activity. *shh* expression is also lost in the ventral telencephalon in *ace*<sup>-</sup> embryos. A role for *shh* in patterning of the ventral telencephalon in mice has been suggested but no role for axon guidance has yet been proposed (Ericson *et al.*, 1995; Kohtz *et al.*, 1998).

**Six3**, the zebrafish homologue of *Drosophila sine oculis*, has been demonstrated to have roles in controlling the size of the forebrain (Kobayashi *et al.*, 1998) and in medaka, eye induction and growth (Loosli *et al.*, 1999). In *ace*<sup>-</sup> embryos, *six3* is lost in cells adjacent to the optic recess, which suggests *six3* expression in this territory may play a role in establishing an axon negative territory. This may be achieved in combination with other transcription factors or signalling molecule as POC axons grow through a *six3* domain of expression.

It may be argued that the commissural phenotype seen in *ace*<sup>-</sup> embryos is a result of loss of the optic stalk territory. Indeed in 72hpf embryos, the preoptic area is greatly reduced in size and brain morphogenesis altered. However I believe the gene expression changes observed at prim 10 stage and the corresponding commissural defects observed at prim 20 are due to incorrect patterning. Examination of gene expression reveals that not all genes, expressed in the hypothalamus underlying the position where POC axons cross the midline, were expanded into the optic stalk region, as would be predicted if the area was absent or smaller in size. There is also differential regulation of the genes examined, which can not be accounted for by the simple loss of the optic stalk territory. During the time period examined, no increased cell death was observed but a corresponding decrease in cell proliferation was detected, suggesting a combination of normal programmed cell death and decreased proliferation could, in time, give rise to a smaller preoptic area.

### 4.3.2. *Ace* is required for formation of the optic chiasm.

In *ace*<sup>-</sup> embryos, retinal axons frequently fail to cross the optic chiasm and instead project to the ipsilateral tectum and/or forebrain. On occasions when the retinal axons do cross the optic chiasm, pathfinding errors within the contralateral forebrain still occur. *ace* expression in the eye does not appear to affect the layering of the eye. In addition, transplant experiments suggest that errors at the optic chiasm dominate the *ace*<sup>-</sup> retinal nerve phenotype. Nasotemporal patterning changes occur within *ace*<sup>-</sup> eye but these may contribute
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to pathfinding within the tectum itself and are unlikely to affect optic nerve guidance over the optic chiasm (Reifers et al., 1998; Picker et al., 1999).

Although *ace*, like *noi*, is expressed in the choroid fissure, only mild coloboma was observed in *ace<sup>-</sup>* embryos (Macdonald et al., 1997). The loss of *Pax2/Noi* is associated with coloboma in zebrafish, mice and humans, suggesting a pivotal role for *Pax2/Noi* in choroid fissure closure (Sanyanusin et al., 1995; Torres et al., 1996; Macdonald et al., 1997). The normal expression of *noi* in the choroid fissure of *ace<sup>-</sup>* embryos may override any subtle effects *ace* signalling may have in the choroid fissure area.

In zebrafish, the optic fibres cross the midline of the diencephalon in separate channels lined by immature glial cells (Burrill and Easter, 1995; Marcus and Easter, 1995). The glial cells play a crucial role in guiding retinal axons across the midline territory. The optic stalk gives rise to thin glia limitan, which mature to form reticular astrocytes, that encase the optic nerve. In *noi<sup>-</sup>* embryos, the initial steps of astrocyte differentiation fail and as *noi* is not expressed in the *ace<sup>-</sup>* midline tissue, we can assume astrocytes probably also fail to differentiate in *ace<sup>-</sup>* embryos (Macdonald et al., 1997). The failure to compartmentalise the nerve using glial cells may lead to the ipsilateral projection frequently observed in *noi<sup>-</sup>* embryos (Burrill and Easter, 1995; MacDonald et al., 1997). A similar explanation could be suggested for the more severe pathfinding defects made by the *ace<sup>-</sup>* optic nerve. A disorganisation of the optic chiasm may allow the optic nerve to wander into regions previously unavailable to it, such as the basal telencephalon. Once in the basal telencephalon, the nerve may follow the most favourable axon guidance cues to project to the dorsal telencephalon and terminalise there.

In addition to glial guidance of the retinal nerve, other cues are probably important in axon pathfinding across the midline (Booth et al., 1999). Several studies have investigated the role of FGFs in optic nerve pathfinding to the tectum. From these studies, a potential role for bFGF in pathfinding within the diencephalon has emerged (McFarlane et al., 1995; McFarlane et al., 1996). bFGF is expressed in the *Xenopus* optic tract and reciprocally, Fgfr is expressed on retinal nerve axon growth cones. Inhibition of Fgfr function leads to a delay in axon outgrowth and ectopic target recognition upon arrival at the tectum; overexpression of bFGF has the similar results. These studies have addressed the role of Fgfs in the optic tract prior to entry into the tectum. Our study has focused on the role of *ace* in retinal axon pathfinding over the optic chiasm. As *ace* is expressed in the ventral optic stalk territory and later in the optic chiasm (Reifers et al., 1998), it may act directly to attract retinal axons towards the optic chiasm midline.

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The Netrin gene family has been proposed to play an important role in the different stages of optic nerve guidance. Retinal growth cones, in vitro, display a DCC mediated outgrowth response towards a netrin1 source, indicating a functional requirement of net1 in optic nerve guidance (de la Torre et al., 1997). Denier and colleagues (1997) have demonstrated that net1 is required for the normal entry of retinal ganglion cells into the optic stalk and a loss of net2 in the choroid fissure in noi~ embryos indicate that zebrafish retinal axons are potentially responsive to netrin molecules (Macdonald et al., 1997). Expression of net2 in the ace~ choroid fissure is normal but the loss of net1, within the optic stalk in ace~ embryos, may cause the retinal axons to lose direction within the optic chiasm. The combination of failure of the glial cells to differentiate and more perturbed optic chiasm may be responsible for the more severe retinal axon pathfinding defect in ace mutants than noi mutants.

4.3.3. Ace is required for forebrain patterning but not induction.

Previous studies reported Fgf8 signalling is required for telencephalic patterning (Shimamura and Rubenstein, 1997) and forebrain dopaminergic cell specification (Ye et al., 1998). The loss of ace signalling leads to early patterning defects in cells predicted to form the basal telencephalon. When later patterning of the basal telencephalon was examined regional identity, as demarcated by genes like dlx2, was unaffected but minor changes in shh, net1, sema3D and ephrin-A-L4 were detected. In general, regional patterning of the telencephalon does not appear to be significantly affected in ace~ embryos. This suggests that Ace alone is not required for induction or broad regional patterning of the forebrain. There are many Fgf genes and so a combination of Fgfs may play a more general role in forebrain patterning. However, although by no means conclusive, the observation that more extensive in vivo blocking of Fgf receptor activity with XFD or SU5204 still gives rise primarily to midline defects does suggest that Fgfs play roles in patterning the forebrain rather than in early induction of forebrain fates.

More subtle defects in the telencephalon are observed in ace mutants, including changes in neuronal specification. Telencephalic neuronal differentiation was differentially affected in ace~ embryos. Although ventral isl1 or caudal lim5 populations of neurons were not affected in ace mutants, the dorso-anterior neuronal groups defined by expression of the Lim homeodomain proteins, lim1 and 6, were reduced or lost in ace~ embryos (Toyama and Dawid, 1997). The lim1 and lim6 population of neurons are in close proximity to the dorsal midline expression of ace, which suggests that ace could act over a relatively short distance.
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The apparent normal expression of proneural genes like, ngn1, support a role for ace in differentiation of dorsal neurons but not in the early stages of telencephalic neurogenesis. Our study suggest that although differentiating neurons have a regional identity, as assayed by expression of a broad regional telencephalic markers, they only differentiate into specific neurons once they have reached their target area.

Dopaminergic cell specification was assayed in the hypothalamus in ace− embryos and was apparently normal. Limitations of the antibody protocol prevented analysis of dopaminergic cell specification in the telencephalon. Fgf8 has been shown to regulate the expression of other Lim homeodomain genes in the pituitary and branchial arch (Ericson et al., 1998; Tucker et al., 1998). The regulation of Lim homeodomain proteins by Fgf8 may indicate its conserved role in differentiation in different tissues.

4.3.4. Ace and the development of the olfactory system.

The current hypothesis regarding olfactory axon pathfinding suggests that the odorant receptors expressed by olfactory sensory neurons are responsible for pathfinding to an invariantly positioned glomeruli (Wang et al., 1998). This pathfinding appears to be governed by the sensory neuron sensing positional cues within the bulb, with no activity dependent refinement of the projections (Dynes and Ngai, 1998; Brunet et al., 1996).

The olfactory axons, in ace− embryos, enter the olfactory bulb but make fewer terminalisations. The Zns2 antibody labels an olfactory pioneer axon population. Failure of these pioneers to pathfind correctly leads to errors in pathfinding by later olfactory axons (Whitlock and Westerfield, 1998). Preliminary results indicate mistargeting of these Zns-2 positive pioneer neurons also leads to olfactory axon pathfinding mistakes in ace− embryos (data not shown).

Recent work has described how olfactory axon pathfinding can occur correctly in mice lacking projection neurons or interneurons (Bulfone et al., 1998). Although my findings contradict those observed by Bulfone and colleagues, there are significant differences between the two studies. The dlx1/2 and tbrl knockout mice affect either the interneurons or the projection neurons, other cell types such as radial glia are unaffected. In ace− embryos, the radial glia cells may be absent leading to a loss of positional cues for olfactory axon guidance. Although the expression of axon guidance cues at the olfactory axons' point of entry, the dorsal telencephalon, is not perturbed in ace− embryos, it is possible that later cues within the olfactory bulb are perturbed. The olfactory bulb defects
observed as a result of a reduction of Fgf8 signalling in mice support a role for ace/Fgf8 in olfactory bulb development (Meyers et al., 1998).

The odorant receptor, expressed by a particular olfactory neuron, may be the crucial determinant in olfactory axon pathfinding (Mombaerts et al., 1996; Wang et al., 1998). Although similar neurogenic cascades occur in the olfactory neuroepithelium as in other systems, little is known about how the different odorant receptor neurons are specified (Cau et al., 1997). Recent insight into how different olfactory neurons express different odorant receptors (OR) has come from the cloning of Drosophila odorant receptors (Clyne et al., 1999). Clyne and workers showed that in a mutant of the Acj6 POU domain transcription factor, a subset of olfactory receptor neuron are not correctly expressed and the mutant animals display abnormal odor sensitivity (Clyne et al., 1999a). Ace mutants have reduced OR2.0 expression but not OR2.4 or OR13.0 expression. Odorant receptors display zonal organisation within olfactory epithelium but are randomly distributed within the zones (Ressler et al., 1993; Weth et al., 1996). Zonal expression of odorant receptors has not been analysed at 48hpf and conclusions can not be drawn on whether ace plays a role in differentiation of ORs in a particular zone. The timing of OR expression in the olfactory epithelium has been demonstrate to be asynchronous in nature (Barth et al., 1996). The transient expression of ace in the olfactory epithelium may provide a molecular mechanism for achieving this asynchronous activation. OR2.0 is expressed relatively early around prim 26, approximately when ace expression is found in the nasal placode, whilst OR2.4 and OR13.0 are expressed later at 48hpf, when ace is no longer detected in the olfactory placodes (Barth et al., 1997). The control of OR2.0 expression by ace appears to be a precise role in differentiation as ace does not appear to affect patterning, as ascertained by markers such as eyab, or early neurogenic steps, as determined by the expression of ngn1 and neuroD.

As defects exist in both the olfactory bulb and epithelium, it is difficult to distinguish where the predominant fault lies in ace mutants' olfactory axon pathfinding. Indeed a crucial role for the olfactory placode in controlling olfactory bulb development has been described in Xenopus and mice, suggesting the olfactory placode and/or the arrival of olfactory axons within the bulb direct proliferation and differentiation (Graziadei and Graziadei, 1992; Gong et al., 1995). Two scenarios may explain the olfactory phenotype seen in ace embryos, either aberrant olfactory axon pathfinding leads to glomerular disorganisation or vice versa. A number of experiments can be performed to distinguish between the possibilities, including olfactory placode transplants, drug blocking experiments and ablation of the
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pioneer axons in wildtype embryos. Further examination of the ace− defect will provide a greater insight into the molecular mechanisms controlling olfactory axon pathfinding.
Chapter Five

Forebrain development in embryos lacking Ace/Fgf8 and Syu/Shh signalling

5.1. Introduction

The subdivision of the nervous system into discrete regions along the anterior-posterior and dorsoventral axes is achieved through the action of planar and vertical signals. In the forebrain, planar signals originate from the anterior neural ridge (ANR) and the non-neural ectoderm and vertical signals from the anterior visceral endoderm, axial mesoderm and midline neural tissue (Rubenstein and Beachy, 1997; Beddington and Robertson, 1999). The molecules involved in signalling from the ANR and non-neural ectoderm include Ace/Fgf8 and Bmp2/4. In the previous chapter, I have described how the loss of Ace signalling perturbs patterning, neurogenesis and axon pathfinding in the developing zebrafish forebrain.

A number of signalling molecules have been implicated in signalling from axial midline tissue, including Shh, Oep, Cyc, Gsc and Gli2/Yot. Of these molecules, the role of Shh in patterning a number of different tissues has been extensively studied. In the forebrain, overexpression of shh in zebrafish embryos leads to an expansion of ventral cell fates at the expense of more dorsal tissues (Barth et al., 1995; Ekker et al., 1995). Similar overexpression studies have been performed in mice and chicks, implying an evolutionary conserved role for Shh in ventral forebrain patterning (Ericson et al., 1996; Dale et al., 1997; Shimamura et al., 1997). In mice and chick, overexpression of Shh leads to an ectopic induction of ventral cell fates, as assayed by ventral forebrain markers, like nkx2.1, and an equivalent suppression of dorsal cell fates, indicated by markers such as BFl or Pax6. Loss of function experiments also indicate a role for Shh in forebrain patterning. Shh knockout mice are cyclopic, lack ventral forebrain tissue and optic stalks (Chiang et al., 1996). If Shh activity is blocked in vitro using anti-Shh-N antibodies, there is a corresponding reduction in forebrain ventral cell specification. Furthermore in chicks, Shh activity in patterning the ventral forebrain is modified by the action of BMP7 (Dale et al., 1997).

The expression patterns of Fgf8 and Shh in developing embryos suggests they may act in conjunction to control the development of a number of different tissues (Bueno et al., 1996). In the vertebrate limb, Fgf8 and Shh are expressed in close proximity, with Shh expressed in the polarising zone and Fgf8 in the apical epidermal ridge (Johnson and Tabin,
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Embryological manipulations and overexpression studies in chick embryos suggest Fgf8 may be required for Shh induction (Crossley and Martin, 1996). The actions of Shh and Fgf may be co-ordinated during the patterning of the midbrain and anterior hindbrain, as Fgf8 is expressed in the isthmic region whilst Shh is expressed in the notochord and the floor plate at the base of the mid-hindbrain boundary (Ye et al., 1998). In postnatal mice, Shh and bFGF are expressed in Purkinje cells in the cerebellum and the combinatorial action of the two molecules regulates the number of cerebellar granule cells produced (Wescher-Reya and Scott, 1999).

