Molecular Mechanisms of Neural Induction and Patterning in the Zebrafish Embryo

Submitted by Carlos Manuel Pereira da Cruz to the University of Exeter

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: …….. Carlos Cruz

Abstract

The brain is our most complex organ, with an estimated 10^{11} neurons. With the spinal cord, it forms the central nervous system which controls our movements and our senses, holds our memories and creates our thoughts. Because of this, neurodegenerative disorders can be extremely distressing and a thorough understanding of how the nervous system develops is essential if progress is to be made in finding ways to treat them. Critically, this includes understanding how the nervous system forms, i.e., the nature of the signals that promote neural identity (neural induction) and determine correct positional information (patterning).

The zebrafish (*Danio rerio*) has become established as a model for embryological studies due to ease of experimental manipulation. Taking advantage of this, the aims of this PhD were to contribute to unravelling the molecular mechanisms of neural induction and patterning, using a variety of embryological and molecular methods. In the first project, functional analyses of the *eve1* gene identified a key factor for posterior neural development. Eve1 was found to be a critical posteriorising factor, with an additional role in posterior neural induction. An outstanding question in neural induction is the relative contribution to this process of two key developmentally important signalling pathways, Bmp and Fgf. In the second project, differential analyses of maternal versus zygotic Bmp and Fgf signalling revealed crucial maternal roles for these two pathways in neural development as neural and epidermal capacitators. The results further suggested that Fgf signalling may be the critical neural inducer. Finally, as a third project, a zebrafish ectodermal explant assay was developed using the organiser-deficient *ichabod* mutant. The aim was to develop a system to analyse how key molecules directly affect ectoderm and neural development, free of mesoderm and endoderm influences, as signalling from these layers can directly or indirectly influence neural development.

Acknowledgements

First of all, I would like to dedicate this thesis to my father, 'Joe' Cruz, who very sadly passed away during the course of my PhD. It is a great shame that he never got to see me get my doctorate. However, he was a great inspiration in my life and for that he will never be forgotten. May he be in a great place.

Secondly, I would like to express my greatest appreciation and gratitude to my supervisor, Dr Tetsuhiro Kudoh, for all the guidance and help that he has provided throughout my PhD. I thank him from the bottom of my heart for the patience he has shown through good times and bad and for always having his door open for me. And thank you for teaching me to focus my mind on what is important.

A huge thank you too for Aya Takesono for being a lovely, calm person who was always there to help and provide guidance even through times of her own personal hardship. Thank you, Aya.

I would also like to thank Anke Lange and Ronnie Van Aerle for all the help with the molecular stuff, as well as everyone who has had to put up with all my endless questions during the past four years. And a great thank you too to Gregory Paull in the fish facilities as well as Jan Shears for all their help and patience.

Last, but most certainly not least, a huge hug and anything else for my lovely wife Geraldine for putting up with me specially during the writing up stages and for always being there for me. Thank you for all the lovely Irish stews.

And very lastly, thank you to everyone in Hatherly for being such great people.

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Research Papers and Author's Declaration.

Statement: I, Carlos Cruz, was involved in the following parts of the presented papers: In conjunction with my supervisor, Dr Tetsuhiro Kudoh, I planned and carried out most of the experiments and subsequent analyses for both paper 1 and paper 2. In addition, and again together with my supervisor, I helped plan and write both papers. For paper 1, we further received valuable input from Dr Igor Dawid and Professor Steve Wilson who co-authored the paper.

A version of the published paper (research chapter 1) is included in the Appendix.

Descriptions

Terms used

Epiboly – cytoskeleton-dependent process characterised by the thinning and spreading of the blastoderm cell layers over the egg yolk and eventually covers it completely.

Gastrulation – where cells movements, characterised by internalisation of cells from the surface of the embryo, lead to a massive reorganisation of the embryo and formation of the three germ layers (ectoderm, mesoderm and endoderm).

Mesendoderm – embryonic tissue that will differentiate into mesoderm and endoderm.

Neural Induction – in the context of this thesis, neural induction refers to the acquisition of neural identity and specification of the neural tissue during gastrulation. It is characterised by the expression of neural-specific genes.

Neural Patterning – refers to the acquisition and maintenance of anterior or posterior identity within the neural plate and how subsequently cells acquire positional information within the emergent central nervous system along the A-P axis.

Neurogenesis – prospective neuronal cells begin to differentiate into neurons. In zebrafish this process begins at the end of gastrulation and continues throughout somitogenesis and beyond and is characterised by neuron-specific gene expression.

Neurulation – infolding of the neural plate in vertebrates leading to formation of the neural tube. Results in the formation of the spinal cord and the brain. In zebrafish, this process begins at the end of gastrulation and is completed by the end of somitogenesis (~24 hpf).

Posteriorisation – specification of posterior neural fate during gastrulation. It is generally assumed that posterior is 'dominant' to anterior and that acquiring anterior character requires keeping anterior neural cells away from posteriorising signals.

Shield – a tissue in teleost fish (such as zebrafish) that is equivalent in function to Spemann's Organiser.

Somitogenesis – although timing may vary in different vertebrates, it refers to the developmental stage at which the somites (muscle precursors) are formed. The

start of somitogenesis coincides with the end of epiboly and gastrulation in zebrafish and is complete by ~24 hpf. It is therefore a useful staging point for characterising zebrafish development.

List of General Abbreviations.

A-P	Antero-Posterior
Bmp	Bone morphogenic protein
DM	Dorsomorphin
D-V	Dorso-Ventral
eve1	*even skipped-like 1* – a zebrafish homologue of the *Drosophila melanogaster even-skipped* gene.
Fgf	Fibroblast growth factor
hpf	hours post fertilisation
Ich	*Ichabod*
RA	Retinoic Acid
Wnt	Wint – name is derived from the *Drosophila melanogaster wingless* gene and the vertebrate INT genes.
WT	Wild Type

Chapter 1. INTRODUCTION

Development among vertebrates proceeds along very similar lines, although the timing of these events, and how they are achieved, may differ in some respects, mainly due to differences in early developmental strategies (Stern et al., 2006). Briefly, in zebrafish, external fertilisation is followed by a cleavage stage, whereby cells divide synchronously and without any increase in cell mass atop an acellular yolk (see Fig. 1 for a more detailed description of early zebrafish development). At this stage, maternal factors control development and there is none or little zygotic gene expression (Kimmel et al., 1995). The end of cleavage marks the midblastula transition (MBT), when the blastula stage begins and the bunched group of cells that sits on top of the yolk is called the blastoderm and the cells blastomeres (Fig. 1). The MBT is also when zygotic gene expression begins. By the late blastula stage epiboly has begun, whereby the blastoderm begins to thin and spread over the yolk, a process that will eventually lead to the blastoderm covering the whole egg. And at the 50% epiboly stage, when the blastoderm covers approximately half the egg (Fig. 1), gastrulation begins during which time the three embryonic germ layers are specified: The ectoderm, mesoderm and endoderm. The signalling activities of, and interactions between, these three cell layers will give rise to the whole vertebrate body plan during embryogenesis. The ectoderm will develop into the complete nervous system, as well as epidermis (skin) and its derivatives. Endoderm will give rise to such diverse tissue as the stomach, liver and pancreas while muscle and heart, among others, will develop from mesodermal cells.

Via mechanisms of involution/ingression, as well as complex convergence and extension movements, mesoderm and endoderm come to underlie the ectoderm, including prospective neural plate. Signals from mesendodermal (mesoderm and endoderm) this will then induce formation of the complete nervous system from the overlying neural plate, which in adult organisms comprises, in its simplest form, of brain, spinal cord and associated neuronal networks. For specification of the nervous system, cells in the ectoderm must acquire neural identity and this is achieved by molecular mechanisms that eventually lead to the dorso-ventral (D/V) patterning of the ectoderm

Figure 1. Important Stages of Early Zebrafish Development

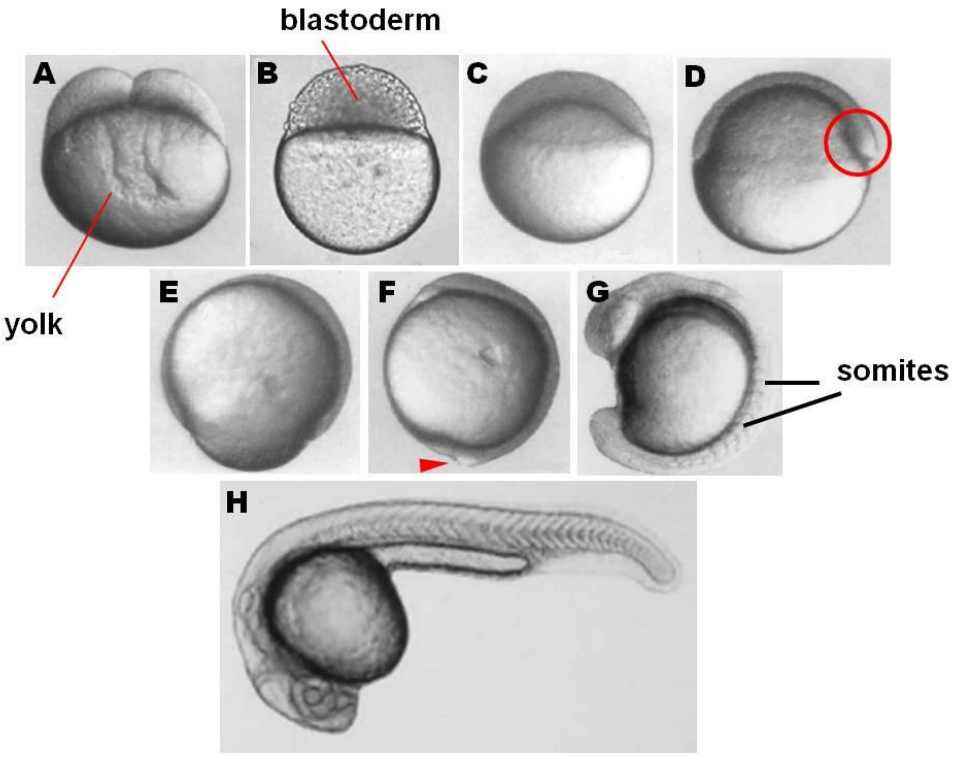

Fig. 1. (A) Two cell stage, cleavage. Egg has been fertilised, cells divide synchronously atop an acellular yolk and with very short or no gap phases cells don't increase in volume. Development under the control of maternally deposited determinants, no zygotic gene expression. **(B) 1024 cell stage, blastula.** Zygotic gene expression begins at the MBT, the 10th cell cycle which occurs at the 512-1024 cell stage. Yolk Syncytial Layer (YSL) forms at the boundary between the blastoderm and yolk cell (margin) as cells at the edge collapse and lose their membranes releasing their nuclei. YSL is an important signalling centre during early development. **(C) 30% epiboly stage.** Epiboly is under way, expression of genes with roles in mesendoderm and posterior neural development have begun to be expressed along the margin. **(D) Shield stage (~50% epiboly).** Morphological organiser is evident at the dorsal margin (red circle), germ ring forms (a thickening of cells at the margin) and gastrulation begins. **(E) Mid-gastrula stage.** Blastoderm covers about three quarters of the egg, neural plate has been specified, local organisers of neural regional patterning are becoming established (see text). **(F) Tail Bud (red arrowhead) stage.** Epiboly is complete, somitogenesis and elongation of the posterior body (somites are muscle precursors) is beginning. Neurulation (neural tube formation) begins, first neurons differentiate. **(G) 15 somite stage (~mid-somitogenesis).** Eyes have begun to form, CNS divisions begin to appear, otic vesicle (future ear) is evident, posterior body, including spinal cord, continue to elongate. **(H) 24 hpf.** Divisions of the

CNS apparent, somitogenesis ends, spinal cord is specified, neural tube has closed and is formed. Neuronal differentiation is well under way. (With the exception of B and H, all pictures were adapted from Kimmel et al., 1995).