In the forebrain, fgf8 is initially expressed the anterior neural plate and shh in the axial mesoderm and midline neural tissue (Krauss et al., 1993; Reifers et al., 1998). As development proceeds, fgf8 expression spreads to the optic stalk region, shh is found in the axial mesoderm and later in the ventral telencephalon. Recent experiments performed by Ye and colleagues (1998) suggest that Fgf8 and Shh are both required to specify dopaminergic neurons in the telencephalon.

To further examine the role that Fgf8 and Shh play in patterning the forebrain, I generated embryos lacking normal Ace and Shh signalling. To achieve this, I utilised the acerebellar(ace) and sonic you (syu) mutants. Homozygous ace mutants make a truncated, non-functional Fgf8 protein (Brand et al., 1996; Reifers et al., 1998). syu mutants, a member of the you-type mutant class identified in the Tübingen, carry a mutation in Shh (Haffter et al., 1996). A number of alleles have been described in which the Shh gene is either not transcribed or is incorrectly spliced (Schauerte et al., 1998).

Generation of the double mutants reveals that although patterning and neurogenesis of the forebrain do not depend on the combinatorial action of Ace and Syu, later proliferation of the ventral forebrain does. Early pathfinding defects in the anterior and postoptic commissure (AC and POC respectively) in the double mutant are similar to those observed in the single mutations. However optic nerve pathfinding is more severely affected in the mutant, with the optic nerve exiting the eye at incorrect points. I have shown a downregulation of axon guidance cues within the eye maybe responsible for the retinal nerve pathfinding errors. I thank Paul Chadwani for his assistance in performing some in situ hybridisations.

5.2. Results

5.2.1. ace and syu are expressed in close proximity in the developing forebrain
Comparison of \textit{fgf8} and \textit{shh} expression patterns in other species had suggested that the two molecules may play an integrated role in patterning a number of different tissues, including the limb bud, midbrain, anterior hindbrain and forebrain (Bueno \textit{et al.}, 1996). To address, whether expression in zebrafish also suggests a combinatorial role in forebrain development, I compared the spatial and temporal expression of the two genes.

\textit{ace} expression in the forebrain is initiated at 95\% epiboly in the rostral margin of the anterior margin (Fig.5.1A). At this stage, \textit{syu} is expressed in the migrating axial mesoderm and midline neural plate (Fig.5.1A). The anterior tip of \textit{syu} expression is only a small number cells away from the rostral tip of \textit{ace} expression. As the neural plate closes, \textit{ace} expression is localised to the dorsal forebrain midline and optic stalk territory (see section 4.2.1). \textit{syu} continues to be expressed in midline neural tissue throughout the embryo. By prim 5, \textit{syu} is expressed in a dorsal hypothalamus domain but is excluded from a small group of cells in the anterior diencephalon through which the POC axons will cross the midline (Fig.5.1B).

At the ventral flexure, \textit{syu} expression is observed in the zona limitans intrathalamica (ZLI) and weaker expression domain is observed in the posterior hypothalamus. However \textit{syu} mRNA is not expressed throughout the dorso-ventral axis at the ZLI, as it is not expressed by a group of cells in the dorsal most aspect of the ZLI. At the equivalent stage, \textit{ace} is expressed in a basal telencephalic domain, directly dorsal to the position where the AC will cross the midline. In the diencephalon, \textit{ace} is expressed in the optic stalk region and a dorsal territory, thought to give rise to the habenular nuclei (Fig.5.1B). \textit{ace} and \textit{syu} expression domains are adjacent but do not overlap to a significant extent in either the optic stalk or dorsal diencephalic region at this stage.

By prim 20, \textit{ace} expression in the midline optic stalk territory is significantly reduced but expression is observed in the lateral optic stalk region and basal telencephalon (Fig.5.1C and data not shown). \textit{syu} expression in the hypothalamus and ZLI persists at prim 20 stage and expression is also weakly detected in the basal telencephalon dorsal to the position where the AC will cross the midline (Fig.5.2E). \textit{ace} and \textit{syu} expression in the basal telencephalon probably overlap but double whole mount in situ hybridisation need to be performed to confirm this finding. By 48hpf, \textit{shh} is expressed in the ventral midline of the neural plate and the basal telencephalon (Fig.5.1D). \textit{ace} is expressed in the telencephalon, in cells above the AC, and expression in the optic stalk is significantly reduced from earlier stages (Fig.5.1D).
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Fig5.1. Wildtype expression of ace/fgf8.1 and syu/shh

(A) Dorsal view of a tailbud embryo labelled with digoxygenin RNA probes for ace and syu. syu is detected in the axial midline tissue, whilst ace is expressed at the presumptive mid-hindbrain boundary (MHB) and weakly in the anterior margin of the neural plate. (B-D) Lateral views of (B) prim 5, prim 14 and 48hpf embryos labelled with digoxygenin RNA probes for ace and syu. By prim 5 stage, syu is detected in ventral midline tissue throughout the brain. A marked inflexion of syu expression is observed at the ZLI. syu transcripts are not detected in the telencephalon or optic stalk region. However ace is expressed in the basal telencephalon (marked by asterix) and in the optic stalk (see Fig4.1). Additionally ace is also expressed in the MHB, dorsal diencephalon, pituitary and posterior hypothalamus. (C) By prim 20 stage, ace and syu maybe expressed in overlapping domains in the basal telencephalon in cells directly dorsal to the position of the AC. In the diencephalon, ace expression has diminished in the optic stalk region and syu remains limited to ventral midline tissue. ace continues to be expressed in the MHB and at other sites, not shown. (D) In the 48hpf embryo, ace and syu continues to be expressed in cells bordering the AC. In midline optic stalk tissue, ace expression is almost extinguished but it continues to be expressed in more lateral optic stalk regions. syu continues to be expressed in ventral midline tissue. Scale bars represent 100µm (A), 75µm (B,C) and 100µm (D).

Abbreviations: ac, anterior commissure, dd, dorsal diencephalon, fb, forebrain, hy, hypothalamus, mhb, mid-hindbrain boundary, os, optic stalk, poc, postoptic commissure, t, telencephalon, zli, zona limitans intrathalamica.

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5.2.2. Generation and phenotype of embryos lacking ace and syu signalling

5.2.2.1. Double mutant carriers were generated by mating heterozygote carriers of the single mutations.

Having established that ace and syu are expressed in close proximity in the zebrafish forebrain, I generated double mutants to investigate potential combinatorial roles for ace and syu in the developing embryos. In order to generate embryos lacking functional Fgf8 and Shh signalling, heterozygote ace and syu fish were mated. ace mutants have a point mutation in the intron splice site after the first exon of the Fgf8 gene, which leads to incorrect splicing and a truncated, non functional protein being synthesised (Reifers et al., 1998). I used the syu^bx392 allele, which has a G to A change in the conserved splice donor site after the first intron of the syu gene (Schauerte et al., 1998). The failure to remove the first intron in the syu^bx392 mutant leads to an ectopic stop site and a truncated protein being synthesised. Progeny of the cross were grown to adulthood and double mutant carriers identified by incross analysis.

5.2.2.2. Double mutant embryos can be identified by morphological defects in the mid-hindbrain boundary, somites and hypothalamus.

Double mutant (ace^−:syu^−) embryos display a phenotype which possesses characteristics of both the single mutations. By prim 6, ace^- embryos lack a cerebellum and have a slightly expanded tectum. In addition the somites do not possess the characteristic chevron shape but are slightly more rounded (Fig.5.2A,B,E,F). syu^- embryos can be identified at this stage by the U shaped somite and perturbed formation of the vasculature in the trunk (Fig.5.2A,C,E,G). syu^- embryos have no detectable disruption in brain morphology at prim 6. At prim 6, ace^−:syu^− embryos lack a cerebellum, have U shaped somites and show severe disruption in the formation of the trunk vein, such that blood accumulates at the caudal tip of the yolk plug extension (Fig.5.2A-H and data not shown). A similar accumulation of blood occurs in prim 6 syu^- embryos and later in prim 22 ace^- embryos. In addition, the yolk plug extension is malformed (Fig.5.2E-H). As with ace and syu mutants, there is variability in the severity of the double mutation. The development of the otic vesicle in ace:syu mutants resembles the ace^- phenotype, which has smaller ears with frequently only one otolith present (Whitfield et al., 1996).

5.2.2.3. The hypothalamic region is smaller in the ace:syu mutant.
Fig5.2. Phenotypes of ace, syu, ace:syu mutants

(A-D) Lateral views of prim 6 stage live wildtype and mutants showing that wildtype and
syu" embryos have a normal cerebellum, whilst ace" and ace":syu" embryos lack
cerebellums. (E-F) Lateral views of the developing somites in prim 6 stage live embryos,
individual somites have been outlined. In wildtype embryos somites are formed in
characteristic chevron shapes, whilst ace" embryos are slightly more rounded. syu" and ace" :
syu" embryos have U-shaped somites. (I-L) Anterior views of live prim 6 stage embryos
showing that the ventral forebrain region, in ace:syu mutants, is reduced when compared to
wildtype and single mutant embryos (double headed arrows indicate comparative sizes). In
contrast, the eyes and dorsal forebrain of ace":syu" embryos do not show a similar reduction
in size when compared to wildtype, ace" and syu" embryos. Scale bars represent 50µm (A-
H) and 25µm (I-J)

Abbreviations: cb, cerebellum, hy, hypothalamus, n, notochord, op, olfactory placode, t,
telencephalon, yp, yolk plug extension.
By prim 6, the hypothalamic region of ace:syu mutants is smaller than in the wildtype, ace and syu mutants (Fig.5.2I-L). Although the whole ace:syu brain becomes smaller during development, the hypothalamic region is always more severely affected when compared to the wildtype and single mutant situations. By 54hpf, the optic chiasm and hypothalamic regions in ace:syu embryos are greatly reduced in size and the nasal placodes closer together in the double mutant (Fig.5.3F-I). The preoptic territory is approximately half the size in the double mutant when compared to the single mutants.

By prim 22, the anterior fin buds have started to grow out but this outgrowth is delayed in syu and ace:syu embryos. All the somites form but the tail is slightly shorter in the double mutant compared to the wildtype, ace and syu mutants. The tail in ace:syu mutants curve downwards like the syu single mutant. The tail in ace embryos also starts to significantly curve downwards after approximately 40hpf. The eyes in ace:syu mutants are smaller than the wildtype and single mutants (Fig.5.2F-I). ace:syu embryos, like syu embryos, do not twitch in response to a stimulus. Extensive cell death in the head of the double mutants starts around 60hpf and the embryo is extremely necrotic around 84hpf.

5.2.3. Forebrain axon pathfinding is perturbed in the ace:syu mutants

5.2.3.1. Early forebrain axonal pathfinding in the ace:syu mutants is more disrupted than in the single mutants.

As ace embryos display ectopic forebrain commissural pathfinding, I examined whether the axonal pathfinding in ace:syu embryos was disturbed to a greater extent. Axon pathways were visualised by using an antibody to N-acetylated tubulin. In wildtype embryos, the anterior commissure (AC) and postoptic commissure (POC) cross the forebrain midline at precisely defined points around the optic stalk region (Chitnis and Kuwada, 1990, Wilson et al., 1990). AC axons project from each telencephalic hemisphere ventrally and cross the midline approximately 3-4 cells dorsal to the optic recess. Similarly axons from the POC project dorsally to cross 3-4 cells ventral to the optic recess (Fig.5.3A).

In ace embryos, at the earliest stage (prim 10) examined, axons from both commissures ectopically project into the optic stalk territory. As described in section 4.2.2., despite the variability of the ace phenotype, axons always invade the optic stalk territory and sometimes axons also fail to cross the midline correctly (Fig.5.3B). When compared to wildtype siblings both commissures, in ace embryos, are defasciculated. By prim 20 stage
5.0 Forebrain development in embryos lacking Ace/Fgf8.1 and Syu/Shh signalling

Fig 5.3. Forebrain axon pathfinding is perturbed in ace:syu mutants

All embryos are labelled with anti N-acetylated tubulin antibody. (A-E) Anterior views of prim 10 stage embryos. In wildtype embryos (A), the AC and POC have formed either side of the optic stalk territory. ace− embryos (B) exhibit variable phenotypes but commonly the AC has not formed, whilst the POC is defasiculated. Both commissures appear to project axons into the lateral optic stalk territory and some axons do appear to wander into more medial optic stalk regions. In syu− embryos (C), the AC forms but the POC is slightly defasiculated and axons from the tract of the POC invade the optic stalk territory. In ace− :syu− embryos (D,E) the axons of tracts of the AC and POC project into lateral optic stalk regions and appear to fuse. Occasional axons project across the midline optic stalk territory.

(F-I) Ventral views of 54hpf embryos. (F) By 54hpf, retinal axons have exited the eye and traversed the midline to form the optic chiasm. Retinal axons eventually project to the contralateral tectum. In ace− embryos (G) the retinal axons frequently fail to cross the midline and project to the ipsilateral tectum or forebrain. Ectopic tracts between the AC and POC are also observed. By 54hpf, syu− embryos have formed a thin POC and retinal axons manage to project out of the eye and towards the midline. Retinal axons are sometimes defasiculated (see arrow in (H)). In ace−:syu− embryos, the retinal axons fail to exit the eye into the preoptic area. The preoptic area is disorganised with the AC and POC entangled within each other.

(J-M) Lateral views of 54hpf embryos. (J,K) In ace− embryos, the preoptic area is reduced and the AC and POC are closer together. The olfactory bulb and telencephalon have started to separate in the single mutants (J-L). syu− embryos maybe delayed slightly. In the double mutant (M) the olfactory bulb and telencephalon have not clearly separated. The preoptic area is highly disorganised and specific axon tracts are hard to distinguish.

(N-Q) Ventral views of 72hpf embryos. By 72hpf (A), the AC and POC are well established in their respective positions in the wildtype telencephalon and diencephalon. The optic chiasm has fully formed. In ace− embryos (O), the AC and POC are close set (or fused) with ectopic tracts forming between them. Retinal axons exhibit similar phenotypes to those observed at earlier stages. In syu− embryos (P), the general tracts appear to have formed but are frequently thinner than in wildtype siblings. By 72hpf, ace−:syu− embryos have undergone cell death such that the olfactory placodes almost fuse and axon tracts can not be clearly distinguished. Scale bars represent 30μm (A-E) and 50μm (F-Q).

Abbreviations: ac, anterior commissure, hy, hypothalamus, ob, olfactory bulb, oc, optic chiasm, os, optic stalk, poc, postoptic commissure, r, retina, t, telencephalon.
ectopic axonal tracts in the optic stalk territory are observed in all ace~ embryos and three phenotypic classes can be defined upon whether the AC and POC cross at the midline. Forebrain commissural projections, in syu~ embryos, are usually normal at prim 10 but in 40% of cases (n=10), axons from the POC are defasiculated and appear to wander into the optic stalk territory and rostro-ventral diencephalon, areas from which POC axons are usually excluded (Fig5.3C).

ace~:syu~ embryos display an exaggerated ace~-like phenotype with the axons always failing to cross the midline correctly and instead projecting laterally in the optic stalk territory. Occasionally axons cross the midline but it is difficult to ascertain from which commissure these axons originate (Fig5.3D,E). In ace~:syu~ embryos, axons remain well fasciculated and the wandering of axons is reduced compared to the single mutants, suggesting the AC and POC maybe fused due to a loss of optic stalk tissue.

5.2.3.2 The optic chiasm is severely affected in ace: syu mutants.

The expression patterns of ace and syu suggest they may act in combination to specify the optic stalk region. Evidence for functional interactions in the forebrain between Ace and Syu come from microinjection studies in zebrafish. Injection of shh into wildtype embryos results in an expansion of noi/pax2 expression throughout the optic stalk and into the eyes. Similar injections into ace~ embryos lead to a reduction in the lateral expansion of noi expression in the optic stalks compared similar injections (Barth and Wilson, unpubl.). If ace and syu do indeed act combinatorially to specify the optic stalk territory, pathfinding by the retinal, AC and POC axons should be significantly more perturbed than in the single mutants. By 54hpf, the midline optic stalk region has developed into the preoptic area which is composed of the optic chiasm, over which the optic nerve projects to the contralateral tectum (Fig5.3F). The AC and POC remain in their original positions straddling the optic chiasm territory.

ace~ embryos exhibit two late AC/POC pathfinding phenotypes. Either an ectopic tract forms between the two commissures or the commissures are closer together and eventually fuse by 72hpf stage (Fig5.3G,K,O). Ectopic tracts between the AC and POC are also observed at 72hpf in 35% of ace~ embryos (Fig5.3O). In 54hpf syu~ embryos, the AC and POC appear to project relatively normally, the earlier postoptic commissure defect has been corrected but the POC appears slightly thinner when compared to wildtype embryos (Fig5.3H,L). In 72hpf syu~ embryos, the axons of the AC and POC also appear to project normally but are thin and the brain generally appears to be disorganised (Fig5.3P).
5.0 Forebrain development in embryos lacking Ace/Fgf8.1 and Syu/Shh signalling

In 54hpf ace\textsuperscript{−}:syu\textsuperscript{−} embryos, the preoptic area is significantly smaller than that observed in the single mutants or wildtype embryo and the AC and POC appear to have fused. The AC is also defasiculated but does cross the midline. The POC fails to cross the midline properly and some POC axon bundles appear to fuse with the AC. Other tracts are seen in between the AC and POC, which project in a haphazard manner (Fig5.3I,M). By 72hpf, the AC and POC are apparently fused but extensive cell death does not allow a sensible interpretation of the N-acetylated tubulin result (Fig5.3Q).

5.2.3. Optic axons fail to exit the eye at the choroid fissure in ace\textsuperscript{−}:syu\textsuperscript{−} embryos.

The optic nerve exits the eye at the choroid fissure before crossing the preoptic midline to project to the contralateral tectum (Fig5.3F,N). In ace\textsuperscript{−} embryos, the optic nerve exits the eye at the choroid fissure but appears to project ectopically in the optic chiasm territory (see section 4.2.3.). Once in the optic chiasm region, the ace\textsuperscript{−} optic nerve in most cases (65% of all embryos examined) projects to the ipsilateral tectum and forebrain (Fig5.3G,O). In cases where the ace\textsuperscript{−} optic nerve successfully crosses the midline of the optic chiasm, it frequently projects to the contralateral forebrain.

In all syu mutants examined, the optic nerve exits approximately near the choroid fissure and projects successfully over the optic chiasm to the contralateral tectum (Fig5.3H,P). The optic nerve does not always exit the eye into the preoptic area at the correct point but projection within the optic chiasm region is always correct in syu\textsuperscript{−} embryos. By 72hpf, the retinal nerves observed in syu\textsuperscript{−} embryos are thinner than those observed in wildtype embryos and some cell death has occurred in the syu\textsuperscript{−} brains. N-acetylated tubulin staining also reveals the presence of a normal neural retina in 54hpf and 72hpf single mutants (Fig5.3F-H,N-P).

Optic axons appear to converge in the retina in ace\textsuperscript{−}:syu\textsuperscript{−} embryos but the nerve fails to exit the eye at the choroid fissure. Instead the nerve projects anteriorly within the eye to varying degrees (Fig5.3I). Upon ectopically exiting the eye, the ace\textsuperscript{−}:syu\textsuperscript{−} optic axons frequently projects to the rostral forebrain or may project to the ipsilateral tectum (Fig5.3M, arrows point out optic axons exiting the eye). The neural retina appears to be disrupted, as N-acetylated tubulin staining is weaker in ace\textsuperscript{−}:syu\textsuperscript{−} embryos (Fig5.3Q). By 72hpf, the optic nerve can not be detected in ace\textsuperscript{−}:syu\textsuperscript{−} embryos, probably due to cell death in the head of the embryo.
5.0 Forebrain development in embryos lacking Ace/Fgf8,1 and Syu/Shh signalling

5.2.3.4. N-acetylated tubulin reveals disorganisation of the olfactory bulb, telencephalon and preoptic area in ace-:syu- embryos.

In 54hpf wildtype embryos, the olfactory bulb has started to bud off from the telencephalon and the telencephalic axons, which contribute to the anterior commissure, can be seen using N-acetylated tubulin (Fig5.3J). In addition, N-acetylated tubulin staining can provide an insight into the overall morphology of the developing zebrafish CNS. Our initial studies suggested the loss of Ace and Syu signalling affects the morphology of the brain at early stages and so N-acetylated tubulin was used to investigate how later morphological movements were affected.

By 54hpf ace- embryos show clear separation of the olfactory bulb from the telencephalon but the preoptic territory, in some embryos, is reduced (Fig5.3K). In syu- embryos, the separation of the olfactory bulb appears to be delayed but the bulb and the telencephalon can be delineated (Fig5.3L). The preoptic area does not appear to be affected in 54hpf syu- embryos. In ace-:syu- embryos, the separation of bulb and telencephalon is unclear and the axons that contribute to the anterior commissure appear to be disorganised (Fig5.3M). The entire preoptic area is lost as anterior and postoptic commissures are fused.

5.2.4. Disruption of axonal pathfinding in the forebrain and hindbrain of ace-:syu- embryos

5.2.3.1. Perturbation of hindbrain development in ace-:syu- embryos.

ace and syu are expressed in close proximity in other areas of the brain, such as the mid-hindbrain boundary and around the ZLI in the dorsal diencephalon. In the 54hpf wildtype hindbrain, tubulin labelled processes are found in well defined positions and numbers (Fig5.4A). No change in number or position of these hindbrain projections is seen in either ace- and syu- embryos (Fig5.4B,C). In most ace-:syu- embryos, the number of axon bundles appears to be altered (Fig5.4D). A less commonly observed phenotype in ace-:syu- embryos results in not only the number of axon bundles being altered but the bundles are also not correctly spaced. This maybe due to either loose fasiculation of the axons or that they no longer project in a discrete dorsal to ventral manner but instead appear to wander whilst maintaining a general dorsoventral direction (Fig5.4E). Little is known about the hindbrain axon bundles detected using N-acetylated tubulin but an examination of genes involved in neurogenesis would provide a greater insight into the phenotype observed in ace-:syu- embryos.
5.0 Forebrain development in embryos lacking Ace/Fgf8.1 and Syu/Shh signalling

Fig5.4. Axon pathfinding defects in the hindbrain and dorsal diencephalon

(A-I) Dorsal views of 54hpf embryos labelled with anti N-acetylated tubulin antibody. At 54hpf (A) the wildtype hindbrain is characterised by evenly spaced axon bundles (marked by arrows). (B,C) These bundles are also observed in ace^- and syu^- embryos at the same stage. In ace^-syu^- embryos (D,E) the numbers and spacing of these bundles are perturbed. In 54hpf wildtype and syu^- embryos (F,H) the habenular commissure has formed in the dorsal diencephalon. As previously described, ace^- embryos (G) typically lack or have reduced habenular commissures. A similar loss of the habenular commissure is observed in ace^-syu^- embryos (I). The habenular commissure is not affected in syu embryos. Scale bars represent 60µm.

Abbreviations: cb, cerebellum, e, epiphysis, hb, hindbrain, hc, habenular commissure, te, tectum.
5.2.4.2. The habenular commissure is not formed in ace\textsuperscript{−}:syu\textsuperscript{−} embryos.

The habenular commissure normally projects over the dorsal diencephalon, anterior to the epiphysis (Fig.5.4F). In ace\textsuperscript{−} embryos, this projection is sometimes reduced or absent (see section 4.2.6.) but in less severely affected embryos, appears normal (Fig.5.4G). Similarly syu\textsuperscript{−} embryos have a normal habenular commissure. ace\textsuperscript{−}:syu\textsuperscript{−} embryos always lack this commissure (Fig.5.3I). Although the origin of the habenular commissures is unknown in zebrafish embryos, the defect in ace\textsuperscript{−}:syu\textsuperscript{−} embryos maybe due to disruption of the midline dorsal diencephalon as both ace and syu are expressed near this region.

5.2.5. Altered expression of axon guidance cues in ace\textsuperscript{−}:syu\textsuperscript{−} embryos

5.2.5.1. The altered expression of netrins correlates to the axon pathfinding defects observed in ace\textsuperscript{−}:syu\textsuperscript{−} embryos.

A number of molecules have been implicated in guiding axons of the anterior and postoptic commissure across the midline. The expression patterns of netrins in wildtype and mutant embryos have implicated them in playing an important role in forebrain axon guidance (Macdonald et al., 1997; Strâhle et al., 1997). As early axon guidance was perturbed in ace:syu mutants, I examined the expression of two netrin genes, netrin 1 and 2 (net1 and 2).

net1 is expressed ventrally along the optic recess and anteriorly at the midline in the telencephalon in prim 6 wildtype embryos. Although the ventral telencephalic expression is present in ace\textsuperscript{−}, syu\textsuperscript{−} and ace\textsuperscript{−}:syu\textsuperscript{−} embryos, the anterior expression is reduced in ace\textsuperscript{−}:syu\textsuperscript{−} embryos but not significantly in either single mutant (Fig.5.5A-D). net1 is also expressed in the ventral optic stalk in wildtype prim 6 embryos. net1 expression is reduced or lost in the ventral optic stalk territory in ace\textsuperscript{−}, syu\textsuperscript{−} and ace\textsuperscript{−}:syu\textsuperscript{−} embryo (Fig.5.5A-D).

Another netrin, net2, is also expressed ventrally along the optic recess in wildtype prim 6 embryos. In ace\textsuperscript{−} and syu\textsuperscript{−} embryos, this expression is slightly reduced but almost lost in ace\textsuperscript{−}:syu\textsuperscript{−} embryos (Fig.5E-H). net2 is also expressed in cells lining the choroid fissure in prim 6 wildtype embryos, in a similar domain to noi. In ace\textsuperscript{−} and syu\textsuperscript{−} embryos, net2 expression in the choroid fissure is slightly reduced at the same stage. In ace\textsuperscript{−}:syu\textsuperscript{−} embryos, net2 expression in the choroid fissure is greatly reduced (Fig.5I-L).
Fig5.5. Perturbed netl, net2 and twhh expression in mutant embryos

(A-H) Lateral views of prim 6 stage embryos labelled with digoxygenin RNA probes for (A-D) netl and (E-H) net2. In wildtype embryos (A) netl is expressed in telencephalic cells lining the optic recess, basal telencephalic midline and in the ventral optic stalk (as show by arrows). In ace~ embryos (B), expression in the basal telencephalic midline and ventral optic stalk is reduced, a reduction in ventral optic stalk expression of netl is also observed in syu~ embryos (C). In ace~:syu~ embryos (D) netl expression is reduced in all forebrain domains. net2 is also expressed in telencephalic cells lining the optic recess and weakly in the ventral optic stalk (E). In ace mutants (F), net2 expressed is reduced in the telencephalon whilst in syu mutants (G) expression is reduced in the telencephalon and ventral optic stalk. As with netl expression, net2 expression is reduced in the telencephalon and diencephalon of ace~:syu~ embryos (H). (I) net2 is also strongly expressed in cells lining the choroid fissure. Choroid fissure net2 expression in ace and syu mutants (I,J) is reduced but almost lost in ace: syu mutants (L). In wildtype prim 6 stage embryos (M) twhh is expressed in a small cluster of cells ventral to the position where the POC crosses the midline. In ace~, syu~ and ace~:syu~ embryos (N-P) twhh expression in this rostral diencephalic domain is reduced. Scale bars represent 50µm.

Abbreviations: b, brain, cf, choroid fissure, e, eye, hy, hypothalamus, op, olfactory placode, or, optic recess, os, optic stalk, t, telencephalon, te, tectum.
5.0 Forebrain development in embryos lacking Ace/Fgf8.1 and Syu/Shh signalling

5.2.5.2. Loss of twhh expression in ace~:syu~.

Another hedgehog family member, twhh, is expressed in the developing zebrafish forebrain. Schauerte and colleagues suggested that the phenotype of syu embryos was less severe than expected due to compensatory signalling through twhh in the rostral brain. In wildtype embryos, twhh is expressed in rostral diencephalic cells adjacent to the optic stalk and in dorsal cells of the ZLI. Expression of twhh, in rostral diencephalic cells, is reduced or lost in ace~, syu~ and ace~:syu~ embryos (Fig5.5M-P). Expression in the dorsal ZLI is relatively unaffected in the single mutants and slightly reduced in ace~:syu~ embryos (data not shown).

5.2.6. Proliferation within the ace~:syu~ forebrain is reduced

5.2.6.1. ace~:syu~ embryos have a marked reduction in the number of proliferative cells within the forebrain.

As the morphological and axon pathfinding phenotypes suggest a reduction in the size of the hypothalamic and optic stalk regions, proliferative and apoptosis studies were performed to assess the relative contributions of each process towards the size reduction.

An antibody to phosphorylated Histone H3 (PH3) was used to mark cells which were undergoing mitosis. Cells were counted in frontal views of whole embryos encompassing a territory bounded dorsally and ventrally by the anterior and postoptic commissures and laterally by the position at which the optic stalk separates from the forebrain neuroepithelium. At prim 5, a 35% decrease in the average number of proliferative cells was detected in ace~:syu~ embryos, when compared to wildtype embryos. At the same stage ace~ embryos had 17% and, syu~ embryos, 24% fewer PH3 positive cells within the optic stalk region (Table5.1).

As the embryo develops, the number of proliferative cells within the optic stalk region decreases. By prim 22, ace~ x syu~ embryos had on average 60% fewer PH3 positive cells when compared to wildtype embryos. On average ace~ embryos had 42% fewer PH3 positive cells and syu~ 30% fewer proliferative cells at prim 22 (Table5.1).

TUNEL staining was performed to assess apoptosis in the ace~:syu~ mutants. TUNEL positive cells were counted in a similar territory as for the cell proliferation assay.
Table 5.1. Cell proliferation and apoptosis in ace⁻:syu⁻ embryos

To assess whether cell proliferation was responsible for the loss of the preoptic territory, cells in a proliferative state, as marked by PH3 antibody, were counted in the optic stalk territory. Similarly to assess apoptotic cells, we used TUNEL staining. ace⁻:syu⁻ embryos have reduced cell proliferation compared to the wildtype and single mutant situations but do not show an increase in cell death.
described above. No significant increase in TUNEL was seen in either prim 5 stage single or the double mutants when compared to the wildtype embryos (Table 5.1). By prim 22 apoptosis within the forebrain, as marked by TUNEL staining, is virtually non-existent in wildtype, single and double mutants.