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into neural and non-neural domains during gastrulation (Fig. 1 and 2). It now seems that his process begins well before gastrulation, and, in addition, is also known to require vertical signals that emanate from the other germ layers and likely planar signals from within the ectoderm itself. In zebrafish, specification of the neural plate becomes apparent soon after the onset of gastrulation and can be visualised by analyses of neural-specific gene expression (Fig. 2). This shows that, by midgastrula stage, the neural plate is specified in a broadly dorsal domain (but also ventro-vegetally). But in addition, it also shows that, by this stage, the neural plate is already subdivided into broad anterior (head) and posterior (trunk and tail) domains (Fig. 2) implying that acquisition of neural fate and initial patterning of the central nervous system (CNS) along its antero-posterior (A-P; head to tail) patterning are tightly linked. However, to what extent this is so is still hotly debated (see later). As gastrulation proceeds, the CNS is further regionalised along its A-P axis: the head is subdivided into forebrain, midbrain and anterior hindbrain territories while posterior hindbrain and spinal cord will form from the posterior neural plate (Fig. 2). And by the end of gastrulation, several 'organising' centres have been established along the A-P axis that will establish and pattern these subdivisions of the CNS and further locally pattern the subdivided ectoderm into their constituent parts. Also at the end of gastrulation, signals from underlying mesendoderm induce infolding of the neural plate and formation of the neural tube, or neurulation, so that by the end of somitogenesis (Fig. 1) the neurulation is complete and the neural tube then comprises the primordia of most of the presumptive neural regions that will eventually give rise to the mature CNS (Kimmel et al., 1995).

Although in recent years many advances have been made in our understanding of how the vertebrate nervous system develops, much still remains to be learnt. Importantly, even after decades of research, the

Figure 2. Regionalisation of the Early CNS in Zebrafish

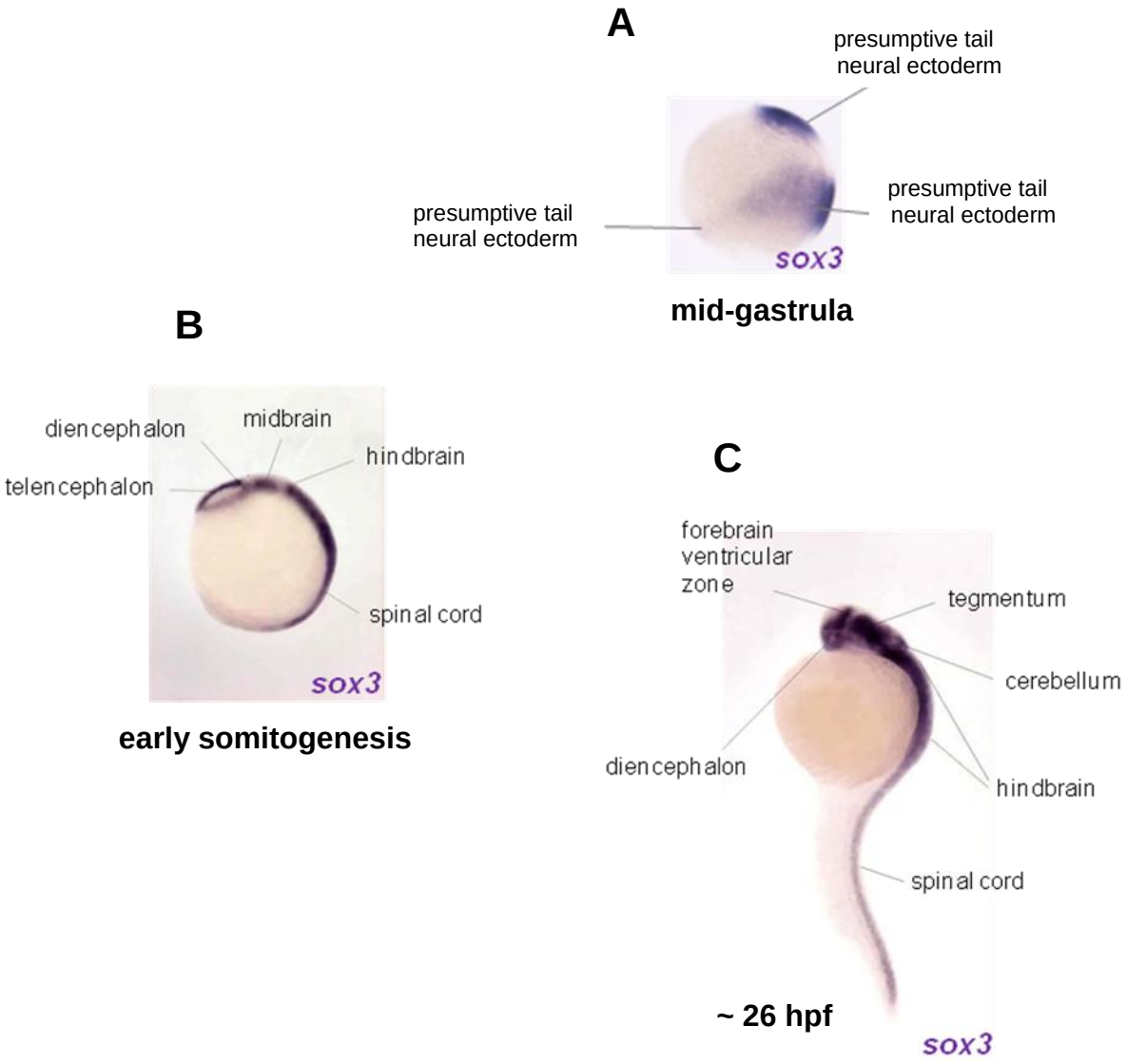

Fig. 2. *In situ* hybridisation staining of the neural marker gene, *sox3*. In all cases, anterior is to the top, dorsal to the right. (A) At the mid-gastrula stage (75% epiboly) there are only broad divisions of the neural ectoderm: anterior (head) and posterior (trunk/tail) (please note: although anterior neural plate is a continuous tissue without obvious divisions, *sox3* at this stage does not mark the future midbrain). (B) At early somitogenesis, when neurulation is well under way, local divisions within the CNS are apparent: the forebrain, consisting of the telencephalon and diencephalon, as well as the midbrain, hindbrain and spinal cord. (C) At 26 hours post fertilisation (hpf), after the neural tube is formed and neurogenesis is well under way, further regionalisation of the CNS is apparent. *Sox3* staining reveals the presence of further subdivisions in the midbrain, such as

the tegmentum, as well as the cerebellum of the hindbrain. (B,C) Adapted from Thisse et al., 2001 and are deposited on the zebrafish information network (ZFIN).

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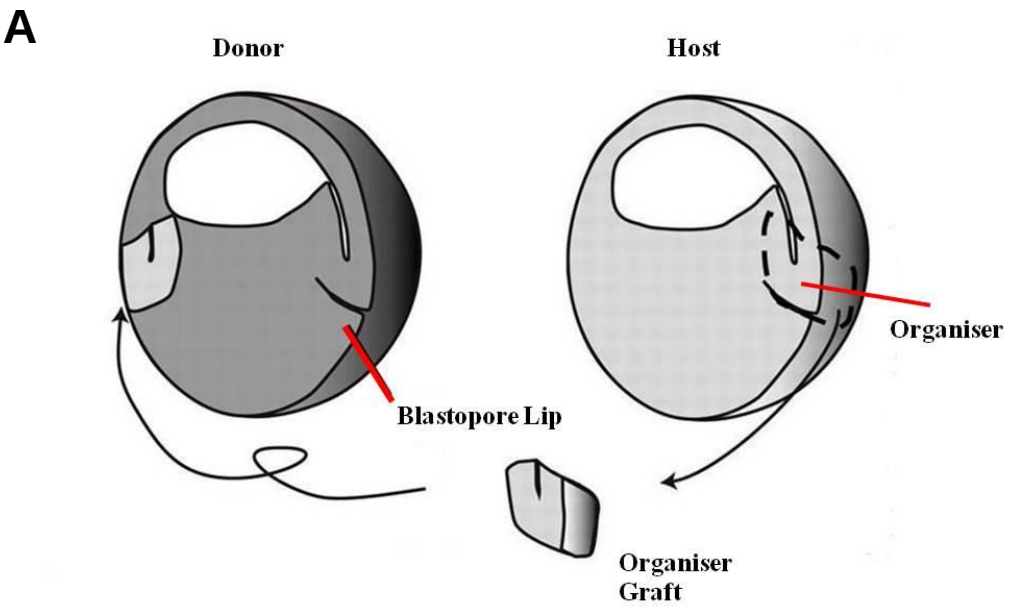
question of which molecular mechanisms are responsible for the initial acquisition of neural cell fate remains unanswered. In addition, many of the molecules involved in early neural development remain to be characterised. Using zebrafish, the objective of this thesis was to contribute to our understanding of the initial stages of neural development in vertebrates: Acquisition of neural fate and neural plate specification (neural induction) and early A-P patterning of the prospective nervous system.