5.2.7. Regionalisation of the forebrain in ace^-syu^- embryos is relatively normal

5.2.7.1. Telencephalic regionalisation is relatively normal in ace^-syu^- embryos but diencephalic regionalisation is affected.

As both Ace and Syu have been implicated in patterning the forebrain, I examined whether broad gene expression domains were affected in ace^-syu^- embryos. Examination of broad gene expression territories will give a clear indication of whether particular forebrain regions are severely affected. *emx1* is expressed in a broad dorsal telencephalic domain in prim 12 stage wildtype embryos (Fig 5.6A). Expression is not altered in ace^- or syu^- embryos and although the brain is smaller in ace^-syu^- embryos, expression appears to be restricted to the same region (Fig 5.6B-D).

In prim 12 stage wildtype embryos, *dlx2* has a broad ventral telencephalic and an anterior diencephalic domain of expression, these two domains line either side of the optic recess (Fig 5.6E). In ace^- embryos, neither domain is severely affected but syu^- mutants display a marked reduction in the anterior limit of the diencephalic expression domain (Fig 5.6F,G).

A similar reduction in the anterior limit of the diencephalic expression domain is seen in ace^-syu^- embryos (Fig 5.6H). The telencephalic *dlx2* expression domain is normal in syu^- embryos and appears to be reduced in ace^-syu^- embryo. This reduction may result from a loss in basal telencephalic tissue in ace^-syu^- embryos.

Another gene that is expressed in a broad diencephalic domain, and thought to convey ventral forebrain identity is *nk2.1* (Sussel et al., 1999). *nk2.1* is expressed in the hypothalamus of prim 12 stage wildtype embryos but is excluded in a region approximately 3-4 cell diameters ventral from the optic recess (Fig 5.6I). The region of cells not expressing *nk2.1* in prim 22 ace^- embryos is reduced to a 2 cell layer (Fig 5.6J). syu^- embryos show no perturbation of *nk2.1* expression. The region of diencephalic cells not expressing *nk2.1* is completely lost in ace^-syu^- embryos (Fig 5.6L).
Fig5.6. Expression of regionally restricted genes in wildtype and mutant embryos

(A-D) Lateral views of prim 12 stage embryos labelled with RNA probes for *emxl*. In wildtype embryos *emxl* is expressed in the dorsal telencephalon and expression appears to be normal in both single (B,C) and double (D) mutants. (E-H) Lateral views of prim 12 stage embryos labelled with *dlx2* RNA probes. In wildtype and *ace~* (E, F) embryos, *dlx2* is expressed in ventral telencephalic cell running parallel to the optic recess and in the dorsal hypothalamus (arrows indicate position of optic recess). In *syu* mutants (G), telencephalic *dlx2* expression is relatively normal but expression in the rostral hypothalamus is lost. A similar situation is observed in *ace~:syu~* mutants (H) but telencephalic expression also appears to be reduced. (I-L) Lateral views of prim 12 stage embryos labelled with digoxygenin RNA probes for *nk2.1*. In wildtype and *syu~* (I,K) embryos *nk2.1* is expressed in the hypothalamus but is excluded from cells lining the optic recess and posterior tuberculum. In *ace~* (J) embryos, *nk2.1* expressed spreads towards the optic recess at this stage, whilst in *ace~:syu~* (L) embryos *nk2.1* expression is detected in cells lining the optic recess. (M-P) Lateral views of prim 12 stage embryos labelled with digoxygenin RNA probes for *six3*. In wildtype (M) embryos, *six3* transcripts are detected in cells in the rostral diencephalon and weakly in the telencephalon. In *ace* and *ace:syu* (N,P) mutants *six3* expression in the ventral optic stalk territory is reduced and in *syu* and *ace:syu* (O,P) mutants expression in the ventral diencephalon is slightly reduced. (Q-P) Dorsal views of 10 somite embryos labelled with probes for *noi/pax2.1*. In wildtype embryos (Q) *pax2.1* is expressed in the anterior eye and optic stalk cells. Embryos lacking Ace activity can be detected through a marked reduction in *pax2.1* expression in the mid-hindbrain boundary. In such embryos (R), *pax2.1* mRNA is lost from the optic stalk tissue and (P) some embryos have reduced expression in the eye. Scale bars represent 60µm (A-P) and 25µm (Q,R).

Abbreviations: ey, eye, hy, hypothalamus, mhb, mid-hindbrain boundary, or, optic recess, os, optic stalk, pt, posterior tuberculum, t, telencephalon.
5.0 Forebrain development in embryos lacking Ace/Fgf8.1 and Syu/Shh signalling

In prim 12 stage wildtype embryos, six3 is expressed in a more restricted diencephalic domain, being limited to a thin layer of cells at the rostral diencephalon. As shown earlier the postoptic commissure will cross the midline within this six3 territory approximately 4 cells ventral to the optic recess (see section 4.2.5). In ace~ embryos, six3 expression is reduced in cells adjacent to the optic recess and a similar reduction in expression is seen in ace~:syu~ embryos. A slight reduction in the ventral portion of diencephalic six3 expression is observed in syu~ embryos (Fig 5.6M-P).

In wildtype embryos, noi/pax2.1 is expressed in the anterior eyes and in cells across the optic stalk. I have previously shown noi is lost from the medial optic stalk in ace~ embryos from the 18 somite stage (see section 4.2.5.). At 10 somite stage, it is not possible to identify ace~:syu~ embryos by morphology but embryos lacking Ace signalling can be identified by a marked reduction in noi expression in the mid-hindbrain boundary and optic stalk. Of the 15/62 embryos identified to have a reduction of noi in the mid-hindbrain boundary, 3 embryos displayed a loss of noi in the optic stalk and a reduction of noi expression in the eyes (Fig 5.6Q-R). This number of embryos is close to the expected frequency of double mutants within such a cross. The other 12 embryos, with reduced optic stalk midline noi expression, have relatively normal expression in the eyes. No change of noi expression was observed in syu~ embryos.

5.2.8. ace~:syu~ embryos have reduced neuronal differentiation in the forebrain

5.2.8.1. Proneural gene expression is reduced in ace~:syu~ embryos.

Although regional patterning is not severely affected in ace~:syu~ embryos, other developmental processes such as neurogenesis maybe be affected by the loss of Ace and Syu signalling. We examined the expression of a number of genes to discover whether neurogenesis was affected in ace~:syu~ mutants. ngnl is expressed in cells which have the potential to become neurons. In prim 5 stage wildtype embryos, it is expressed along the optic recess in the telencephalon, in a group of dorso-anterior telencephalic cells and in a small group of cells in the hypothalamus (Fig 5.7A). In ace~ embryos, ngnl is expressed normally in all these domains. In both syu~ and ace~:syu~ embryos, ngnl expression is expressed weakly in the telencephalon and is strongly reduced in the anterior hypothalamic region (Fig 5.7B-D).

5.2.8.2. Neuronal differentiation is also reduced in ace~:syu~ embryos.
5.0 Forebrain development in embryos lacking Ace/Fgf8.1 and Syu/Shh signalling

Fig 5.7. Analysis of neurogenesis with single and double mutants

(A-D) Lateral views of prim 5 stage embryos labelled with RNA probes for ngnl. In wildtype and ace\(^{-}\) embryos (A,B) ngnl is expressed in telencephalic cells lining the optic recess and in another group of dorsal cells. In the diencephalon ngnl is expressed in the posterior tuberculum and in cells lining the otic recess. In syu and ace: syu mutants, ngnl expression is reduced in the telencephalon and lost in rostral diencephalic cells. (E-H) Anterior views of prim 10 stage embryos labelled with anti-islet-1 antibody. Expression of isl-1 in the basal telencephalon does not appear to be affected in the single or double (E-H) mutants but rostral diencephalic expression of isl-1 is reduced in syu\(^{-}\) and ace\(^{-}:\)syu\(^{-}\) embryos (G,H-indicated by arrows). (I-L) Lateral views of prim 5 stage embryos labelled with liml probes. Expression of liml in two telencephalic domains (see arrows) does not appear to be affected in syu\(^{-}\) embryos (I,K) but the anterior domain of liml expression is reduced in ace\(^{-}\) and ace\(^{-}:\)syu\(^{-}\) embryos (J,L). In the diencephalon liml is expressed in cells lining the tract of the POC (I) and expression is reduced in rostral diencephalic cells in syu and ace: syu mutants (K,L). (M-P) Lateral views of prim 8 stage embryos labelled with probes against lim6 mRNA. In wildtype (M) embryos lim6 is expressed in a group of anterior telencephalic cells (see arrows). In both the single mutants (N,O), this domain of lim6 expressing cells is reduced and almost lost in the double mutant (P). (Q-T) Lateral view of prim 10 stage embryos labelled with probes against lim5. (Q) lim5 is expressed broadly in the caudal telencephalon and dorsal hypothalamus. Although no change in telencephalic lim5 expression is detected in either the single or double mutants, lim5 expression in the rostral diencephalon is lost in syu\(^{-}\) and ace\(^{-}:\)syu\(^{-}\} (R-T; see arrows) embryos. (U-X) Ventral views of prim 22 stage embryos labelled with anti-tyrosine hydroxylase (TH) antibody. In wildtype (U) embryos TH is detected in a row of cells in the hypothalamus, adjacent to the tract of the POC. Hypothalamic cell expressing TH are also detected in ace\(^{-}\) and syu\(^{-}\} (V,W-see arrows) embryos. A marked reduction in the number of cells expressing TH is observed in ace\(^{-}:\)syu\(^{-}\} (X-see arrows) embryos. Scale bars represent 50\(\mu\)m.

Abbreviations: e, eye, hy, hypothalamus, op, olfactory placode, or, optic recess, os, t, telencephalon.
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During late somitic stages, the telencephalon can be broadly subdivided into four domains depending on the combinations of LIM homeodomain genes expressed (see Chapter Three). To ascertain whether neuronal differentiation was affected in ace−:syu− embryos, I examined the expression of a number of LIM genes.

Isl-1, a marker for motor neurons, is expressed in the ventral telencephalon and rostral most diencephalon in wildtype prim 10 stage embryos (Fig5.7E). Telencephalic expression of isl-1 is normal in ace−, syu− and ace−:syu− embryos. Diencephalic expression of isl-1 is not perturbed in ace− embryos but is slightly reduced in both syu− and ace−:syu− embryos (Fig5.7F-H).

Anterior telencephalic neurons can be distinguished by their expression of liml and lim6. lim1 and lim6 are expressed in broadly overlapping domains in wildtype embryos older than prim 5 stage. In addition, lim1 is expressed in a dorsal telencephalic group of cells and in the diencephalon in cells coincident with the postoptic commissure (Fig5.7I,M). Anterior telencephalic lim1 and 6 expression in ace− embryos is either reduced or absent; dorsal lim1 expression is also slightly reduced (Fig5.7J,N). Although lim1 is relatively unaffected in syu− embryos, lim6 expression is reduced when compared to wildtype expression (Fig5.7K,O). In ace−:syu− embryos, telencephalic lim1 is reduced, as in ace− embryos. lim6 expression in ace−:syu− embryos is more markedly reduced compared to sibling wildtype, ace− and syu− embryos (Fig5.7L,P). lim1 expression in the anterior diencephalon is reduced in a similar fashion in syu− and ace−:syu− embryos. lim5 is a good marker for dorso-caudal telencephalic neurons. Telencephalic expression of lim5 is unaffected in the single and double mutants (Fig5.7Q-T).

Expression of eome in the dorsal telencephalon is also unaffected in the single and double mutants (data not shown). However lim5 expression in the anterior diencephalon is reduced in syu− and ace−:syu− embryos, when compared to the wildtype and ace− situation (Fig5.7S,T).

Fgf8 and Shh have been proposed to act combinatorially to specify tyrosine hydroxylase (TH) positive neurons in rat forebrains (Ye et al., 1998). In fish, TH positive neurons are first detected in the posterior hypothalamus at prim 5, the olfactory bulb at 60hpf and shortly afterwards telencephalon (Guo et al., 1998). As ace−:syu− embryos usually undergoes cell death after 60hpf, we chose to examine whether the hypothalamic domain of TH positive neurons were affected in prim 22 stage ace−:syu− embryos (Fig5.7U-X). There
is a reduction in the number of TH positive neurons in ace\textsuperscript{−}:syu\textsuperscript{−} embryos compared to the wildtype, ace\textsuperscript{−} and syu\textsuperscript{−} phenotypes.

5.3. Discussion

In this study, I investigated possible interactions between two signalling molecules, Ace/Fgf8 and Syu/Shh, in the development of the zebrafish forebrain. Although the two genes are not required to specify a particular forebrain region or nuclei, the combinatorial action of the genes is required for maintaining proliferation within the forebrain. In addition, the interaction between the two genes may be important in ganglion cell differentiation and pathfinding within the eye.

5.3.1. Ace and Syu control proliferation in the zebrafish forebrain

The most striking phenotype observed in ace\textsuperscript{−}:syu\textsuperscript{−} embryos is the reduction in the size of the forebrain. Initially the hypothalamus is reduced in size but as the embryos develop, the entire forebrain decreases in size, such that the eyes become extremely close set and the olfactory placodes almost fuse. An overall reduction in brain size is observed but this phenotype occurs later, after prim 20 stage, than the reduction in forebrain size. The Shh knockout mouse is cyclopic but this is probably due to a lack of ventral forebrain midline tissue which would normally bifurcate the single eye-field (Chiang et al., 1996). Similar cyclopic phenotypes are observed in the zebrafish mutants cyclops and one eyed pinhead, which lack ventral forebrain midline tissue (Hatta et al., 1991; Schier et al., 1996). In contrast the eyes are apparently normally separated in ace\textsuperscript{−}:syu\textsuperscript{−} embryos at early stages of development and come closer together later. Although extensive cell death occurs after 60hpf in the double mutants, a loss of cell proliferation appears to be the primary cause of the reduction of forebrain size in double mutants. A marked reduction in the number of proliferating cells is observed at prim 5 stage and the further reduction is noted at prim 22, when the hypothalamus has become significantly smaller than the single mutants and wildtypes. Apoptosis within the forebrain is not significantly greater in prim 5 stage ace\textsuperscript{−}:syu\textsuperscript{−} embryos than in wildtype or single mutants. The relatively small number of double mutants does not allow us to perform a meaningful statistical analysis at this stage and greater numbers of embryos must be collected before firm conclusions can be drawn.

Fgf8 has been shown to be a powerful mitogen in a number of different systems. Fgf8 is required for the outgrowth of the chick limb bud, overexpression of Fgf8 in the mouse Rathke's pouch leads to pronounced enlargement of the pituitary and a consequent...
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reduction in cell differentiation (Crossley et al., 1996a; Ericson et al., 1998; Treier et al., 1998). In the forebrain, Fgfs are potent mitogens of mouse telencephalic neural stem cells (Tropepe et al.; 1999). The brains of mice with reduced Fgf8 signalling are smaller than their wildtype siblings (Meyers et al., 1998). The ace− brain also becomes smaller relatively late in development. The reduction in the number of mitotic cells in ace− embryos and the corresponding loss or reduction of the preoptic area suggests that Ace is required for correct proliferation in the zebrafish forebrain.