1.1 Neural Induction

Induction is defined as an instructive interaction emanating from one group of cells that causes another group of cells to adopt a fate different to what it would normally do, and pioneering work by Spemann and Mangold in newts in the 1920s first suggested an instructive nature for neural specification (Harland, 2008). They transplanted cells from the dorsal region (notochord, derived from mesoderm) of one species of newt gastrula stage embryo to the ventral side (prospective epidermis) of another species of newt at a similar stage of development and found that the donor cells induced an almost complete second nervous system which, importantly, consisted mainly of cells recruited from the host embryo (Fig. 3). This dorsal region with neural inducing capacity subsequently became known as Spemann's organiser and in the following years equivalent regions were discovered in a variety of other vertebrates, such as Hensen's node in birds and mammals and the Shield in teleost fish (Stern, 2005 and references therein). Since the organiser was shown to be capable of inducing a neural plate across different classes, it suggested that the molecular mechanisms involved in neural induction were conserved in the vertebrates: Neural inducing signals from a dorsally positioned organiser instructed adjacent

Figure 3. Induction of Secondary Axis by a Grafted Organiser



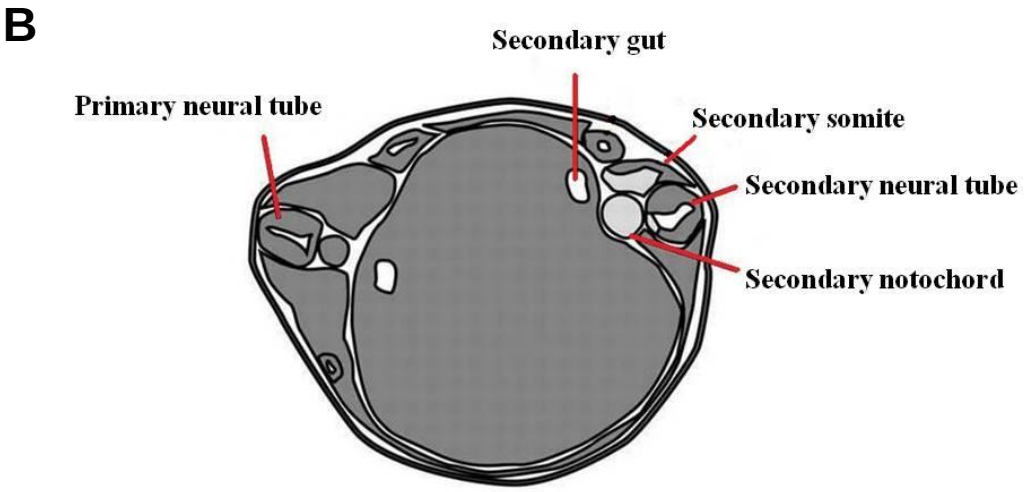


Fig. 3 Spemann and Mangold's discovery of the Organiser. At the gastrula stage, they removed a piece of tissue from the blastopore lip (where gastrulation begins in amphibians – see Fig. 2) of a donor newt embryo and transplanted it to the ventral side (prospective belly) of a another newt embryo (host), which was differently pigmented for easy identification of host and donor cells (A). They found that the graft of donor tissue induced a complete secondary axis (B,C), and importantly much of the secondary axis, including most of the neural tube, consisted mostly of cells derived from the host embryo (B). The region from the dorsal blastopore lip from which the graft came (A) was later called Spemann's Organiser. This experiment as also established the concept of the instructive nature of neural induction (Stern, 2005). (C) A twinned frog embryo from a similar experiment to that performed by Spemann and Mangold. (Adapted from Harland, 2008).

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ectoderm to adopt a neural fate, while more ventrally placed ectodermal cells adopted an epidermal fate in the absence of neural inducing signals. The instructive nature of the organiser identified in Spemann's experiments, meanwhile, also led to the idea that epidermal was the default state of naïve ectoderm, as it was assumed that cells that did not receive instructive signals from the organiser automatically adopted an epidermal fate (Hemmati-Brivanlou and Melton, 1997; Weinstein and Hemmati-Brivanlou, 1999). Since organiser grafts from Spemann's experiments could induce a complete secondary axis, and based mainly on experiments by Mangold which showed that progressively older organiser grafts induced progressively more posterior (trunk and tail) regions, it was proposed that multiple organising centres were responsible for inducing different regions of the nervous system in a temporal-dependent manner (see Foley et al., 2000; Stern, 2001; Stern, 2005 and references therein). Later, however, Nieuwkoop proposed the 2-step (activation/transformation) model for the induction of neural tissue. He had found that artificial neural inducers (activators) induced neural tissue with only forebrain character, which could nevertheless be caudalised by addition of identified transforming factors that induced neural tissue of posterior character. This issue remains unresolved to this day (discussed later), but since then the molecular mechanisms involved in neural development have been extensively studied in a variety of model vertebrate organisms, including the frog, chick, mouse and zebrafish, and many of the signalling pathways involved have been shown to be conserved. This also appears to be true of basal chordates as has been shown for amphioxus (Bertrand et al.,

2011; Yu et al., 2007), suggesting a much wider conservation of mechanisms of neural development.

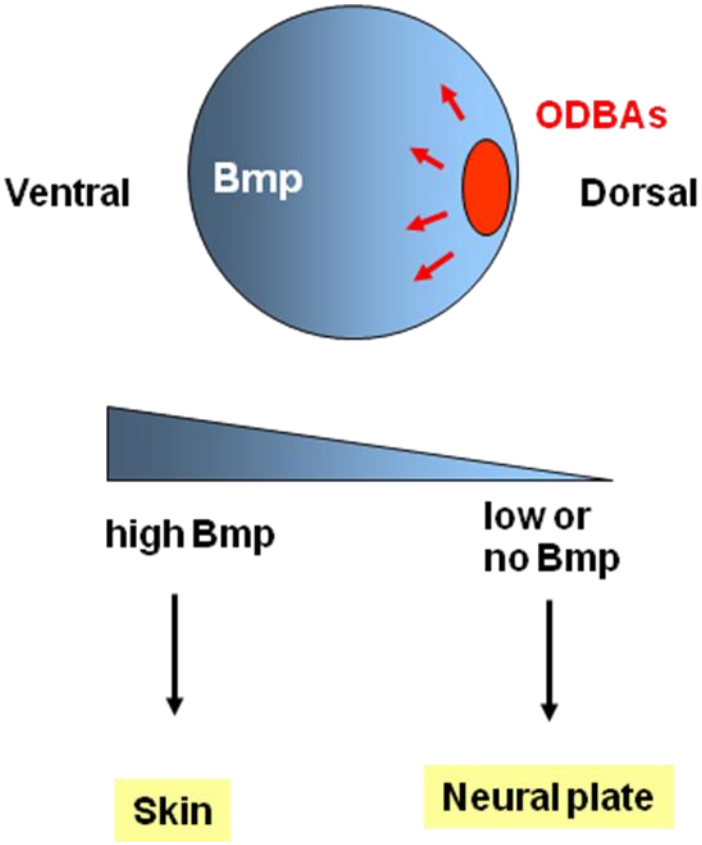
However, apparent differences have led to differing models being proposed for the initial steps in neural fate determination, i.e., how ectodermal cells first acquire neural identity. Since the early 1990s, *Xenopus* has taken centre stage in the study of the molecular events underlying neural induction in vertebrates, particularly due to the use of ectodermal explants in embryological experiments. These are groups of cells excised from the animal pole of blastula/early gastrula embryos (animal caps) which theoretically should consist purely of ectoderm as excision occurs before specification of mesendodermal tissue. This was critical, as mesendoderm, from which organiser tissue is derived, could induce neural tissue. These explants, as well as *Xenopus* embryos, have been instrumental in our current understanding of neural development and the concept of the 'Default Model' of neural induction arose from experiments on the frog.

1.1.1 The 'Default Model' of neural induction.

In the late 1980s and early 1990s data from experiments involving both *Xenopus* embryos and ectodermal explants began to emerge which challenged the idea that epidermis was the default state of the ectoderm. These experiments showed that although explants developed as epidermal when cultured alone, as had previously been shown by Spemann (Hemmati-Brivanlou and Melton, 1997a), dissociation and subsequent reaggregation of animal cap cells at gastrula stages neuralised the explants (Godsave and Slack, 1989; Grunz and Tacke, 1989), and the same result was obtained with dissociation of whole *Xenopus* embryos at early stages of development (Sato and Sargent, 1989). It was also found that misexpression of a dominant-negative type II receptor for the activin ligand, a member of the TGF-β superfamily, also had not only a neuralising effect on ectodermal cells that would normally adopt an epidermal fate (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou and Melton, 1994), but also suppressed mesoderm formation. These results challenged the idea that an instructive signal from the organiser was responsible for the induction of neural tissue, since no mesoderm is formed either in the explants or in embryos expressing the truncated Activin receptor. As a result, the observed neuralisation was likely to be direct and involve cell-cell signalling. Together, these observations suggested that inhibition of diffusible epidermis-inducing factor(s), likely a TGF-β related ligand, caused

neuralisation and led to the proposal of the 'Default Model' of neural induction as an explanation for the neuralisation of dissociated ectodermal cells (Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou and Melton, 1997b) (Fig. 4). In its simplest form, it implies that suppression of an epidermis-inducing signal is a necessary and sufficient condition for neural induction to occur and thus that neural, and not epidermis, may be the default state of the ectoderm: Since ectodermal explants normally develop as epidermis, cell signalling within the ectoderm should normally inhibit neural induction. Removal of the neural inhibitor, and in the absence of any further signalling, leads ectodermal cells to acquire a neural fate, their 'default' state. In the case of amphibian embryonic development, this meant that antagonists of a neural suppressant, derived from a dorsal organiser, would induce neural tissue dorsally, whilst ventrally expression of the same epidermal-inducing factor(s) would specify epidermis in the absence of antagonistic factors. Subsequently it was found that Bone Morphogenic Proteins (Bmps), and not Activin, restored epidermal fate when added to dissociated animal cap cells (Suzuki et al., 1997a; Wilson and Hemmati-Brivanlou, 1995) (See Box1). Similarly, epidermis was induced and neural fate suppressed in dissociated cells excised from embryos overexpressing many downstream components of the Bmp signalling pathway, including a constitutively-activated form of the Bmp Type I receptor, Alk2 (Suzuki et al., 1997b), as well as nuclear effectors of Bmp signalling, Smad1 (Wilson et al., 1997) and Smad5 (Suzuki et al., 1997a). This conforms to one of the predictions of the default model, that since removal of a neural-inhibiting/epidermis promoting factor is predicted to neuralise dissociated ectodermal cells, addition of said epidermal inducer should inhibit neural induction and promote epidermal fates. That Bmps may be the postulated neural suppressors was supported by experiments that suppressed Bmp signalling in intact *Xenopus* ectodermal explants. Inhibition of members of the Bmp signalling pathway neuralised explants that would otherwise exhibit epidermal character;

Figure 4. The 'Default Model' of Neural Induction.