Until recently, Shh was thought of as predominantly being involved in patterning structures as diverse as the ventral neural tube and the limb bud (Ericson et al., 1995; Johnson and Tabin, 1997). However recent work in Drosophila and vertebrates have implicated the hedgehog family as a being powerful mitogens in the skin, eye and cerebellum (Parisi and Lin, 1998; Jensen and Wallace, 1997; Wechsler-Reya and Scott, 1999). The role of Shh in cell proliferation in the cerebellum is particularly interesting, as basic Fibroblast Growth Factor (bFGF) is implicated in modulating the response of cells to Shh signalling. Purkinje cells in the postnatal cerebellum express shh and downstream targets of Shh, like gli1, gli2 and ptc2, are expressed in granule cell precursors. Overexpression of the active N-terminal of Shh leads to an increase in the numbers of granule cells produced and application of an antibody blocking Shh activity leads to a reduction in the expression of ptc2 and a marked reduction in the external germinal layer, which is composed of granule cells (Wechsler-Reya and Scott, 1999; Wallace, 1999; Dahmane and Ruiz-i-Altaba, 1999). bFGF is produced by Purkinje cells and astrocytes and can stimulate proliferation of granule cell precursors (Hatten et al., 1988; Matsuda et al., 1994). However Wechsler-Reya and Scott show bFGF can inhibit the Shh stimulated proliferation and suggest that bFGF acts as a molecular check to prevent overproduction of granule cells. Additional evidence for a role for Shh in proliferation of cerebellar granule neurons comes from studies of Basal Cell Nevus Syndrome, which is associated with an increased frequency of medulloblastomas. Medulloblastomas often consist of small granule cell precursor like cells and heterozygote mice with mutations in ptc and humans with PTC mutations develop sporadic medulloblastomas (Hahn et al., 1996; Goodrich et al., 1998).

Interactions between syu and ace maybe responsible for controlling proliferation of cells in the zebrafish forebrain. The single mutants show a small reduction of mitotic cells in the forebrain territory but the double mutant phenotype appears to be more severe than either two single mutations. The reduction in mitotic cells observed in the double mutant appears to suggest an additive or synergistic phenotype. In contrast to the cerebellum, where bFGF tempers Shh stimulated proliferation of granule cell precursors, the forebrain study
suggests both molecules are required to maintain correct proliferation. A more accurate study of proliferation in ace~:syu~ embryos, using BrdU pulse studies combined with neuronal cell markers, would give a clearer understanding of the proliferation phenotype observed.

5.3.2. Patterning and neurogenesis in the forebrain of ace~:syu~ embryos

Ace/Fgf8 and Syu/Shh have been implicated in gross patterning of the different embryonic forebrain regions. Fgf8 signalling from the ANR can induce the telencephalic gene, BF-1, in neural plate explants (Shimamura and Rubenstein, 1997) and Shh has been implicated in patterning the ventral forebrain (Ericson et al., 1995; Barth and Wilson, 1995; Ekker et al., 1995; Shimamura and Rubenstein, 1997; Dale et al., 1997; Kohtz et al., 1998). In the Shh knockout mouse, emx1 expression spreads ventrally and ventral forebrain appears to be lost (Chiang et al., 1996). A loss of Ace and Syu signalling would be expected to result in severe forebrain patterning defects.

My studies indicate that although Ace is required for correct patterning of the optic stalk territory (see chapters 4 and 5), regionalisation of the telencephalon and rostral diencephalon is relatively unaffected in ace~ embryos. Patterning of the caudal diencephalon is not greatly affected in syu~ embryos and telencephalic patterning appears to be normal. Alterations of dlx2, six3, net2, ngn1, lim1 and lim5 expression in the rostral diencephalon are observed in syu~ and ace~:syu~ embryos. The alterations suggest Syu is required for patterning and neurogenesis in this region of the zebrafish ventral forebrain. The loss of gene expression in ace~:syu~ embryos is probably due to the loss of Syu activity and of tissue in this region. Not all genes in the rostral diencephalon are regulated by Syu, as shown by the normal hypothalamic expression of nk2.1 in syu mutants.

Regional forebrain specification in ace~:syu~ embryos maybe perturbed to a greater extent than in either single mutant. Ventral optic stalk tissue, as demarcated by non-nk2.1 expressing cells, appear to be lost in ace~:syu~ embryos. Coupled to this loss of diencephalic tissue, a loss of correct anterior hypothalamic patterning, similar to that observed in syu~ embryos, is also detected in ace~:syu~ embryos. A reduction in dlx2, net1 and net2 expression in the ventral telencephalon of ace~:syu~ embryos implicate Ace and Syu in the combinatorial patterning of the ventral telencephalon. However the reduction in these genes may reflect a loss of ventral tissue and further experiments must be performed to assess patterning and specification of this territory in ace~:syu~ embryos.
Although forebrain patterning defects are observed in ace<sup>~</sup>, syu<sup>~</sup> and ace<sup>~</sup>:syu embryos, the observed defects are not as severe as would be predicted from in vitro and knockout studies (Chiang et al., 1996; Shimamura and Rubenstein, 1997; Huh et al., 1999). The lack of phenotypic severity in these mutants suggests that other important molecules may functionally compensate the loss of Ace and Syu signalling. One molecule that may compensate lack of Syu signalling is the hedgehog family member, twhh (Ekker et al., 1995; Schauerte et al., 1998). However in ace<sup>~</sup>, syu<sup>~</sup> and ace<sup>~</sup>:syu<sup>~</sup> embryos, transcription of twhh is reduced in the anterior hypothalamus. Loss of twhh expression in ace<sup>~</sup>:syu<sup>~</sup> embryos would suggest that non-hedgehog family molecules, such as TGF-B family members, may play a more significant role in ventral forebrain patterning in the zebrafish (Rebagliati et al., 1998; Feldman et al., 1998; Sampath et al., 1998). Analysis of the gli2 mutant, you-too (yot), also suggests that non hedgehog family member are important in patterning the zebrafish ventral forebrain (Karlstrom et al., 1999). Gli2 is a downstream effector of hedgehog genes and although the mutants display more severe ventral forebrain patterning defects than the non deletion alleles of syu, the severity of the yat mutant is still less pronounced than the mouse Shh knockout. No forebrain defect has yet been described in gli2 knockout mice (Ding et al., 1998).

Proneural gene expression in the ventral optic stalk region is reduced in syu<sup>~</sup> and ace<sup>~</sup>:syu<sup>~</sup> embryos and subsequent neuronal differentiation steps, as marked by expression of Lim homeodomain genes, are also reduced in this territory. However loss of neurogenesis in this region probably reflects a loss of patterning in this territory as observed by the loss of dlx2 expression. Ace/Fgf8 and Syu/Shh are required for specifying forebrain and midbrain dopaminergic neurons in vitro (Ye et al., 1998). Analysis of ace<sup>~</sup>:syu<sup>~</sup> embryos revealed there is a reduction in the number of hypothalamic tyrosine hydroxylase positive neurons but ace<sup>~</sup> and syu<sup>~</sup> embryos do not have significant reductions in these neurons. The reduction in the number of tyrosine hydroxylase in ace<sup>~</sup>:syu<sup>~</sup> embryos may simply be a consequence of the reduction in the size of the hypothalamus in these embryos. Gene expression changes in the hypothalamic region of the single and double mutants do not support Ye and colleagues' assertion that Fgf8 and Shh act specifically in the differentiation of dopaminergic neurons.

The reduction of ngn1 expression within the telencephalon in syu<sup>~</sup> embryos does not appear to affect general neurogenesis. The apparently normal expression of genes involved in the differentiation of neurons, in syu<sup>~</sup> embryos, is probably due to compensation for the reduced ngn1 expression by other proneural genes. In the batch of embryos examined, anterior telencephalic lim6 expression in ace<sup>~</sup>:syu<sup>~</sup> embryos was more severely affected than in the single mutants. As expression of lim6 and not lim1 is affected in syu<sup>~</sup> embryos, it is
possible that Ace and Syu may act together to specify \textit{lim6} expression. However a severe reduction of \textit{lim6} expression has been previously observed in ace\textsuperscript{-} embryos (see section 4.2.7.) and the variability of single mutant phenotypes maybe to explain.

\subsection*{5.3.3. Forebrain axon guidance in the \textit{ace\textsuperscript{-}:syu\textsuperscript{-}} embryos}

Early defects in AC and POC guidance in \textit{ace\textsuperscript{-}:syu\textsuperscript{-}} embryos resemble defects seen in ace\textsuperscript{-} embryos. In addition both commissures in \textit{ace\textsuperscript{-}:syu\textsuperscript{-}} embryos appear to be more tightly fasciculated than in ace\textsuperscript{-} embryos. Examination of genes and proliferation suggests that the optic stalk territory may already be significantly reduced in prim 10 \textit{ace\textsuperscript{-}:syu\textsuperscript{-}} embryos. The tighter fasculation and reduced wandering of the axons in the midline territory may also reflect the loss of this optic stalk territory, as the commissures appear to fuse together rather than form ectopic tracts in the optic stalk region.

Although rostral diencephalic patterning is perturbed in syu\textsuperscript{-} and \textit{ace\textsuperscript{-}:syu\textsuperscript{-}} embryos, POC pathfinding does not appear to greatly affected. Initially POC axons appear to wander into the ventral optic stalk territory, with only a handful of axons correctly crossing the midline. By 54hpf, the POC has formed but is slightly thinner in syu\textsuperscript{-} embryos than in wildtype siblings. Correction of POC pathfinding severity is also observed in ace\textsuperscript{-} embryos, where failure to cross the midline by POC axons is rectified in later embryos. Early POC defects in \textit{yor} embryos resemble those observed in syu\textsuperscript{-} mutants, suggesting that the two molecules act in the same pathway (Karlstrom \textit{et al.}, 1999). Gli1 is also expressed in the ventral neuroectoderm of zebrafish embryos and may also mediate Syu and Twhh signalling (Karlstrom, Talbot and Schier, unpubl.). However \textit{yor} embryos also have a reduced AC, which is not observed in syu\textsuperscript{-} embryos. The gene expression changes, described in the previous section, in the ventral optic stalk region of syu\textsuperscript{-} embryos probably cause the defasculation of the POC. Axons still reach the midline in syu\textsuperscript{-} embryos but the loss of patterning within the ventral optic stalk causes the axons to wander in the midline region, even invading the optic stalk territory.

Defasculation of the POC is also observed in noi\textsuperscript{-} embryos but surprisingly noi expression is not affected in 10 somite stage syu\textsuperscript{-} embryos, Schauerte and colleagues (1998), also report no change in noi expression in syu\textsuperscript{-} embryos. During early axon pathfinding, Noi is thought to repulse axons from the ventral optic stalk region. As axon guidance occurs through an interaction between attractive and repulsive cues, a Noi independent pathway probably also operates in the ventral optic stalk (MacDonald \textit{et al.}, 1997). The defects seen
5.0 Forebrain development in embryos lacking Ace/Fgf8.1 and Syu/Shh signalling

in ace⁻ embryos also appear to be caused through defective patterning through Noi dependent and independent pathways.

Zebrafish retinal axons exit the eye at the choroid fissure and project over the optic chiasm to the contralateral tectum (Burrill and Easter, 1994). In ace⁻ embryos, the retinal axons sometimes enter the preoptic area at ectopic locations. Once within the optic chiasm, ace⁻ retinal axons project to the ipsilateral or contralateral forebrain and tectum. In the syu⁻ embryos examined, all retinal nerves projected to the contralateral tectum after entering the optic chiasm at various ectopic points. Retinal axons in ace⁻:syu⁻ embryos fail to exit the eye at the choroid fissure and instead project anteriorly within the eye. N-acetylated tubulin staining also reveals the neural retina of the eye is slightly disrupted in ace⁻:syu⁻ embryos. The defect in retinal axon pathfinding maybe due to disruption of axon cues in the choroid fissure of the eye. net2 expression is greatly reduced in ace⁻:syu⁻ embryos at the choroid fissure, previous reports have implicated netrins in retinal axon pathfinding out of the eye and across the optic chiasm (Deiner et al., 1997; MacDonald et al., 1997; de la Torre et al., 1997).

In noi⁻ embryos, the choroid fissure does not close properly and the retinal axons are defasiculated as they exit the eye. Similar defects in choroid fissure formation and optic nerve trajectory are detected in mice lacking Pax2 activity (Torres et al., 1996). A reduction in noi activity in ace⁻:syu⁻ embryos could partially explain the phenotype observed but the severity of the ace⁻:syu⁻ retinal axon pathfinding phenotype suggests other faults also exist in the ace⁻:syu⁻ embryos. As I have previously described in section 4.3., the control of noi expression is tightly linked to axon pathfinding at the forebrain midline. Previous reports have described how Shh is required for optic stalk formation and in particular for the optic stalk expression of pax2/noi. (Macdonald et al., 1995; Ekker et al.; 1995; Chiang et al., 1996; see section 4.3.). However I find that mutations in the syu gene and subsequent loss of twhh expression in syu⁻ embryos does not lead to a loss of noi expression in the optic stalk. In zebrafish, it appears that Hh regulation of noi maybe an artefact of overexpression studies and other molecules, such as eye⁷, probably play a more instructive role in noi expression (Regliabiati et al., 1998). In mice, mutations in the pax2 gene do not lead to the complete loss of the optic stalk and the downregulation of pax2 in Shh deficient mice may merely reflect the loss of the optic stalk territory (Torres et al., 1996). Ace clearly regulates noi, with the loss of midline noi expression observed in ace⁻ embryos during somitogenesis. noi expression in lateral optic stalk and choroid fissure is also reduced in post somitogenesis stage ace⁻ and ace⁻:syu⁻ embryos (Russell, Wilson, unpubl.).
Surprisingly a deletion allele (t4) of syu, which begins 4.0kb before the initiation codon and extends 2.2kb beyond the stop codon, displays a similar reduction in forebrain size and axon pathfinding to the ace−:syu− mutant (Schauerte et al., 1998). Although the study did not address the errors which lead to the reduced head size in the syu^d4 mutant, it is possible that a reduction in proliferation also occurs. Autocatalytic cleavage of the shh protein occurs to produce a 200 amino acid N-terminal fragment, which has been shown to mediate all the biological activity of the protein (Ekker et al., 1995). A 100 amino acid N-terminal fragment would be predicted to be synthesised in syu^bx392 embryos. Functional studies of the truncated Syu protein produced in syu^bx392 mutant have not been performed and it is possible that the truncated syu^bx392 protein may have some activity. Such residual activity would explain why the syu^bx392 allele is less severe than the phenotype described for the syu^d4 allele.