ODBAs – Organiser Derived Bmp Antagonists

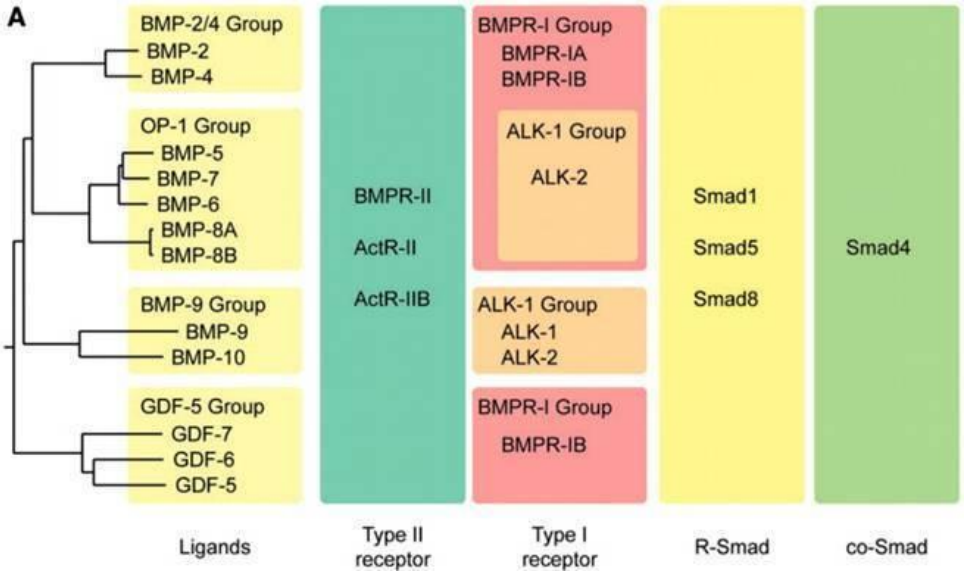
Fig. 4 The 'Default Model' of neural induction proposed that as the organiser formed (red circle), it expressed antagonists of epidermis inducers, the Bmps. Secreted molecules, such as Chordin and Noggin, diffused ventrally (red arrows) and antagonised Bmp activity on the dorsal side. This created a ventral to dorsal Bmp expression gradient and it was proposed that this was necessary and sufficient for induction of the CNS. Where Bmp signalling was high on the ventral side, epidermis (skin) would be induced. Neural plate would be induced on the dorsal side in areas of low or no Bmp activity. (Adapted from a Figure kindly supplied by Dr Tetsuhiro Kudoh).

Box 1

Bone Morphogenic Proteins

Bmps, along with Growth Differentiation Factors (Gdfs) constitute the Bmp subfamily belonging to the TGF-β superfamily of secreted growth factors. To date, more than 20 members of the Bmp subfamily have been identified (Bragdon et al., 2011) and are further divided into subgroups, the best characterized of which are shown in Figure 1. As homo or heterodimers, Bmp ligands assemble heterodimeric complexes of type I and type II transmembrane serine-threonine kinase receptors at the cell surface and, upon complex formation, type I receptors are phosphorylated and activated by constitutively active type II receptors. Although the type II receptors are ubiquitous for all the Bmps, type I receptor is ligand/subgroup dependent (Fig. 1). Activated type I receptors then phosphorylate and activate the principle, but not exclusive, effectors of Bmp (and all Tgf-β) signaling, the Smads. Receptor-activated Smads (R-Smads) 1, 5 and 8 are exclusive effectors of Bmp subfamily signaling and require Smad4 (co-Smad) for translocation to the nucleus (Fig. 1), as do all Smads. In the nucleus, Smad activity is known to affect a wide variety of developmental and cellular processes, such as differentiation,

Fig. 1 Bmp subgroups and members of the Bmp signal transduction pathway.



(Adapted from Miyazono et al., 2009)

organogenesis, apoptosis, cell signaling, cell fate specification and cell proliferation (Massagué, 1998; Shi and Massagué, 2003). Responses to Bmp signaling are regulated by a variety of means (Bragdon et al., 2011; Massagué, 1998), including ligand-receptor specificity, intracellular regulation of Smad activity, tissue/temporal specificity of ligand as well as downstream molecules and extracellular regulation which involves inhibition of ligand-receptor binding by molecules such as Chordin and Noggin (for a comprehensive list, see Bragdon et al., 2011).

Although many Bmp ligands have been identified, only the Bmp-2/4 group and Bmp7 from the OP-1 group have been shown to be expressed at the right time and place and to be involved in neural induction in organisms where they have been found. In addition, in zebrafish, maternal Gdf6/Radar has been shown to function upstream of Bmp2b and Bmp4 in ectodermal patterning (Sidi et al., 2006).

uncleavable forms of Bmp ligands, which fail to form active dimeric complexes, induced neuralisation and suppressed epidermal induction (Hawley et al., 1995); and a dominant-negative form of a Bmp receptor (Sasai et al., 1995; Xu et al., 1995) and expression of Bmp-4 antisense RNA (Sasai et al., 1995) had a similar effect, as did manipulation of other downstream components of the Bmp signalling pathway (see Weinstein and Hemmati-Brivanlou, 1999). Furthermore, although Bmps were found to be ubiquitously expressed initially in the entire ectoderm, it was observed that expression was suppressed dorsally as the organiser formed (Fainsod et al., 1994; Hawley et al., 1995). Since organiser grafts could induce a complete nervous system when transplanted ventrally, presumably by suppressing a suppressor of neural induction, another prediction of the default model was the presence of inhibitor(s) of epidermal inducer(s) in the dorsal organiser region that could directly induce neural tissue in ectoderm. A number of factors were indeed discovered which were expressed in the organiser and could induce neural tissue in ectodermal explants without inducing mesoderm, such as Noggin (Lamb et al., 1993) and Chordin (Sasai et al., 1995), which were subsequently shown to be selective antagonists of Bmp ligand-Bmp receptor interactions (Piccolo et al., 1996; Zimmerman et al., 1996), showing that suppression of BMP signalling is a necessary step in neural induction.

Numerous experiments have since confirmed that epidermis-inducing and neural suppressing effects of Bmp overexpression in the ectoderm in other

vertebrates (Aberdam, 2004; Finley et al., 1999; Hild et al., 1999; Kishimoto et al., 1997; Ying et al., 2003). Furthermore, with the possible exception of the chick, it has been shown that downregulation of antagonists of the Bmp pathway leads to suppression of neural tissue formation. For example, in mice with double knockout of the *noggin* and *chordin* genes, anterior nervous system structures do not form (Bachiller et al., 2000). Likewise, in *Xenopus tropicalis*, simultaneous suppression by morpholino knockdown of three Bmp antagonists, Follistatin, Chordin and Noggin (FCN morphants) leads to an almost complete loss of neural plate specification with concomitant dorsal expansion of epidermis (Khokha et al., 2005), and use of a Chordin morpholino completely blocks the ability of an organiser graft to induce neural tissue (Oelgeschläger et al., 2003). In zebrafish, meanwhile, triple knockdown by morpholinos of chordin, noggin and a follistatin-related gene, *fstl2*, led to a severely ventralised phenotype (Dal-Pra et al., 2006). These data all indicate that the Bmp signalling pathway must be suppressed for normal neural development in vertebrates.

However, at about the time that the Default Model was gaining acceptance, evidence was emerging that suggested that although Bmp inhibition was necessary for proper neural development, it may not be sufficient or indeed necessary for neural induction. In *Xenopus*, It had been observed that although inhibiting Bmp activity could induce neural tissue in ectodermal explants, it could not do so in the ventral side of the embryo, which is normally fated to give rise to epidermis (Hawley et al., 1995; Wilson and Hemmati-Brivanlou, 1995), although epidermal induction was suppressed. Similarly, in the chick, ectopic Chordin expression could not induce neural genes in competent ectodermal tissue (Streit et al., 1998) and furthermore, addition of Bmp protein was found not to inhibit formation of the neural plate. Instead, the authors suggested that the role of Chordin, and presumably other Bmp antagonists, was to stabilise a neural state rather than being required for neural induction. In support of this idea, downregulation of Bmp signalling in the developing chick neural plate was shown to occur only after the initiation of neural-specific neural gene expression (Streit et al., 1998; Streit and Stern, 1999b), although this is the opposite of what is observed in amphibians where restriction of Smad activity to the ventral side of *Xenopus* embryos is observed by the late blastula (pre gastrula) stage (for example, see Kurata et al., 2000). Indeed, other data from the chick has suggested that Bmp signalling is downregulated before neural plate formation (Wilson et al.,

2000). Further challenging the Default Model is evidence that suggests that organiser activity is not required for neural induction. For example, mouse HNF3β mutants which do not form a node (organiser) or its derivatives still develop a neural plate (Ang and Rossant, 1994; Dufort et al., 1998; Weinstein et al., 1994), while there is also evidence to suggest . that neural induction may be initiated prior to gastrulation and before organiser formation (Delaune et al., 2004; Kuroda et al., 2004; Linker and Stern, 2004; Streit et al., 2000; Wilson et al., 2000). These observation appear to contradict one premise of the Default Model, which proposes that organiser-derived Bmp antagonists are the signals that are required for neural induction. However, again with the possible exception of the chick, antagonism of Bmp signalling has remained a central theme in the explanation of the process of neural induction, even though it is generally accepted that neural induction begins before the appearance of the organiser, at least for the anterior central nervous system. In *Xenopus*, expression of *chordin* and *noggin* is first transiently activated, downstream of maternal *β-catenin* signalling, just after the MBT in dorsal-anterior cells, in what has been called the Blastula Chordin and Noggin-Expressing centre (BCNE) (Kuroda et al., 2004; Wessely et al., 2001). Cells of the BCNE will eventually contribute to the anterior CNS (forebrain, midbrain and most of the hindbrain), organiser and notochord, and when cells of the BCNE are removed the brain does not form (Kuroda et al., 2004). Furthermore, although explants of BCNE cells cultured in saline were shown to auto-differentiate into neural tissue, they differentiated into epidermis instead when *chordin* expression was inhibited by injection of a morpholino (Kuroda et al., 2004). Likewise, in zebrafish, factors that suppress Bmp activity such as *bozozok/dharma*, which has been shown to represses *bmp2b* transcription (Leung et al., 2003), are induced downstream of maternal *β-catenin* signalling in the blastula. Furthermore, mitogen-activated protein kinase (MAPK) is detected in *Xenopus* blastula embryos at the onset of the MBT and Mapk activity was shown to inhibit Smad activation via the phosphorylation of the C-terminus region of Smad1, while in contrast, the Bmp receptor phosphorylates a linker region, which is associated with Smad activation (Pera et al., 2003) . These early Bmp inhibitory mechanisms in the blastula may explain why neural plate still forms after removal of organiser tissue or suppression of organiser formation and further suggests that the Default Model may indeed provide a viable molecular model for neural induction in vertebrates, although the initial steps in neural induction may

not reside in the organiser. It seems probable, nonetheless, that organiser activity is still critical for proper neural development as many factors present in the organiser can still have critical effects on neural specification (see De Robertis and Kuroda, 2004).

These and other data that show that neural induction may begin in the early blastula also imply that some D-V patterning already exists at this stage of development, and calls into question the viability of using ectodermal explants as a model to study neural development, since animal cap cells may already be predisposed to neural inducing signals. Some evidence exists that a Bmp gradient (and hence competence for both neural and non-neural fates) exists in both *Xenopus* prospective epidermal cells and chick explants, which were shown to express neural crest, but not neural, markers in response to Bmp inhibition (Linker et al., 2009). It has also been suggested that only cells close to the neural/non-neural border can be induced to a neural fate by inhibition of the Bmp signal in both *Xenopus* (Linker et al., 2009) and chick explants (Linker et al., 2009; Linker and Stern, 2004; Streit et al., 1998; Streit and Stern, 1999b) and these data would again argue against the Default Model. However, a more recent paper has reported contradictory results in *Xenopus* (Wills et al., 2010) which implies that the results are due to 1) different experimental approaches, 2) there may be species-specific differences or 3) the *Xenopus* explants really are not suitable for research into neural induction, as they may not reflect the reality *in vivo*.

Whether the Default Model is a sufficient mechanism to explain neural induction or not, neural induction is certainly a more complicated process than was originally proposed. In addition to Bmp antagonism, a number of other signalling pathways have been shown to have roles in the process of neural induction, including Wnt, Nodal and most critically, Fgf signalling (see De Robertis and Kuroda, 2004; Stern, 2005). Although the roles of Wnts and Nodal signalling in neural induction remain unconfirmed, the finding that Fgf signalling may play a critical, instructive role in neural induction has provided the main challenge to the premise that antagonism of Bmp signalling is sufficient for neural induction to occur.

1.1.2 Fgf signalling in neural induction.

Fibroblast Growth Factor ligands are secreted, diffusible molecules that signal through Fgf-specific cell surface receptors Fgf-Rs, and impact on a number of intracellular signalling cascades such as the ERK/MAPK and the PLC/Ca^{2+} pathways. These mechanisms are conserved in vertebrates, as are their multiple functions in embryonic development which include vital roles in mesoderm, endoderm and neural development (Böttcher and Niehrs, 2005). Whether Fgfs are sufficient for neural induction remains an unresolved issue, possibly due to species-specific differences and/or differences in experimental approaches. The Default Model continues to have its proponents, mainly in *Xenopus* research (for example, see Marchal et al., 2009; Wills et al., 2010), but research in chick and zebrafish has tended towards greater support for the notion that suppression of Bmp signalling is not a sufficient mechanism for neural plate specification, at least not in its entirety. With the mouse the evidence for either model is more complicated. Although the function of Bmps as epidermal inducers as well as the function in anterior neural development of their node-derived (node is the mouse organiser) antagonists appear to be conserved, there is as yet not enough experimental data to make a judgement. As an example, it had been reported that Bmps can inhibit neural differentiation (Ying et al., 2003), as can Wnt signalling (Aubert et al., 2002). However, addition of Noggin to culture media could not rescue neural induction in the presence of an Fgf-R inhibitor, SU5402 (Ying et al., 2003), and this was interpreted as a requirement for autocrine Fgf signalling in neural induction, independently of Bmp signalling, so arguing against the Default Model. However, Fgf/Erk signalling is required for mESCs to exit the self-renewal cycle, and all lineage differentiation, including epidermal, is blocked in cells treated with Fgf inhibitors (Kunath et al., 2007). This makes it difficult to distinguish between the possible roles of Fgf signalling in neural induction and stem cell self-renewal and this subject is very controversial in the mouse. As such, and as most of the available evidence for a requirement for Fgf signalling in neural induction has come from frog, chick and fish, this introduction will concentrate mainly on data gathered from these model organisms.

In *Xenopus*, it had been observed that Fgf could induce neural tissue when overexpressed in animal cap cells (Cox and Hemmati-Brivanlou, 1995; Holowacs and Sokol, 1999; Hongo et al., 1999; Kengaku and Okamoto, 1995; Lamb and Harland, 1995). But as only posterior neural genes were induced in many cases, it remained elusive as to whether Fgf signalling had a role in neural induction and

often this was attributed to the role of Fgfs in A-P neural patterning rather than as neural inducers (Gamse and Sive, 2001). But in addition, some experiments showed that expression of a dominant-negative Fgfr1 (XFD), which lacked the intracellular domain, could block neural induction in both Xenopus embryos and ectodermal explants by Noggin and organiser tissue (Launay et al., 1996), as well as by Chordin (Sasai et al., 1996). Although contradictory results were also obtained with XFD expression, it was later found that loss of neural induction was more sensitive to another Fgf receptor, Fgfr4 (Hardcastle et al., 2000; Hongo et al., 1999; Umbhauer et al., 2000) and even more importantly, it suggested that intact Fgf signalling was required for induction of tissue of an anterior character (Hongo et al., 1999). Further evidence for a requirement for Fgf signalling in both anterior and posterior neural induction in *Xenopus* has come from experiments showing that exposure of *Xenopus* embryos to the Fgfr inhibitor, SU5402, led to the suppression of all neural tissue in a concentration-dependent manner (Delaune et al., 2004). Also importantly, Delaune et al showed that suppression of Fgf signalling from the midblastula transition, when zygotic genes expression commences, leads to the severest phenotypes observed with exposure to SU5402. However, the phenotypes were less severe the longer from the MBT the exposures were started. These data also implied that organiser-derived Bmp antagonism was not sufficient for neural induction, since it suggested that neural induction was initiated prior to gastrulation and presumably organiser formation, a possibility that had already been proposed.

It is in the chick that the Default Model gained least acceptance, due to the inability of Bmp antagonists to induce neural tissue under most experimental conditions, the inability of Bmp overexpression to suppress neural tissue formation and a temporal regulation of Bmp signalling that may not conform to the Default Model (Stern, 2002; Stern, 2005). Fgf signalling has, however, been shown to be important for chick neural induction. Ectopic Fgf can induce neural tissue in chick embryos (Alvarez et al., 1998; Storey et al., 1998; Streit et al., 1998; Streit et al., 2000; Wilson et al., 2000) and in addition, a screen for early-response genes in neural induction identified a number of genes, among them *erni* and *churchill*, which were subsequently shown to be induced by Fgf but not by inhibition of Bmp (Sheng et al., 2003; Streit et al., 2000). Furthermore, although grafted organiser tissue could induce neural markers on its own, this induction was inhibited by suppression of the Fgf pathway both by overexpression of a

dominant–negative Fgf receptor or by exposure to SU5402 (Streit et al., 2000). Similarly, exposure of chick neural plate explants to SU5402 inhibited the induction of neural genes, and this inhibition could not be lifted by overexpression of Noggin (Wilson et al., 2000). However, it is by no means clear if there is a direct requirement for Fgf signalling in chick neural induction. In the chick neural plate explants, exposure to SU5402, apart from blocking neural induction, also led to the maintenance of Bmp expression which is normally downregulated in developing neural plate tissue (Wilson et al., 2000). And in tissue that is competent to become neural, neither Bmp inhibition alone, nor Bmp inhibition in conjunction with overexpression of several Fgf ligands was shown to be sufficient to induce the definitive neural marker, *sox2*, implying that signals other than Fgfs were required for neural induction (Linker and Stern, 2004). However, it is worth noting that this differs from *Xenopus* where injection of Fgf4, together with the Bmp signalling inhibitor Smad 6, into blastomeres normally fated to become epidermis, could induce neural markers (Delaune et al., 2004).

To try to reconcile the different results that implicate Bmp antagonism or Fgf signalling in neural induction, it has been suggested that Fgf signalling may neuralise via the suppression of Bmp activity, and thus the neural inducing properties of Fgf may be indirect. As previously mentioned, Mapk phosphorylates Smad1 thus inactivating it and, in addition, overactivation of Fgf signalling was shown to suppress transcription of Bmp ligands (Londin et al., 2005; Wilson et al., 2000). But although this may account for certain aspects of the neural inducing properties of Fgf, it is not a sufficient mechanism to explain all the observations. In the ascidian *Ciona intestinalis*, although not a vertebrate, neural induction is initiated via Fgf signalling and not Bmp inhibition (Bertrand et al., 2003; Hudson et al., 2003; Hudson et al., 2001). In *Xenopus*, injection of mRNA coding for the frog homologue of Fgf4, eFgf, was able to induce the definitive neural marker, *sox2,* in prospective epidermal cells. *Sox2,* however, could not be induced by suppression of Bmp signalling alone, either by use of a dominant-negative Bmp receptor or overexpression of the inhibitory Smad6, which blocks signalling downstream of the Bmp receptor (Delaune et al., 2004). Furthermore, inhibition of Bmp was unable to restore expression of neural genes in embryos and explants exposed to SU5402, a result that was recapitulated with use of the dominant-negative Fgf receptor, XFD (Launay et al., 1996; Sasai et al., 1996). And in chick explants, neither ectopic Noggin nor Chordin could induce a neural

fate in the presence of SU5402 (Streit et al., 2000; Wilson et al., 2000). These data provide evidence for the neural inductive properties of Fgf signalling independently of Bmp antagonism, and that it is necessary for neural induction. However, whether Fgf signalling alone is a sufficient inducer of both anterior and posterior neural tissue *in vivo* is a question that remains unanswered. In a further twist to the story, Marchal and others (Marchal et al., 2009) recently concluded that neural induction was initiated by Fgf signalling in response to Bmp inhibition, as Fgf4 expression, along with neural markers, was induced in prospective epidermis by injection of a dominant-negative form of Smad5 (Smad5-sbn) and activation of neural gene expression by Smad5-sbn was shown to be dependent on Fgf4 activity. In addition, in the basal chordate amphioxus, neural fate is suppressed by overactivation of the Bmp pathway (Onai et al., 2010; Yu et al., 2007) but not by suppression of Fgf signalling with exposure to SU5402 (Bertrand et al., 2011).

1.1.3 Neural Induction and Zebrafish.

The findings in the chick embryo imply that Bmp inhibition is neither necessary nor sufficient for neural induction, and that although Fgf signalling plays an important early role in this process, it is not a sufficient neural inducer. Instead, it is postulated that this function may be under the control of other important, and as yet unidentified, signals. This contrasts with *Xenopus* and mouse, where Bmp antagonism appears to be at least necessary, if not proven to be sufficient, for neural induction. On the other hand, there is also increasing evidence for a requirement for early (blastula stage) Fgf signalling in neural induction in the frog, although like in the chick, it has not been established whether Fgf signalling is a sufficient neural inducer.

In zebrafish, the mechanisms of neural induction have been analysed and the signalling pathways that have been uncovered, both intra and extracellular, have been shown to be conserved with other model organismsOverexpression of Bmp ligands and downstream components suppress neural induction and ventralise embryos, with concomitant expansion dorso-anteriorly of epidermis (Dee et al., 2007; Kudoh et al., 2004; Rentzsch et al., 2004), and inhibition of components of the Bmp signalling pathway have the opposite result (Dee et al., 2007; Dick et al., 1999; Hild et al., 1999; Kramer et al., 2002). Further, and although temporal and spatial expression patterns may vary, most of the Bmp antagonists identified in

other vertebrates are also expressed in zebrafish, where they perform similar functions: They expand neural tissue at the expense of epidermis when overexpressed and show loss of neural tissue, with expansion of epidermis, in loss-of-function experiments (Dee et al., 2007; Gonzalez et al., 2000; Kudoh et al., 2004; Londin et al., 2005; Rentzsch et al., 2004; Schulte-Merker et al., 1997). So it appears that in zebrafish, as is also the case in the frog and mouse, but unlike in the chick, suppression of the Bmp pathway is necessary for neural induction. However, research using zebrafish has shown that, even in the severest cases of overactivation of the Bmp pathway, posterior neural plate specification is not abolished, but rather preferentially suppresses anterior neural markers at the late gastrula stage (Dee et al., 2007; Fürthauer et al., 2004; Kudoh et al., 2004; Rentzsch et al., 2004). These data imply that suppression of Bmp is necessary for induction of anterior, but not posterior, neural tissue in zebrafish (Table 1).