Ventral forebrain syu expression, in zebrafish embryos, depends on upstream enhancer sequences and it is possible downstream effectors of Ace may regulate syu through these sequences (Müller et al., 1999). Such a mechanism may help explain the late proliferation defects observed in ace− and ace−:syu− embryos. If Ace regulates shh transcription, within zebrafish embryos, a downregulation of shh transcription should be observed in ace− embryos. In ace− embryos, shh expression is lost or reduced in the basal telencephalon but is relatively unaffected in the hypothalamus, suggesting that Ace does not regulate shh transcription in the hypothalamus. An alternative interpretation involving Ace regulation of Syu processing, diffusion or action may explain the similarities between the ace−:syu− and the syu^d4 deletion allele mutants.
Chapter Six

Patterning and neurogenesis of the rostral neural plate is not discernibly affected in embryos overexpressing zebrafish *emx* genes

6.1. Introduction

Powerful insights into the mechanisms underlying segmental organisation of the nervous system have come from the analysis of vertebrate homologues of genes first identified in *Drosophila*. The expression domains of many these genes, notably the *Hox* genes, along the main body axes appear to be conserved throughout evolution. Although the control of trunk segment identity by *Hox* genes has been established in invertebrates and vertebrates, the factors governing the formation of the head segments or subdivisions remain elusive.

In *Drosophila*, mutagenesis screens, which proved invaluable for identifying genes for many developmental processes, failed to uncover homeotic selector genes responsible for conveying specific identity for anterior regions. Additionally, no pair rule genes were discovered, which could interpret anterior segmental patterning established through the action of the maternal and gap genes. However the discovery of overlapping expression patterns of the cephalic gap genes, *orthodenticle (otd)*, *empty spiracles (ems)* and *buttonhead (btd)*, suggested that segmental identity maybe established through the combinatorial expression of these genes (Cohen and Jürgens, 1991). In this model, cells expressing different combinations of the genes would give rise to different segments, for example, cells expressing *ems* and *btd* would contribute to the intercalary segment. Loss of these genes would lead to the loss of certain segments and transformation of others. Indeed, mutations in *ems* lead to a loss of the intercalary and antennal segment and disruption to the preantennal region (Dalton *et al.*, 1989, Hirth *et al.*, 1995). Similarly overexpression of these genes should lead to the transformation of segment identity. However overexpression of *otd* and *btd* do not lead to an alteration of head segment identity (Wimmer *et al.*, 1997; Gallitano-Mendel and Finkelstein, 1998).

Analysis of vertebrate homologues of genes involved in *Drosophila* head segmentation, such as *otd* and *ems*, also revealed zonal, nested expression patterns suggestive of a segmental organisation of the vertebrate brain (Simeone *et al.*, 1992). Members of the *Otx* gene family are expressed throughout the dorsal forebrain and midbrain region, whilst *Emx* gene family members are mainly restricted to the telencephalic region (Simeone *et al.*, 1992; Briata *et al.*, 1996; Gulisano *et al.*, 1996). As with *Drosophila*,

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vertebrate head and trunk segmentation appear to be governed by different processes. In the caudal neural tube, up to the midbrain, \textit{Hox} genes appear to dictate segmental identity (Lumsden and Krumlauf, 1996). In contrast, no \textit{Hox} gene expression has been detected in the anterior neural plate (Prince \textit{et al}., 1998).

In mice, the combinatorial model for head segmentation does not completely explain the results observed in loss of function studies. Mutations in \textit{Otx2} result in the complete loss of anterior head structures and this appears to result from the loss of \textit{Otx2} expression in the anterior visceral endoderm, and not within the neural plate (Acampora \textit{et al}., 1995; Rhinn \textit{et al}., 1998). More anteriorly, transgenic mice lacking \textit{Emx1} or \textit{Emx2} activity do not display a general loss or transformation of telencephalic structures, but rather lose specific dorsal telencephalic structures, such as the hippocampus (Pellegrini \textit{et al}., 1996; Yoshida \textit{et al}., 1997). A more precise critique of the combinatorial model would be facilitated by analysis of the \textit{emx1:emx2} knockout mouse. The mechanisms through which \textit{Emx} genes may specify dorsal telencephalic territories are also poorly understood. A study of the promoter region of \textit{Wnt1} revealed the presence of \textit{Emx} binding sites. Negative regulation of \textit{wnt1} by \textit{Emx} genes is supported by the expansion of \textit{wnt1} expression in \textit{Emx2} knockout mice (Iler \textit{et al}., 1996; Yoshida \textit{et al}., 1997). Downregulation of the other genes examined in the \textit{emx2} knockout mouse, imply a loss of limbic structures and do not provide an insight into the molecular mechanisms utilised by \textit{Emx} genes to specify this territory.

The functions of the \textit{Drosophila} head cephalic genes are starting to be elucidated and may also provide important clues into how their vertebrate homologues work. Yousnossi-Hartenstein and colleagues (1997) have described how the loss of cephalic head genes leads to loss of specific neuroblast populations in the head segments. In particular, loss of \textit{ems} results in the loss of neuroblasts in the deuterocerebrum and tritocerebrum. In the vertebrate caudal neural plate, neurogenesis is specified in three stripes representing the motor neuron, interneuron and sensory neuron populations of the neural tube. The positions of the three stripes appear to be determined through a step-wise process, involving an initial interpretation of the BMP gradient from the ventral to dorsal axis of the gastrulating embryo (Barth \textit{et al}., 1999; Chitnis, 1999). In the forebrain a comparative delay in neurogenesis is observed, such that the first signs of neurogenesis are only detected at the end of gastrulation (Papalopulu and Kintner, 1996; Blader \textit{et al}., 1997).

The factors underlying this delay and molecules required to initiate neurogenesis are starting to be elucidated. A special population of anterior neural plate cells, called row1 cells, have been demonstrated to influence patterning and neurogenesis in the zebrafish...
6.0 Misexpression of zebrafish *emx* genes

forebrain (Houart *et al*., 1998). Recent work has also identified the *Xenopus* homologue of the winged helix transcription factor, *BF-1*, as a potential mediator of the neurogenic activity of the rowl cells (Bourguignon *et al*., 1998). Misexpression studies revealed that *XBF-1* has dual activity, able to act as both a transcription activator or repressor. Furthermore cells expressing high levels of *XBF-1* inhibit neuronal differentiation whilst cells expressing low levels of *XBF-1* activate proneural gene expression. Additional evidence for the localised activation of neurogenesis in the forebrain has also come from studies of epiphysial neuronal specification in the zebrafish mutants, *floating head* and *masterblind* (Masai *et al*., 1997). Due to the highly conserved nature of neurogenesis across species, forebrain neurogenesis in vertebrates maybe expected to be regulated by *Emx* genes.

A greater understanding of the role of *Emx* genes in specifying rostral brain identity and regulation of forebrain neurogenesis would be facilitated by in vivo misexpression studies. In the zebrafish embryo, a number of different factors allow such studies to be performed with relative ease. In this study, I report that the misexpression of *emxl* or *emx2* does not affect regionalisation of the forebrain or neurogenesis in the zebrafish embryo. However preliminary results suggest that *emxl* may play a role in positioning the forebrain and eye field domains within the early neural plate.

6.2. Results

6.2.1. Spatial and temporal expression of *emx* genes suggest a crucial role in zebrafish telencephalic development

6.2.1.1. *emxl* expression is mostly restricted to the dorsal telencephalon.

Despite a number of different approaches, only a few genes have been isolated which display restricted expression in the early telencephalic anlage (Xuan *et al*., 1995; Bulfone *et al*., 1995). Of these *emxl*, one of the earliest genes, whose expression demarcates the prospective telencephalic territory in a number of species (Briata *et al*., 1996; Gulisano *et al*., 1996; Pannese *et al*., 1998). Zebrafish *emxl* expression mirrors the expression of the *emxl* genes across different species. *emxl* mRNA is first detected, at the 90% epiboly stage, in the anterior margin of the neural plate. Expression is strongest in two bilateral stripes, with a weaker domain at the rostral tip of the anterior neural plate (Fig6.1A). Following neurulation, *emxl* transcripts are detected in the dorsal telencephalon (Fig6.1B-D). Within the dorsal telencephalon, *emxl* is expressed mainly in post mitotic cells but expression is also detected in mitotic cells earlier in development (Fig6.1E). As the embryo develops, *emxl*
Fig6.1. Wildtype expression of $emx1$

All embryos have been labelled with digoxigenin RNA probes for $emx1$. (A) Animal pole view of tailbud stage embryos, showing strong $emx1$ expression in lateral edges and weaker expression at the most rostral of the anterior neural plate. Lateral views of (B) 18 somites, (C) prim 5, (D) prim 20 and (F) 48hpf embryos showing restriction of telencephalic $emx1$ expression to the dorsal half of the embryos. $emx1$ is also expressed in the posterior tuberculum. (E) Dorsal view of prim 20 stage embryo. $emx1$ expression is detected in ventricular cells and in post mitotic cells away from the ventricular zones. By 48hpf (F), $emx1$ expression is also detected in cells of the evaginating olfactory bulb. Scale bars represent 50µm.

Abbreviations: d, diencephalon, hy, hypothalamus, ob, olfactory bulb, op, olfactory placode, or, optic recess, pt, posterior tuberculum, t, telencephalon, te, tectum.
expression is maintained in the dorsal telencephalon and also spreads to cells in the olfactory bulb (Fig 6.1F). emx1 is also expressed in the posterior tuberculum and in the branchial arches (Fig 6.1C,D and data not shown).

6.2.1.2. Expression of zebrafish emx2 is more widespread than the emx1.

Of the two vertebrate homologues of Drosophila empty spiracles isolated in most species, emx2 displays a more widespread expression than emx1. In mouse and chicks, emx2 is also expressed earlier than emx1 (Gulisano et al., 1996; Fernandez et al., 1998). In order to establish whether this spatio-temporal regulation of emx gene expression was conserved in the zebrafish, whole mount in situ hybridisation was performed using a digoxygenin labelled RNA probe to emx2.

emx2 expression is first detected at the 1 somite stage in a broad dorsal rostral brain territory (Fig 6.2A). Comparative expression studies using markers for presumptive midbrain territories, reveal that early emx2 expression has a caudal boundary at the mid-hindbrain boundary (Delaney, unpubl). Subsequent restriction of emx2 expression to the developing telencephalon is observed (Fig 6.2B). Within the telencephalon, emx2 mRNA is detected in a dorsal territory similar to the emx1 expression domain (Fig 6.2C-E). emx2 is also apparently detected in proliferating and post mitotic cells (Fig 6.2D). Around prim 18 stage, emx2 expression is upregulated in the midbrain region. By prim 22 stage, weak emx2 expression is detected in the zona limitans intrathalamica (ZLI), tectum and tegmentum and, strong dorsal telencephalic expression is maintained (Fig 6.2E). However by 48hpf, the situation has reversed with high levels of emx2 expression detected in the tectum, expression in the dorsal telencephalon is greatly diminished with only a small number of cells weakly expressing emx2 (Fig 6.2F).

6.2.2. Regionalisation of the neural plate is unaffected in embryos transiently overexpressing emx genes

6.2.2.1. Rostral neural plate regionalisation appears to be unaffected when emx genes are overexpressed.

The early expression of the zebrafish emx genes in the telencephalic anlage suggests these genes may play an important regulatory role in its development. This is supported by analysis of transgenic mice lacking emx gene activity (Pellegrini et al., 1996; Yoshida et al., 1997). Mice lacking emx2 lose dorsal telencephalic structures, such as the dentate gyrus and...
Fig6.2. Wildtype expression of *emx2*

(A-C,E,F) Lateral views of wildtype embryos labelled with digoxygenin RNA probes for *emx2*. *emx2* is first detected at the 2 somite stage (A) in a broad dorsal territory, expression is weaker in the rostral parts of the embryos. By 10 somites (B), *emx2* mRNA is localised to the dorsal telencephalon. Strong expression is detected in the dorsal telencephalon at prim 5 (C) and prim 22 (E) stages. (D) Dorsal view of a prim 5 stage embryo reveals *emx2* is also expressed in ventricular proliferating cells and post mitotic cells. By 48hpf (F), *emx2* is only weakly detected in the telencephalon and higher levels of expression are found in the midbrain territories. Scale bars represent 50μm.

Abbreviations: cb, cerebellum, fb, forebrain, hy, hypothalamus, me, mesencephalon, or, optic recess, pt, posterior tuberculum, t, telencephalon, te, tectum, tg, tegmentum, zli, zona limitans intrathalamica.
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hippocampus. More subtle defects, such as the failure of callosal commissure axons to cross
the telencephalic midline, are observed emx1 knockout mice (Yoshida et al., 1997). If the
combinatorial model is correct, overexpression of these genes should lead to the formation
of ectopic rostral brain territories. In order to test this hypothesis, we overexpressed the emx
genes throughout the neural plate during development by microinjecting capped RNA into
one cell of 1-4 cell stage zebrafish embryos. In vitro transcribed capped RNA encoding full
length emx1 or emx2 was co-injected with green fluorescent protein RNA (GFP). Injected
embryo were analysed for GFP fluorescence to ensure successful microinjection had taken
place, embryos were also fixed at late gastrulation stages and labelled with an anti-GFP
antibody to visualise the distribution of the RNA in the injected embryos (data not shown).

The transcription factor, anf, is expressed in the anterior margin of the neural plate,
in a territory thought to contribute to the telencephalic forebrain subdivision (Kazansky et
al., 1997). anf undergoes complex regulation, where it is initially expressed a broad anterior
neural plate domain but is downregulated to leave strong expression in the anterior and
lateral most cells of the anterior neural plate. anf expression was analysed, in tailbud stage
embryos which had been injected with emx1 and emx2 RNA. At tailbud stage, anf
expression is limited to the anterior margin of the neural plate (Fig6.3A). At this stage, shh
expression can also be detected in axial midline cells, with the rostral extent of shh
expression underlying anterior cells expressing anf (Fig6.3A). In emx1 or emx2 injected
embryos, no perceptible change in anf expression is detected (Fig6.3B,C). otx2 is expressed
throughout the anterior neural plate, in territories that will give rise to the forebrain and
midbrain (Li et al., 1994) and mice otx mutants lose anterior head structures (Acampora et
al., 1995). At tailbud stage, otx2 is expressed in a broad domain, thought to encompass the
forebrain and midbrain territories (Fig6.3D). No apparent change in otx2 expression is
detected in emx1 or emx2 injected embryos (Fig6.3E,F).

6.2.2.2. Subdivisions of the forebrain are relatively normal in emx injected embryos.

To investigate more specific areas of forebrain regions, nk2.1 expression was
investigated in emx1 or emx2 injected embryos. nk2.1 expression is detected in midline cells
in the anterior neural plate at late gastrulation stages (Fig6.3G). In the zebrafish, these nk2.1
expressing cells will develop into the hypothalamus. Again no change in nk2.1 expression is
detected in embryos injected with emx1 or emx2 RNA (Fig6.3H,I). ace/fgf8.1 is expressed
in a subset of emx1 cells in the anterior neural plate and has been shown to be required for
early maintenance of the most rostral emx1 expression (see section 4.2.2.). Following
neurulation, ace is expressed in the dorsal telencephalic midline and the optic stalk territory
Fig6.3. Anterior neural plate markers are normally expressed in emx injected

Animal pole views of tailbud (A,D,G) control, (B,E,H) emxl injected and (C,F,I) emx2 injected embryos. (A-C) Embryos labelled with digoxygenin RNA probes for anf and shh. The characteristic horseshoe expression domain of anf is observed in the anterior neural plate of (A) wildtype and (C) emx2 injected embryos; (B) some emxl injected embryos have a smaller domain of anf expression. (D-F) Embryos labelled with digoxygenin RNA probes for otx2 and shh appear to be normal, even when injected with (E) emxl or (F) emx2 RNA. otx2 is expressed in a broad anterior neural plate territory. (G-H) The expression of nk2.1 mRNA, in the anterior axial midline, also appears to be normal in (H) emxl or (I) emx2 injected embryos. (J-K) Lateral views of (J) wildtype, (K) emxl injected and (L) emx2 injected embryos labelled with digoxygenin probes against ace/fgf8.1. (L) emx2 injected embryos apparently correctly express ace but (K) some emxl injected embryos have reduced expression of ace in the forebrain. Scale bars represent 50μm.