Table 1. Organism dependence on Bmp and/or Fgf signalling in neural induction.

Organism	**Required for neural induction**			
	Bmp antagonism		Fgf signalling	
	Anterior neural	Posterior neural	Anterior neural	Posterior neural
Ciona intestinalis	No	No	Yes	Yes
Amphioxus	Yes	Yes	No	No
Fish	Yes	No	No	Yes
Frog	Yes	Yes	Yes	Yes
Chick	No	No	Yes	Yes
Mouse	Yes	?	?	?

Table 1. Differences in the requirement for Bmp antagonism and Fgf signalling, or both, in neural induction. In the ancestral organism, *Ciona intestinalis*, Bmp antagonism does not induce neural tissue, which is achieved by Fgf signalling. This is the opposite to what is observed in *Amphioxus*, next up in the evolutionary line. Differences between fish and frog centre on posterior versus anterior neural

development, whereas in the chick, as with *Ciona*, Fgf signalling rather than Bmp antagonism appears to be the main requirement for neural induction. However, other mechanisms are thought to operate in addition to Fgf signalling. Lack of data in the mouse model stems from the lethality of loss of function of embryonic Bmp signalling. With the remainder, it is not clear if these are real differences or differences due to experimental approaches.

There is genetic evidence in support of this idea in the form of the *ichabod* mutant. The *ichabod* mutation is a maternal-effect mutation in the *β-catenin2* gene (Bellipanni et al., 2006; Kelly et al., 2000), a nuclear effector of the Wnt signalling pathway, and these mutants are thought to be deficient in dorsal nuclear localisation of maternally-derived β-Catenin2 (Kelly et al., 2000). Maternal β-Catenin2 has a conserved role in setting up the vertebrate D-V body axis, and is absolutely required for specification of all dorsal tissue, including organiser formation. Consequently, the most severe *ichabod* mutants lack all anterior and dorsally-derived tissue, including neural, and the latter is thought to be due to non-inhibition of the Bmp pathway: *β-catenin2* is required for activation of molecules important for inhibition of Bmp signalling, such as *chordin* and *bozozok/dharma* (Bellipanni et al., 2006; Maegawa et al., 2006), and it is thought that non-functional β-catenin2 protein leads to ubiquitous Bmp expression in these mutants (Kelly et al., 2000; Maegawa et al., 2006). However, even in the most severe cases, posterior neural tissue is still initially specified (Dee et al., 2007; Kudoh et al., 2004), which implies that signals other than Bmp inhibition are involved, at least in part, in the induction of posterior neural ectoderm. Furthermore, under all these conditions of Bmp overactivation, epidermal (ventral) markers are expanded dorsally at the expense of anterior neural markers, but expression of epidermal markers is never expanded posteriorly (Dee et al., 2007; Kudoh et al., 2004; Rentzsch et al., 2004). Also importantly, the *ichabod* data provide further evidence that the organiser, and consequently Bmp antagonism, is dispensable for posterior neural induction in zebrafish.

The above data imply that signals other than Bmp antagonism act as posterior neural inducers, and Fgf signalling has been identified as a prime candidate to fulfil this role. In support of this, two groups showed that Fgf signalling (*fgf3*) was required for acquisition of trunk and tail neural fate as loss of zygotic Fgf signalling, both with expression of XFD (Kudoh et al., 2004) and SU5402 (Dee et

al., 2007; Kudoh et al., 2004), led to loss of posterior, but not anterior, neural markers. Kudoh and others (Kudoh et al., 2004) showed that, conversely, Fgf overexpression could induce neural markers, which were of a posterior character. They further showed that induction of posterior neural ectoderm by Fgf was independent of the levels of Bmp since Fgf could induce posterior neural genes even in the presence of high levels of injected *bmp2b* mRNA. Furthermore, although overexpression of Fgf could suppress *bmp4* whilst inducing *chordin* expression, it could do neither when *fgf3* and *bmp2b* mRNA were co-injected. These data suggest that Bmp signalling is still active in embryos overexpressing Fgf and that posterior neural induction by Fgf does not depend on abrogation of the Bmp signal. However, this does not rule out the possibility that other mechanisms of Bmp regulation are active in posterior ectoderm. Indeed, it is likely that Bmp antagonism is still important in the specification of the posterior neural plate, possibly to stabilise neural gene expression (Kudoh et al., 2004), as has been proposed for Bmp antagonism in chick neural induction (Linker and Stern., 2004; Streit et al., 1998). In the *ichabod* mutant, for example, although posterior expression of *sox3* and other neural markers is present at the mid to late gastrula stages, expression of these genes is abolished or becomes very faint by the end of somitogenesis and neural tube formation. And very few, if any, neurons are present (Kelly et al., 2000; Kudoh et al., 2004; Maegawa et al., 2006). Nonetheless, these data from zebrafish were instrumental in defining a possible new model for neural induction in vertebrate embryos, which proposes a differential requirement for Bmp antagonism and Fgf signalling for anterior and posterior neural induction, respectively (Fig. 5).

A similar conclusion was arrived at by a different group using a different approach (Rentzsch et al., 2004). They showed that only a combination of SU5402 with a stabilised form of the epidermal-specific and Bmp target, the transcription factor protein P63, could completely abolish the entire neural plate. Overexpression of either *bmp2b* or application of p63 alone could only suppress anterior expression of the neural marker *sox3*, while in embryos injected with mRNA coding for the Bmp antagonist, Noggin, and also exposed to SU5402, only anterior *sox3* expression was induced (Rentzsch et al., 2004). This latter observation certainly suggests that it is Bmp antagonism that is important for induction of anterior neuroectoderm in zebrafish. But there is also some evidence in zebrafish that Bmp antagonism may be sufficient for posterior neural induction.

For example, both *bozozok/dharma* and *chordin* genes have been shown to antagonise Bmp signalling and *boz/dharma; chordin* double mutant analyses showed a synergistic loss of head and trunk tissues, with only the tail remaining, indicative of overactivation of the Bmp pathway in the mutant embryos (Gonzalez et al., 2000). However, apart from the effects on Bmp signalling of double *dharma;chordin* knockdown, *boz/dharma* activity is also required for induction of mesoderm (Fekany-Lee et al., 1999; Hashimoto et al., 2000) and hence indirect effects due to loss of mesoderm, and possible loss of posterior-specific Fgf expression (or other mesoderm-derived signals), cannot be ruled out. There is some evidence in support of a differential model in other vertebrates, and there are some indications that this model may apply to chick and frog. In the chick, where an early role for Fgf signalling in neural induction has been proposed, various experiments have shown that overexpression of Fgf induces tissue of a posterior nature (Muhr et al., 1999; Storey et al., 1998). In the frog, where current evidence suggests that Bmp inhibition is necessary for all neural induction, it is perhaps telling that simultaneous knockdown of three important genes coding for Bmp antagonists (*follistatin, chordin* and *noggin*), although leading to the almost complete loss of neural plate, does not completely abolish induction of the posterior neural plate. Some expression of both *sox3* and the definitive neural marker, *sox2,* still remains near the margin (Khokha et al., 2005), similarly to what is observed with *Xenopus* embryos injected with a β-catenin morpholino (Wills et al., 2010) and also similar to *sox3* and *zic2.1* expression in the zebrafish *ichabod* mutant (Kudoh et al., 2004). In addition, overexpression of Fgf can also induce posterior neural markers in the frog, as is the case with chick and fish (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995), and members of the Fgf family of ligands, such as Fgf8, are expressed at the correct place (marginal mesoderm) (Christen and Slack, 1997), as is the case with the fish.

But although a differential model, as proposed for fish, is a tempting alternative that in many ways reconciles the different results regarding the roles of Bmp antagonism and Fgf signalling in neural induction, the zebrafish results are by no means conclusive regarding the sufficiency of Bmp antagonism in anterior, or Fgf signalling in posterior, neural induction.In zebrafish, experiments involving loss of Fgf function have

Figure 5. Differential Model of Neural Induction in Zebrafish

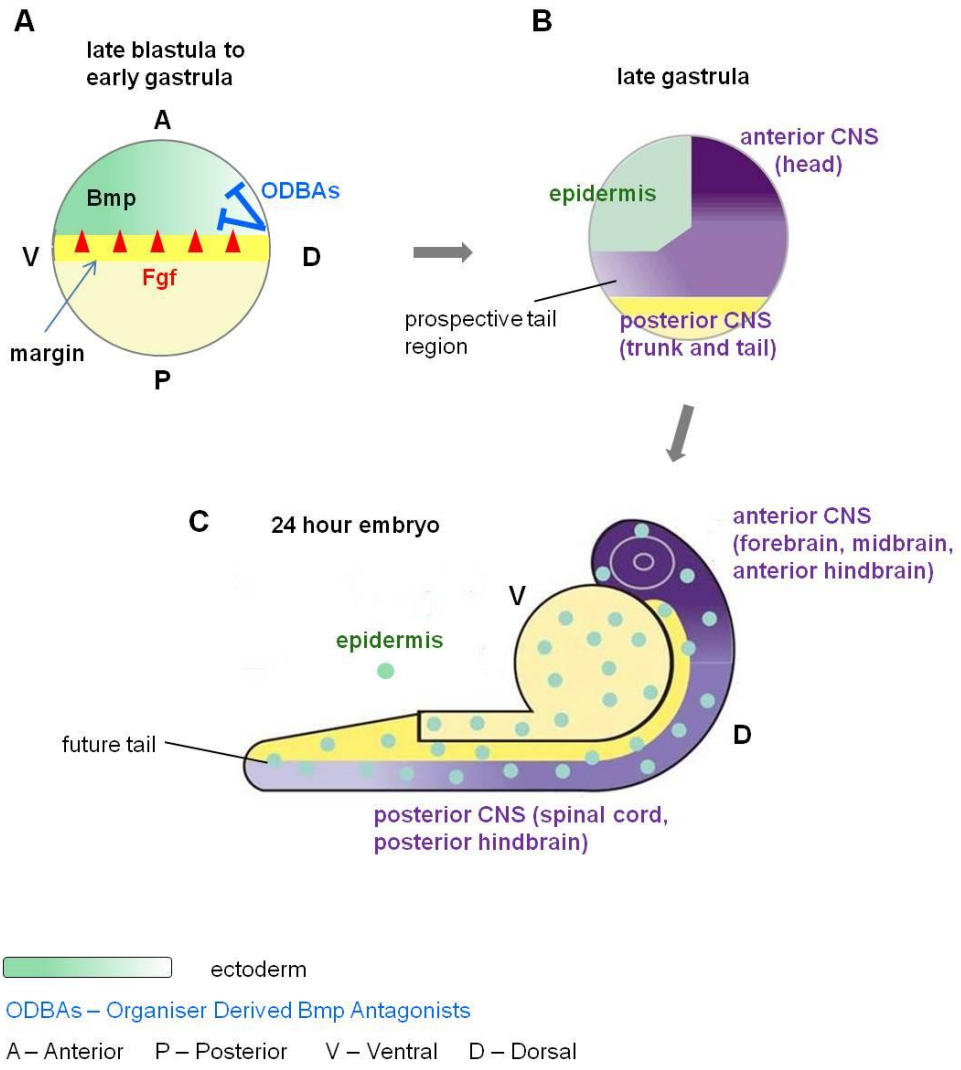


Fig. 5 Model for neural induction in zebrafish. (A,B) Dorsal to the right, anterior (animal pole) to the top. Initially, Bmps are expressed ubiquitously in the blastula ectoderm (green in (A)). At the onset of gastrulation, organiser-derived Bmp antagonists (ODBAs), such as Chordin and Noggin, are secreted dorsally by organiser cells and diffuse ventrally, creating a ventral to dorsal concentration gradient of Bmp expression (green-white gradient in (A)). Low or no Bmp expression on the dorsal side is sufficient for induction of anterior neural tissue (darker purple domain (B,C) but not for induction of posterior neural tissue (lighter purple domain (B,C). This is achieved by Fgf signalling emanating from the margin (red arrowheads in (A) and is independent of levels of Bmp. Epidermis is induced on the ventral side and is dependent on high levels of Bmp signalling (light green in (B), light green dots in (C). Note that there is a 90° turn in the A-P axis of the zefrafish gastrula (B) when compared to the 24 hpf embryo