Abbreviations: fb, forebrain, mhb, mid-hindbrain boundary, s, somites, tb, tailbud.
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(Fig 36J). Although ace expression is normal in emx2 injected embryos, 25% of emxl injected embryos (n=20) have reduced ace expression in the forebrain and expanded expression in the hindbrain (Fig 6.3K,L).

6.2.3. Rostral neural plate neurogenesis in emx injected embryos

6.2.3.1. Establishment of regions competent for neurogenesis is not affected in emx injected embryos.

In the caudal neural plate, zones of neuronal specification can be visualised through their expression of proneural and neurogenic genes during gastrulation. In contrast similar territories in the rostral neural plate, as assayed by the expression of similar markers, are not detected until the tailbud stage. In the anterior neural plate, ngn1 is expressed shortly after emxl expression is initiated (Morita et al., 1995; Blader et al., 1997). ngn1 is also expressed within the emxl domain, suggesting emxl may control neurogenesis within the anterior neural plate (data not shown). Along with the fact ems controls neurogenesis in Drosophila head segment, the spatio-temporal expression of emxl suggests it may play a role in forebrain neurogenesis.

I examined ngn1 expression in emxl injected embryos to see whether ectopic regulation of emxl activity lead to ectopic proneural gene expression in the neural plate. As the activity of XBF-1 was concentration dependent, analysis of ngn1 expression was performed on embryos that were injected with a range of concentrations. Patchy ngn1 expression, in the anterior neural plate, is detected at the tailbud stage (Fig 6.4A). Similar expression is detected in embryos, injected with emxl RNA at a range of concentrations (Fig 6.4B,D,F). Ectopic expression of XBF-1 in Xenopus lead to the suppression of neurogenesis in the caudal neural plate but upregulation of neuronal differentiation in the ventral side of the embryo (Bourguignon et al., 1998). In the zebrafish neural plate neurogenesis, at any position along the anterior-posterior axis, was not affected in emxl injected embryos (data not shown).

ngn1 expression was also examined in emx2 injected embryos, to see whether altering the timing and position of emx2 expression alters neurogenesis within the neural plate. Again ngn1 expression was apparently unaffected by ectopic expression of emx2 (Fig 6.4C,E,G). Expression of the neurogenic gene, delta b, was also examined in emx injected embryos. In the 10 somite embryo delta b is expressed in a salt and pepper manner
Fig6.4. Neurogenesis is unaffected in \textit{emx} injected embryos

(A-G) Animal pole views of tailbud stage embryos stained with digoxygenin labelled RNA probes for \textit{ngnl}. Panel (A) is an example of a control embryo, panels (B,D,F) are examples of embryos injected with 30ng/\mu l, 40ng/\mu l and 60ng/\mu l respectively of \textit{emxl} RNA. Panels (C,E,G) are examples of embryos injected with 30ng/\mu l, 40ng/\mu l and 60ng/\mu l of \textit{emx2} RNA. \textit{ngnl} is expressed in the margin of anterior neural plate at tailbud stage (A) in a domain which includes \textit{emxl} expressing cells (indicated by arrows). \textit{ngnl} expression is also detected in the cells of the presumptive trigeminal ganglion and midbrain. In the caudal neural plate, \textit{ngnl} is expressed in three stereotypical stripes which will develop into the motor, interneurons and sensory neurons. (B-G) In \textit{emx} injected embryos, expression in all \textit{ngnl} domains is normal and no ectopic \textit{ngnl} expression is detected in other regions of the embryo. (H-J) Lateral views of 10 somite stage (H) control, (I,J) \textit{emxl} and \textit{emx2} embryos stained with digoxygenin labelled RNA probes for \textit{delta b} and \textit{shh}. (H) At this stage, \textit{delta b} is expressed throughout the telencephalic vesicle in a salt and pepper manner. (I,J) Again expression of \textit{delta b} expression is not perturbed in \textit{emx} injected embryos. Scale bars represent 40\mu m.

Abbreviations: \textit{mhb}, mid-hindbrain, \textit{tg}, trigeminal ganglion.
Fig6.5. Telencephalic neuronal differentiation is also unaffected in emx injected embryos

Lateral views of prim 5 control (A,C) and emxl injected (B,D) embryos labelled with digoxigenin labelled RNA probes for liml and eome. In wildtype (A) embryos, liml is expressed in two telencephalic populations of differentiating cells, one anterior and the other dorsal (shown by arrows). Embryos injected with emxl RNA (B) showed no ectopic expression of liml in the telencephalon or other areas of the brain. (C) eome is also expressed in some post mitotic cells in the telencephalon and (D) shows no perturbed expression in emxl injected embryos. Scale bars represent 50μm.

Abbreviations: hy, hypothalamus, t, telencephalon, te, tectum, zli, zona limitans intrathalamica.
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throughout the telencephalon (Fig6.4H). Expression of \textit{delta} \textit{b} is not affected in \textit{emx} injected embryos (Fig6.4I,J).

6.2.3.2. Neuronal differentiation is also unaffected in \textit{emx} injected embryos.

\textit{emx1} and \textit{emx2} are expressed in the dorsal telencephalon when neurons first start to differentiate (Macdonald \textit{et al}., 1994). The expression of \textit{emx} genes may convey identity to these neurons to differentiate as dorsal telencephalic genes. To test this hypothesis, expression of genes characteristically expressed in the dorsal telencephalon was examined in \textit{emx} injected embryos. In prim 5 stage embryos, \textit{liml} is expressed in two groups of cells in the dorsal telencephalon, these groups are positioned in a dorsal and anterior territory (Fig6.5A). \textit{liml} is also expressed in the dorsal hypothalamus and tegmentum (Fig6.5A). In \textit{emx1} injected embryos, \textit{liml} is expressed relatively normally and no sites of ectopic expression are detected (Fig6.5B). \textit{eome} expression is expressed in mitotic and post mitotic cells in the dorsal telencephalon and does not appear to ectopically expressed in \textit{emx1} injected embryos (Fig6.5C,D).

6.2.4. Specification of the eyes in \textit{emx1} injected embryos

6.2.4.1. Smaller eyes in \textit{emx1} injected embryos.

Fate mapping experiments have revealed tissue competent to form eyes is positioned immediately caudal to the presumptive telencephalic domain during gastrulation (Eagleson \textit{et al}., 1995; Shih and Fraser, 1995). As the combinatorial model would predict the overexpression \textit{emx1} expression would perturb adjacent domains, the development of the eyes was investigated in \textit{emx1} injected embryos. \textit{emx1} injected embryos were allowed to develop until prim 8 stage and the development of the eyes was examined. In 41\% of embryos examined (n=13/32), eyes were smaller when compared to control embryos (Fig6.6A,B). No change in the size of the eyes was observed in \textit{emx2} injected embryos (data not shown).

6.2.4.2. \textit{six3} expression is reduced in \textit{emx1} injected embryos.

To further analyse the smaller eyes observed in \textit{emx1} injected embryos, \textit{six3} expression was investigated. Zebrafish \textit{six3}, a homologue of \textit{Drosophila sine oculis}, has been described to control the size of eyes and forebrain territories in the developing embryo.
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Fig 6.6. Smaller eyes are observed in emx1 injected embryos
Dorsal views of prim 8 stage (A) control and (B) emx1 injected embryos stained with an anti-GFP antibody. (B) In some embryos emx1 RNA segregates to one half of the embryo. In such embryos the eye is usually smaller, on the side receiving the RNA (see arrows). GFP is not detected at this stage in injected embryos. Animal pole views of tailbud (C) control and (D,E) emx1 injected embryos labelled with digoxygenin labelled RNA probes for six3. (C) six3 is expressed in the anterior neural plate in cells which are thought to give rise to the forebrain and eyes. (D,E) In 51% of emx1 injected embryos, six3 expression in the anterior neural plate is reduced. Scale bars represent 30μm in (A,B) and 50μm (C-E).
Abbreviations: e, eye, fb, forebrain, op, olfactory placode, t, telencephalon.
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(Kobayashi et al., 1998; Loosli et al., 1999). During late gastrulation, six3 is expressed in a broad anterior neural plate domain, which includes the presumptive telencephalon and eye fields (Fig. 6.6C). In 51% of emxl injected embryos (n=23/45), six3 expression is reduced (Fig. 6.6D,E). Without further experimentation, it is not possible to determine whether the reduction in six3 expression is biased in the rostral or caudal domain.

6.3. Discussion

Regionalisation of the apparently amorphous neural plate is first detected through the restricted expression domains of genes, such as Otx and Emx gene family members. Although the role of transcription factors, such as the Hox genes, in specification and patterning of trunk have been extensively described, examination of genes involved in regionalisation of the anterior neural plate is still in its infancy. In mice, loss of function studies have implicated Emx genes in the specification of dorsal telencephalic structures, such as the hippocampus and dentate gyrus, and in neuronal differentiation in the olfactory bulb. I have started to conduct misexpression experiments to study the roles of Emx genes in regional patterning and neurogenesis. Initial results suggest that misexpression of zebrafish Emx genes does not lead to ectopic forebrain patterning or neurogenesis. However emxl may influence the determination the specification and growth of the eyes.

6.3.1. Ectopic expression of emx genes does not alter rostral neural plate regionalisation

The early, restricted expression of the emx genes in the vertebrate forebrain suggests an important role in the regionalisation of the rostral neural plate and specification of the telencephalon (Morita et al., 1995; Gulisano et al., 1996; Pannese et al., 1998; Fernandez et al., 1998). An understanding of the potential roles of Emx genes was gained through the analysis of transgenic mice carrying mutations in the Emx genes. Emx2 is required for the formation of archipallium structures, such as the hippocampus, dentate gyrus and medial limbic cortex. Additionally Emx2 appears to be required for proliferation of the dorsal telencephalon, as knockout mice have reduced evagination of the cerebral hemispheres. Loss of Emx1 does not result in gross telencephalic defects but more subtle defects of axonal pathfinding and neuronal cell type differentiation are observed (Pellegrini et al., 1996, Yoshida et al., 1997). The severity of defects observed in both mutants are relatively minor considering their expression domains, suggesting that emx genes may functionally compensate the loss of each other. In support of this, Yoshida and colleagues noted that
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defects in \textit{emx}2 knockout mice were limited in regions which normally express only \textit{emx}2. Studies of the double knockout mouse would greatly increase our understanding of the role of \textit{emx} genes.

Misexpression studies have proved an invaluable tool to dissect gene activity, allowing the importance of timing and position of expression to be thoroughly assessed. My results indicate that the zebrafish \textit{emx} genes do not have the ability to perturb general forebrain and midbrain patterning when ectopically expressed. The lack of patterning activity upon misexpression may be explained by a number of reasons. In \textit{Drosophila}, the loss of segment identity in mutants of the cephalic gap genes (\textit{ems, otd} and \textit{btd}), along with the absence of pair rule gene activity in head, suggested \textit{ems, otd} and \textit{btd} may be directly responsible for establishing head segments (Dalton \textit{et al.}, 1989; Finkelstein and Perrimon, 1990; Cohen and Jürgens, 1990). However misexpression of neither \textit{otd} and \textit{btd} results in transformation of segment identity (Wimmer \textit{et al.}, 1997; Gallitano-Mendel and Finkelstein, 1998). Consequently there has been a reappraisal the role of these genes and the issue of how head segments are specified in \textit{Drosophila} remains unresolved. In vertebrates, a similar situation may be occurring where the loss of \textit{Emx} and \textit{Otx} results in loss of head structures but the overexpression does not significantly perturb the regionalisation of the neural plate. In \textit{Xenopus} overexpression of \textit{Emx} genes also results in apparently normal brain patterning (G. Lupo, G. Barsacchi, pers. comm.)

The lack of induction of ectopic structure by \textit{Emx} genes in both zebrafish and \textit{Xenopus} may indicate a permissive role for these genes during development. However, the loss of archipallial structure in knockout mice indicate that these genes are necessary for development. The lack of ectopic phenotypes in wildtype embryos overexpressing \textit{btd} also suggest the \textit{Drosophila} cephalic genes may play a general, permissive but necessary role during anterior development (Wimmer \textit{et al.}, 1997). Alternatively \textit{Emx} genes may require a co-factor to regulate downstream targets (Fisher and Caudy, 1998) and the overexpression studies do not produce a phenotype due to the restricted nature of this co-factor.

Another possible explanation for the lack of overexpression phenotype arises from the experimental method employed in these studies. Although successful microinjection and transcription does occur in the embryos analysed, as assayed through green fluorescent protein synthesis, I can not be certain that functional \textit{Emx} protein was synthesised. The plasmid constructs included a FLAG epitope at the 3' end of the gene but experimental difficulties with the anti-FLAG antibody meant that I was unable to assess whether \textit{Emx} protein synthesis had occurred. In early gastrulating stages, in situ hybridisation studies
revealed that emx RNA was present within the microinjected embryos. However further analysis to determine RNA stability and protein synthesis need to be performed.

6.3.2. Emx genes and eye specification

Although general patterning of the neural plate was unaffected in emx injected embryos, smaller eyes were frequently observed in emx1 injected embryos. Smaller eyes are also observed in Xenopus embryos injected with Xemx1 (G. Lupo, G. Barsacchi, pers. comm.). In zebrafish, the reduction in eye size correlates with a reduction in the six3 expression domain at tailbud stages. In early embryos, six3 is expressed throughout the anterior neural plate, in an area thought to give rise to the forebrain and eyes. Misexpression studies in zebrafish and medaka describe a role for six3 in specification of the eye and control of retinal growth (Kobayashi et al., 1998; Loosli, et al., 1999). The reduced six3 expression in emx1 injected embryos further supports this role. It is unlikely that emx1 misexpression causes a reduction of the eye territory, in favour of other forebrain fates, as no detectable increase in telencephalic size was observed in the injected embryos.

It is possible that emx1 inhibits cell proliferation in the eye without necessarily activating other cell fate choices. Overexpression XOptx2, a member of the Six/sine oculis family in Xenopus, results in a dramatic increase in eye size (Zuber et al., 1999). Xoptx is an intrinsic transcriptional repressor, which may act in conjunction with Pax6, to activate cell proliferation within the eye. As overexpression of emx1 results in a downregulation of six3 expression, a similar downregulation of optx2 could be envisaged to explain the smaller eye phenotype observed in emx1 injected embryos. This explanation is favourable given the lack of phenotype throughout the CNS in emx injected embryos.