(C) so that the prospective tail territory is positioned ventrally in the gastrula (B). (Adapted from a figure kindly supplied by Dr Tetsuhiro Kudoh).

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relied on injection of a dominant-negative Fgf receptor, XFD, and exposure chemical SU5402. However, results with XFD have tended to show only partial loss-of-function, while exposure to SU5402 has usually been done after the MBT and the start of zygotic gene expression, thus bypassing maternal Fgf signalling. In these conditions, only induction of posterior neural plate is primarily seen to be affected. As previously stated, in *Xenopus* embryos, inhibiting Fgf signalling by exposure to SU5402 from the MBT, presumably from when Fgf activity either begins or becomes indispensible, the whole neural plate fails to be specified, and this could not be rescued by Bmp co-inhibition, suggesting that continuous Fgf signalling is crucial for both anterior and posterior neural induction (Delaune et al., 2004). Thus in order to identify if Fgf signalling is required for anterior, as well as posterior, neural induction in zebrafish, more research is needed on the effects on neural development of blocking early (maternal) Fgf signalling as several ligands of members of the Fgf family are expressed maternally (Yamauchi et al., 2009; T.K., personal communication).

The data and ideas mentioned above are not fully inclusive but nevertheless serve to define the history and current knowledge of the molecular mechanisms that initiate neural fate determination. Neural induction in vertebrates is currently thought to begin during blastula stages and involves inhibition of the Bmp pathway, Fgf signalling, or both. In frog, fish and the mouse it certainly seems that Bmp antagonism/inhibition is necessary for neural induction, at least in the anterior nervous system, and this has been shown both by genetic and molecular approaches. In the chick, Bmp antagonism has been shown not to affect the induction of the neural plate, and instead it has been proposed that inhibiting the Bmp signal may be required only as a late, maintenance step that stabilises neural gene expression. Further, and although Fgf signalling is required for neural induction in the chick, Fgf signalling is not sufficient to induce the definitive neural marker, *sox2*, even in the presence of Bmp antagonists, although it can induce early neural markers. This has been used to argue that signal(s) other than Fgfs are (is) required for neural induction, but any such signal remains to be

identified in the chick (or any vertebrate). Meanwhile, Fgf signalling has also been shown to be important for neural induction in both fish and frog. In zebrafish, the data favour a differential model of neural induction where marginal Fgf signalling, and not Bmp antagonism, induces posterior neural ectoderm while Bmp antagonism induces anterior neural ectoderm. In the frog, however, there is no direct evidence for such a model, rather the discussion centres on whether it is Bmp inhibition or Fgf signalling that are sufficient for neural induction as a whole. The latest data from the frog proposes that inhibition of Bmp signalling initiates neural induction by activating Fgf expression (Fgf4), evidence that the Default Model in its strictest sense may hold true (Marchal et al., 2009). However, these results have yet to be repeated and the effects of a dominant-negative Smad5-sbn construct are unknown. In zebrafish, for example, the product of an antimorphic *smad5-sbn* allele imparts a different phenotype than that of a null allele, although strong dorsalisation occurs in both instances (Hild et al., 1999; Kramer et al., 2002). Furthermore, the Smad5-sbn protein may also restrict the function of Smad4, which is thought to mediate the activity of all Smad proteins, including Smad2, which functions downstream of Nodal signalling in mesoderm development. And also importantly, it also does not address the question of possible separable anterior and posterior neural induction mechanisms, which is also central to determining the molecular mechanisms that operate in conferring initial A-P polarity to the CNS.

1.2 A-P Neural Patterning

Although some progress has been made in understanding how these mechanisms establish the conditions that lead to A-P patterning of the neural axis, much is still unknown and very often confusing due to overlapping roles of these signalling molecules and downstream effectors in multiple and often overlapping developmental processes. As such, a complete discussion of neural A-P patterning events is beyond the scope of this thesis. Instead this Introduction will provide an overview of the known mechanisms that confer anterior or posterior character to the CNS, how this character is maintained and how conditions are established to further regionalise the CNS along the A-P (and D-V) axis.

1.2.1 Acquiring anterior or posterior identity

An outstanding question in vertebrate neural development is how the A-P (head to tail) axis is initially set up and what molecular mechanisms are involved. Central to this question is when does neural patterning begin and is it separable from neural induction? Although many models have been put forward to explain how neural development is initiated (Stern, 2001), the 'multiple organiser' model first proposed by Mangold in 1933 and Nieuwkoop's two-step (activation/transformation) model are the most prevalent (Foley and Stern, 2001; Niehrs, 2004; Rhinn et al., 2006; Stern, 2001). Mangolds's original idea that there were at least two organising centres, one for the head and one for the trunk/tail, was based on the assumption that different regions of the organiser possess different anterior and posterior inducing and patterning properties in a time-dependent manner. It implies that anterior and posterior neural plate is induced separately and that prospective neural cells acquire either anterior or posterior identity. Thus, the induction process would also impose initial A-P asymmetry to the prospective neural plate. Incompatible with the former, Nieuwkoop's model proposed a two step process for how A-P asymmetry is established in the neural plate, which implied that neural patterning was a continuation of the process of neural induction under the control of only main organising centre: as a first step, early organiser signalling would induce neural identity which is of anterior character (activation) while, as a second step, later signals from a dynamic organiser would 'transform' anterior neural fates to progressively more caudal ones. With current knowledge, however, modifications have necessarily been proposed for both models. Contrary to previous thinking that neural induction began after the appearance of the organiser, current evidence suggests that neural induction begins during blastula stages in vertebrates, at least for prospective anterior ectoderm, and requires Bmp inhibition, Fgf signalling or both. As previously mentioned, this implies that organiser-derived signals are indispensible for neural induction. So in the strictest sense, neither model is likely to be correct. However, a modification to the two-step model, the three-step model, has recently been proposed (Foley et al., 2000): Although similar tissue has yet to be found in either frog or fish, functionally equivalent structures in both the chick and mouse (the hypoblast in the chick and the anterior visceral endoderm (AVE) in the mouse) that are present in blastulae and give rise to extraembryonic structures, have both been shown to be required for anterior neural induction (Foley et al.,

2000; Foley and Stern, 2001; Thomas and Beddington, 1996). In addition, in all vertebrates where this has been studied, presumptive prechordal mesendoderm (PME) cells, which are derived from organiser cells, are the first to involute after the initiation of gastrulation and come to underlie prospective anterior neural tissue, their anterior migration displacing the AVE/hypoblast in chick and mouse (Stern, 2001). They contribute to and are required for head development, including neural, and can induce a stable forebrain fate when grafted to competent ectoderm (Foley et al., 1997; Kinder et al., 2001; Pera and Kessel, 1997; Shimamura and Rubenstein, 1997). But since the hypoblast (and the AVE) can only induce transient expression of supposedly anterior neural markers, its role would be to induce a 'pre-neural/pre-forebrain' state as a first step, which would then be stabilised, possibly by factors from organiser-derived axial tissue such as the PME, as a second step. Caudalisation factors from non-axial mesoderm (discussed later) would then progressively transform an anterior to a posterior neural state as the third step (Foley et al., 2000). Which, if any, of these models best represents early neural developmental processes remains unclear, but these models are prevalent in the discussion of whether neural induction and patterning are separable events or not.

As discussed before, Fgf signalling and Bmp antagonism have important roles in neural induction, but determination of their respective roles has proved elusive. One of the difficulties in determining the role of Fgf signalling in neural induction has stemmed from the premise of the two-step model of neural development and the fact that marginal Fgf signalling is also important for posterior neural patterning (see later). In the frog, for example, fate mapping has revealed that prospective neural tissue arises from dorsally located ectoderm which is relatively close to the organiser and its derivatives (Harland and Gerhardt, 1997), making it easier to relate frog neural induction to the Default Model, as well as the two-step model. In addition, under most experimental conditions, inhibiting the Bmp pathway in the frog, fish and mouse, and overexpressing Fgf in the chick, activates the expression of anterior neural genes (see previous section) and this has tended to support the activation/transformation model. However, these experiments, as well as analyses of the effects of Bmp antagonists, have relied considerably on *Xenopus* ectodermal explants, which are thought to consist only of cells of an anterior character, and the molecular mechanisms that have been uncovered have usually been interpreted as indicative

of induction of the whole neural plate. These and similar experiments in the chick (for example, Streit et al., 2000; Wilson et al., 2000) have also to a large extent been performed with isolated presumptive ectodermal tissue and may thus not reflect the situation *in vivo*. In addition, the expression of some marker genes used to identify anterior neural tissue are not necessarily limited to anterior neural ectoderm, such as *otx2* which is also expressed in anterior mesendodermal cells in both chick and mouse (Ang et al., 1994; Bally-Cuif et al., 1995; Pannese et al., 1995; Tam and Behringer, 1997). Zebrafish may in fact be a better model to use in the study of these early neural induction/patterning events as it has more clearly defined A-P divisions in the expression of neural-specific genes (expressed in both anterior and posterior domains), such as *sox3*. This allows for distinctions between anterior- or posterior-specific effects and can further be analysed in the context of the embryo. It was this peculiarity of zebrafish that allowed for the proposition of a differential model of neural induction in vertebrates, which further suggests that separate signalling centres induce and confer initial anterior (head) and posterior (trunk and tail) CNS character. Indeed, Fgf signalling can induce posterior neural markers without inducing either mesoderm or anterior neural genes in fish, frog and chick (Kudoh et al., 2004; Kuroda et al., 2004; Streit et al., 2000).This shows that posterior neural tissue can be induced without concurrent induction of anterior neural tissue, and furthermore, anterior neural induction can be suppressed without affecting induction of posterior tissue, such as when the Bmp pathway is ectopically activated at low levels (Kudoh et al., 2004; Rentzsch et al., 2004). These are strong arguments against an activation /transformation mechanism for neural induction and patterning.