6.3.3. Spatio-temporal regulation of Emx genes in the zebrafish

Regulation of chick and mouse Emx gene expression appears to be virtually identical. emx2 is expressed first in a broad domain, thought to encompass the presumptive fore and midbrain territories. Shortly afterwards, emx1 mRNA is detected in cells of the presumptive telencephalon (Gulisano et al., 1996; Fernandez et al., 1998). Although early spatial expression is conserved in the zebrafish, temporal regulation of the Emx genes is significantly different. Of the two zebrafish emx genes, emx1 is expressed earlier than emx2. This apparent switch in temporal regulation of the emx genes is also observed in Xenopus (Pannese et al., 1998).
Additional differences in spatial expression of the two *emx* genes in zebrafish and mouse are also observed. In mice, *emx2* expression is maintained in the dorsal telencephalon to adulthood and expression in the mitral cells of the bulb is also detected at later stages. In the zebrafish, *emx2* is downregulated in the telencephalon after prim 26 and increasingly expressed throughout the midbrain domain. *emx2* is also not expressed in the olfactory bulb. Understanding the significance of the spatio-temporal regulatory switch in zebrafish and *Xenopus* will increase our understanding of the evolution of the vertebrate telencephalon.

Differences in gene expression may be a result of the additional genome duplication thought to have occurred in the zebrafish lineage (Prince *et al.*, 1998; Amores *et al.*, 1998). Indeed phylogenetic analysis of the *emx* genes reveals that zebrafish *emx1* may not be the true orthologue of the other vertebrate *emx* genes, further suggesting the presence of other *emx* genes in the zebrafish (Patarnello *et al.*, 1997). Other *emx* genes may play a more pivotal role in zebrafish telencephalic development and the isolation of these genes remains an important goal for those studying forebrain development in zebrafish.
Chapter Seven

General discussion and future directions

In this study I have sought to describe the morphogenetic events and patterning mechanisms underlying the development of the zebrafish forebrain. From late somitogenesis, the zebrafish telencephalon is organised into distinct proliferative and differentiating regions. In the roof of the telencephalon, regionalised proliferation probably combine with morphogenetic movements to initiate the teleostean process of eversion around 48hpf. In addition to the identification of regional histological zones, the pallial and sub-pallial subdivisions of the zebrafish telencephalon were identified through the expression of \textit{dlx2} and \textit{emxl}. By following the expression of these two genes, the pallial and sub-pallial divisions can be delimited until 5days post fertilisation. The late expression of \textit{emxl} in presumptive everted tissue suggests that the adult derivative of everted tissue is exclusively pallial in nature. A third telencephalic domain, which does not express either \textit{dlx2} or \textit{emxl}, is also detected, which may homologous to similar domains uncovered in other species.

Analysis of zebrafish \textit{ace/fgf8.1} mutants revealed ectopic pathfinding of AC, POC and retinal axons. All these axon bundles traverse the optic stalk territory and patterning of this territory is severely perturbed in mutant embryos. Ectopic pathfinding correlates with perturbed expression of a number of transcription factors, including \textit{noi}, and axon guidance cues, such as \textit{net1} and \textit{sema3D}. Patterning of the telencephalon was generally normal in the \textit{ace} mutants but more subtle defects in neuronal differentiation were detected. Ace is also required for correct pathfinding of olfactory axons from the olfactory placodes. However as the \textit{ace} embryos displayed defects in both olfactory sensory neuron and olfactory bulb differentiation, the main cause of the defect has yet to be pinpointed. Potential interactions between \textit{ace/fgf8.1} and \textit{syu/shh} were investigated by generating double mutants. Although general patterning does not appear to be more severely perturbed in the double mutant than the single mutants, other specific defects are detected. For example, failure of the retinal axons to exit the eye into the preoptic area in the double mutant correlates with a reduction or loss of guidance cues within the choroid fissure. Investigation of the role of \textit{emx} genes in the telencephalon suggests that misexpression of these genes does not lead to ectopic forebrain structures. A more detailed investigation into potential defects in eye formation in embryos overexpressing \textit{emx} genes needs to be performed.

7.1. Pallial, sub-pallial and intermediate territories in the zebrafish telencephalon
Attempts to understand how the forebrain evolved have primarily concentrated on comparative analysis of extant adult brains due to the lack of fossil records. In the adult, the identification of homologous structures is crucial for the correct interpretation of evolutionary events. Recently the study of gene expression in the embryo has greatly aided the search for homology. In particular the use of Dlx and Emx genes to identify homologous structures across species is vindicated by the fact that the early expression of these genes is conserved across a number of species (Bulfone et al., 1993; Papalopulu and Kintner, 1993; Akimenko et al., 1994; Simeone et al., 1992; Morita et al., 1995; Fernandez et al., 1998). A detailed examination at the onset of neuronal production further supports the use of Dlx and Emx genes as markers for pallial and subpallial territories. An additional intermediate domain (ID) is detected in chick, frogs, mouse and turtles (Fernandez et al., 1998; Puelles et al., 1999). In this study, I have identified the equivalent domains in the zebrafish embryonic telencephalon (Fig 7.1).

Although the early development of the telencephalic vesicle is conserved across species, later stages of development are not. Perhaps the most striking example of this is the development of the mammalian multi-layered neocortex (Karten, 1997). Non-mammals do not have a structure which can be easily compared to the neocortex. The expression of Emx genes in the dorsal telencephalon of the chick and mouse suggests that the chick Dorsal Ventricular Ridge (DVR) is homologous to the cortex. This observation is further supported by the identification of cell types with similar transmitters, receptors and axonal connections in the DVR and neocortex (Karten, 1991). The characterisation of the zebrafish telencephalon is relatively poor and the extensive studies on cell type and connectivity have yet to be performed. Despite the diverging morphogenetic events underlying the development of the teleostean and other vertebrate telencephalons, our studies indicate that the dorsal telencephalon probably is the equivalent of the mouse neocortex and chick DVR. The dorsal pallium probably compromises the entire area dorsalis and may include dorsal regions of the area ventralis, as emx1 is initially expressed in non-everted tissue and subsequently in everted tissue.

Homologous sub-pallial structures have been delineated through examination of Dlx gene family members across species. During late somitogenesis stages dlx2 expression is restricted to the ventral half of the telencephalon but in post hatching stages, dlx2 expression expands towards the dorsal telencephalon. Extrapolation of this territory would suggest that the adult area ventralis forms the sub-pallium in the zebrafish. The organisation of the amniote subpallium, also known as the basal ganglia, was thought to be significantly different from that of anamniotes (fish and amphibians). However recent studies in
Fig 7.1. **Interpretative scheme of the evolution of telencephalic regionalisation**

The presumptive adult structures produced by the three subdivisions of the developing telencephalic vesicle. The pallial territory is in red, the sub-pallial in green and the intermediate domain in blue. The divisions in adult zebrafish telencephalon represent an initial attempt to classify structures. In the zebrafish, the fate of the pallial and ID regions remains uncertain due to the lack of knowledge about late post hatching forebrain development. Extrapolation of morphological, proliferative and gene expression studies until 5 dpf suggests that the area dorsalis probably forms a major component of the zebrafish pallium. The apparent lack of expansion of the ID during development suggests that the fate of the zebrafish ID may be similar to that of the mouse. In the mouse the ID also does not expand proportionately during development and contributes to the amygdala and parts of the medial septum. Thus the zebrafish ID may also contribute to relatively small adult structure positioned laterally and medially. Abbreviations, ad, area dorsalis, a, amygdala, av, area ventralis, cx, cerebral cortex, dc, dorsal cortex, dp, dorsal pallium, DVR, anterior dorsal ventricular ridge, lp, lateral pallium, mp, medial pallium, ms, medial septum, n, neostriatum, p, paleostriatum, s, septum, st, striatum.

Adapted from Fernandez *et al.*, 1998
Archetypic embryonic stage

- frog
- turtle
- chicken
- mouse
- zebrafish
amphibians suggest that this may not accurately reflect the evolution of the basal ganglia and that a basic level of organisation exists between amniotes and amphibians (Marín et al., 1998). The expression of genes, such as \textit{dlx2} and \textit{nk2.1}, suggest that this level of organisation may be applied to fish (Akimenko et al., 1994; K.Rohr, unpubl.). A thorough investigation of axonal connections between brain divisions and the identification of nuclei using neurotransmitter markers, such as GABA, will reveal whether the organisation of the basal ganglia was established in an anamniote ancestor (Marín et al., 1997).

In mice and chicks, the fate of the ID varies (Fernandez et al., 1998 Puelles et al., 1999). In the chick the intermediate territory continues to expand, along with the rest of the telencephalon, to give rise to the a substantial part of the DVR. In mice the fate of the ID is unclear but it is thought that the ID fails to expand proportionately and will eventually give rise to the parts of the paleocortex, amygdala and medial septum. Although it is uncertain whether the derivatives of the ID in mice and chick are homologous, the identification of the ID has aided the search for the avian homologue of the amygdala (Fernandez et al., 1998). In the zebrafish the ID seems to follow a similar fate to that observed in mice, as it is difficult to distinguish after the prim 20 stage. Fate mapping experiments will enable us to gain a clearer understanding of the final position and size of the zebrafish ID, as well as possibly identifying a potential teleostean homologue of the amygdala.

A number of studies need to be performed on the zebrafish telencephalon before exact homologies can be defined. Further examination of cell type, connectivity and gene expression would greatly increase our understanding of how the telencephalon develops and how it relates to the other telencephali. Cell labelling studies to dissect the mechanisms, which give rise to eversion and other morphogenetic movements need to be investigated to comprehend the development and homologies of the zebrafish telencephalon.

Initial comparative studies between the zebrafish and goldfish brains reveal structurally homologous regions in the tectum and future studies may reveal such domains in the telencephalon (Rupp et al., 1996). Extensive neurobiological studies have been carried out on a number of closely related fish species (Barthfield et al., 1984; Northcutt and Wulliman, 1988; Wulliman and Meyer, 1993). Further comparative neuroanatomy between zebrafish and these species will aid our understanding of the structure and function of the teleostean telencephalon.

7.2. Patterning and axon pathfinding in the zebrafish forebrain
Mutagenesis screens of *Drosophila* have elucidated numerous genes involved in a wide array of processes, including midline development and axon pathfinding (Seeger *et al.*, 1993). Genes involved in vertebrate midline development are now being isolated through mapping and positional cloning of zebrafish mutants. I have characterised the zebrafish mutants, *acerebellar* and *sonic you*, to describe the roles *ace/fgf8.1* and *syu/shh* play in forebrain midline development and subsequently axon guidance. *ace* is required for correct patterning of the optic stalk region and in *ace* mutants, ectopic axon pathfinding correlates with disrupted expression of molecules involved in axon guidance, such as *sema3D* (Fig 7.2). In mutants lacking both *ace* and *syu*, gene expression in the forebrain is perturbed but not more severely than in either single mutant. Additionally, pathfinding defects made by the AC and POC appear to reflect a reduction in brain size, indicating potential interaction between *ace* and *syu* may regulate mitosis in the forebrain (Fig 7.2).

The study of zebrafish mutants, such as *noi* and *yot* mutants (Macdonald *et al.*, 1997; Karlstrom *et al.*, 1999), have provided an insight into the how the forebrain midline is patterned and how patterning may affect axon pathfinding. In combination with studies on other species, a comprehensive picture of molecules required for forebrain midline development can be gained. In the optic stalk the prevailing view that Shh is required for Pax2 expression is challenged by my findings in the zebrafish (Chiang *et al.*, 1996; Huh *et al.*, 1999). A lack of Syu signalling does not affect *noi* expression in the optic stalk. In *noi* and *ace* embryos, expansion of *syu* expression into the optic stalk does not correlate with upregulation of *noi*. The further loss of *twhh* in *syu* mutants suggest that in the zebrafish, Hh independent signals maybe required for the induction and maintenance of optic stalk *noi* expression. For example, Ace is clearly required for the maintenance of optic stalk *noi* expression. In the zebrafish, *cyc* and *oep* are potential regulators of *noi* optic stalk expression (Feldman *et al.*, 1998; Regabliati *et al.*, 1998) and the examination of these genes in other vertebrates will greatly increase our understanding of forebrain midline development.

The value of mutagenesis screens to isolate genes involved in axonal pathfinding was further illustrated by the identification of several zebrafish mutants with retinotectal pathfinding defects (Baier *et al.*, 1996; Karlstrom *et al.*, 1996; Trowe *et al.*, 1996). A rescreen of mutants isolated in the large scale Tübigenen mutagenesis screen has identified a number of interesting forebrain pathfinding defects. Using the N-acetylated tubulin antibody to visualise axonal tracts, members of our laboratory have identified 19 mutants with AC and 11 mutants with POC defects (Houart, Heisenberg, Roldan-Navarro, Schilling and Wilson, unpubl.). For example, *blowout* was initially identified as the retinal axons fail to
Fig 7.2. Schematic representation showing the positions of the AC and POC in relation to four neuroepithelium domains in wildtype and mutant embryos

A) The positions of the AC and POC closely correlate with the expression domains of several genes. For example, *shh*, *ephrin-A-L2* and *ephrin-A-L4* (pink) are expressed ventral to the position of the POC, *noi* (blue) is expressed in the ventral optic stalk dorsal to the POC, *ephA3* (red) is expressed dorsal to the AC and *net1* and *net2* (green) are expressed in the entire optic stalk but more strongly in the basal telencephalon, ventral to the AC. (B) The loss of *noi* activity (shown by pale blue domain) results in axons of the POC invading the optic stalk. Correspondingly *shh* expression spreads dorsally into the ventral optic stalk but no other genes appear to be perturbed. (C) In *ace* mutants, a greater perturbation of forebrain midline patterning correlates with more severe axon pathfinding defects. Axons, which would normally contribute to the AC and POC, projecting laterally within the optic stalk territory and axons are also seen wandering into the medial optic stalk territory. Gene expression in the basal telencephalon and ventral optic stalk are observed (shown by paler expression domains). Expression of axon guidance molecules, such as *net1* and *sema3D*, is reduced, which may reflect the roles of these molecules in attracting axons towards the midline but steering them away from the optic stalk territory. (D) The role of *syu* in forebrain midline patterning and axon guidance is revealed in *syu* mutants. Axons of the tract of POC are defasiculated and project into the ventral optic stalk. Correspondingly the expression of *twhh*, *net1* and *net2* are altered in the optic stalk area. (E) The severe patterning defects in the optic stalk territory and the projection of axons in *ace*:*syu* embryos suggests that optic stalk territory is reduced. A reduction in optic stalk territory may explain the fact that axons of the tracts of the AC and POC project laterally within the optic stalk region but occasional axons do manage to cross the midline. The reduction or loss of gene expression in the optic stalk also correlates with such an explanation.

Abbreviations: ac, anterior commissure, os, optic stalk, poc, postoptic commissure.
cross the midline and project ipsilaterally. A role for blowout in attracting axons towards the midline is further suggested by the loss of the AC (Baier et al., 1996; Karlstrom et al., 1996; Hourt and Wilson, unpubl.). Other mutants, such as otter, have defects in both the AC and POC. The further characterisation and mapping of these mutants will increase our understanding of general forebrain development.
Chapter Eight

Bibliography


Bibliography