1.2.2 Promoting and maintaining anterior neural identity

Whether posterior neural ectoderm is induced or transformed, during vertebrate development there are separable mechanisms that specify anterior or posterior identity. This initial A-P patterning of the vertebrate neurectoderm appears to be dependent on global and local gradients of developmentally important and evolutionary conserved signalling pathways that establish domains of a plethora of gradient-dependent transcription factor activity. It is thought that initially, global gradients, in conjunction with morphogenetic movements occurring during gastrulation, are responsible for the acquisition and maintenance of anterior or posterior fates, thus establishing broad anterior (head) or posterior

(trunk/tail) domains within neural ectoderm (Chen and Shier, 2001; Dosch et al., 1997; Kiecker and Niehrs, 2001; Niehrs, 2004). And at around the time, or soon after, that the events that initiate the process of neural induction are thought to occur, there are areas within the developing embryo that are critical for the development of the anterior CNS. The anterior dorsal BCNE centre of the *Xenopus* blastula, with concomitant gene expression, is transiently induced just after the MBT and it has been shown that when these cells are removed early in development, only the brain does not form (Ishibashi et al., 2008; Kuroda et al., 2004)). In addition, the neural inducing activity of the BCNE was shown to be dependent on the Bmp antagonist Chordin (Ishibashi et al., 2008; Kuroda et al., 2004), suggesting that Bmp antagonism mediates the anterior neural specification activity of the BCNE centre. Evidence gathered mainly from frog and fish has shown that there is a ventral to dorsal Bmp activity gradient that is initially established by the activity of Bmp antagonists dorsally (Dosch et al., 1997; Niehrs, 2004; Wilson et al., 1997), and clearance of the Bmp signal in dorsal areas may be a precondition for head development in all vertebrates as discussed previously. And although no tissue analogous to the BCNE has been found in vertebrates other than the frog, genes that downregulate the Bmp signal on the dorsal side of the embryo, such as *boz/dharma,* are also expressed from a very early stage in the YSL in zebrafish and are required for head development (Leung et al., 2003). Similarly, both the AVE and hypoblast of the mouse and chick are pre-organiser tissues that have been shown to be necessary, although not sufficient, for head induction. Both also express Bmp antagonists, although no direct correlation between this and head induction has been established. But in addition, they are also sources of antagonists of Wnt as well as Nodal signalling, as is the early dorsal margin of both fish and frog, features that are shared with later-developing organiser-derived prospective mesendodermal tissues. These tissues have head inducing activity and are presumed head organisers, such as the PME and prospective prechordal plate in zebrafish (Kiecker and Niehrs, 2001 and references therein; Niehrs, 2004; Perea-Gomez et al., 2002). They expresses genes, such as *cerberus (cer),* which codes for a multifunctional secreted antagonist of Bmp, Wnt and Nodal signalling in *Xenopus* (Bouwmeester et al., 1996; Piccolo et al., 1999) and *dickkopf1(dkk1),* coding for a conserved secreted antagonist of the Wnt pathway (Glinka et al., 1998; Hashimoto et al., 2000; Marvin et al., 2001; Niehrs et al., 2004; Shinya et al., 2000).These molecules have

been shown to be potent head inducers when overexpressed and to be required for normal head development (Ciani and Salinas, 2005). In addition, activity of these secreted factors in vertebrate prospective anterior tissue is thought set up both Wnt and Nodal global (whole organism) signalling gradients along the A-P axis, in a posterior (marginal mesoderm) to anterior direction (Bertocchini and Stern, 2002; Kemp et al., 2005; Perea-Gomez et al., 2002; Piccolo et al., 1999; Skromne and Stern, 2002; Yamamoto et al., 2004), and that these gradients, with their low point in the anterior, promote A-P patterning of the whole neuroectoderm. There is some evidence for these gradients (for example, see Chen and Shier, 2001; Kiecker and Niehrs, 2001; Yamamoto et al., 2004), and Wnt signalling in particular, has been shown to act directly on ectoderm to pattern the neural plate in a graded fashion, activating the expression of patterning genes in a concentration-dependent manner in *Xenopus* (Kiecker and Niehrs, 2001). In the chick and fish, meanwhile, graded Wnt activity has also been observed to pattern ectoderm (Lekven et al., 2001; Nordstrom et al., 2002). And importantly, loss of Wnt function leads to expanded anterior territories with concomitant posterior truncations, effects that are concentration-dependent (Ciani and Salinas, 2005; McGrew et al., 1997; Niehrs, 2004).

Nodal gradients that pattern embryonic axes in all vertebrates are well documented for Nodal signalling (Niehrs, 2004) and initially the *cer* data from *Xenopus* led to the suggestion that triple inhibition of Bmp, Wnt and Nodal signalling was a requirement for head, and hence anterior neural, development (Piccolo et al., 1999). However, although it is likely that antagonism of both Bmp and Wnt activity is required for head development, the case is less clear for Nodal signalling and whether there is a direct requirement for suppression of Nodal signalling for head development is controversial. A direct requirement for Nodal antagonists in anterior neural specification has been difficult to establish due to the multiple functions of Nodal signalling during gastrulation (Niehrs, 2004; Wilson and Houart., 2004). Nodal signalling has a conserved role in mesoderm induction and specification in all vertebrates (Schier and Shen, 2000), and these tissues are where posteriorising signals are expressed. This makes it difficult to separate direct effects on anterior ectoderm from possible indirect effects from mesoderm (Niehrs, 2004; Wilson and Houart., 2004; see later) and currently, the effects of Nodal signalling on head neural specification are thought to be indirect and related to mesendoderm development (Niehrs, 2004; Wilson and Houart.,

2004). Despite this, all the above data suggest that specification of anterior neural tissue results from inhibition of signalling pathways that would otherwise direct prospective anterior ectodermal cells to a different fate. Indeed, it has been suggested that one of the main anterior-specifying roles of the head organiser is to inhibit the activity of factors that promote posterior identity (Foley and Stern 2001; Niehrs, 2004). In support of this idea, evidence from the chick and mouse suggest that one of the roles of the AVE/hypoblast is to direct anterior migration of the overlying prospective ectoderm away from sources of posteriorising activity (Foley et al., 2000; Kimura et al., 2000; Perea-Gomez et al., 2001). Furthermore, it has also been suggested that the role of the organiser-derived PME is to directly antagonise later posteriorising signals emanating from the organiser, as well as from non-axial mesoderm, and help to stabilise anterior neural identity (Foley et al., 2000: Foley and Stern, 2001).

1.2.3 Posteriorising factors

The effects of Wnt and Nodal signalling in directly or indirectly suppressing anterior fates, whilst also promoting posterior fates has led them to being classified as posteriorising factors. In addition, Bmps have also been often classified as posteriorising factors, mostly due to their role in ventral-posterior patterning. And in addition to Wnts, Bmps and Nodals, there are other diffusible factors which have been proposed to act in posteriorising and patterning posterior neural ectoderm, mainly Fgfs (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Koshida et al., 1998; Kudoh et al., 2002; Lamb and Harland, 1995) and RA (Blumberg et al., 1997; Conlon, 1995; Durston et al., 1989; Kudoh et al., 2002; Papalopulu et al., 1996; Sive et al., 1990).

Similarly to what is observed in the anterior for Nodal inhibition, the role of Nodal signalling in posteriorisation of neural tissue has been difficult to separate from its mesoderm-inductive properties. Likewise, a possible the role for Bmp in posteriorisation has been difficult to determine as, although overexpression of Bmp can preferentially suppress anterior development, this is likely due to its D-V patterning properties rather than any posteriorising activity. However, Bmp probably also has a role in posteriorisation, as Bmp activity was shown to promote more caudal fates in the zebrafish posterior ectoderm (Kudoh et al., 2004). This function is probably due to its role in the regulation of convergence movements

during gastrulation (Marlow et al., 2004; Myers et al., 2002; Wilson and Houart, 2004) but may in addition include regulation of *caudal*-related (*cad*/*cdx*) genes, which code for homeodomain-containing transcription factors that are critical for posterior development (see below) (Haremaki et al., 2003). Although both Wnts and Fgfs have functions during gastrulation that encompass developmental processes other than posteriorisation of neural ectoderm, for example both pathways are required for mesoderm specification (Kimelman, 2006), nevertheless initially global gradients of Wnt and Fgf, as well as RA, are established and have all been shown to directly affect cell fate specification and positional information in posterior neural ectoderm. In addition to effects of components of the Wnt pathway discussed previously, Wnt signalling has been shown to activate posterior neural markers at high concentrations while progressively lower levels of Wnt signalling progressively activate the expression of more anterior neural genes (Kiecker and Niehrs, 2001; Nordstrom et al., 2002). Similar to Wnt loss-of-function, loss of Fgf signalling also causes posterior truncations in a concentration-dependent manner in both fish and frog (Delaune et al., 2004; Kudoh et al., 2004). Fgf signalling has also been observed to act in a graded manner in a variety of contexts, in particular in *Xenopus* where experiments with ectodermal explants showed that the *Xenopus* Fgf2 homologue, bFgf, could induce anterior neural genes at low concentrations whilst at high concentrations posterior neural genes were induced (Kengaku and Okamoto, 1995). And consistent with their postulated roles in posteriorisation, ligands of members of both pathways are expressed in marginal mesoderm in all vertebrate species and at the right time (Christian et al., 1991; Isaacs et al., 1994; Kudoh et al., 2001; Ohuchi et al., 1994; Shamim and Mason, 1999; Skromne and Stern, 2002).

RA, meanwhile, is thought to diffuse anteriorly from its site of synthesis in posterior paraxial mesoderm during gastrulation, and its graded activity in the regulation of posterior hindbrain and anterior spinal cord has been well documented (Dupe and Lumsden, 2001; reviewed in Gavalas and Krumlauf, 2000; Godsave et al., 1998; Marshall et al., 1992). Consistent with its role in trunk development, exposure of vertebrate embryos to RA suppresses both head and tail fates (Blumberg et al., 1997; Durston et al., 1989; Maden, 2002) while loss of function of *raldh/aldh* genes, which code for RA synthesising enzymes, as well as exposure to inhibitors of RA signalling such as DEAB and mutations in RA

response elements (RAREs), produces the opposite effect, with embryos showing a reduced hindbrain and anterior spinal cord phenotype (Begemann et al., 2001; Begemann et al., 2004; Grandel et al., 2002; Maden, 2002; Niederreither et al., 2000; Zhao et al. 1996). *Cytochrome P450RA* (*cyp26*)-related genes are initially expressed in anterior neural ectoderm during early vertebrate development (de Roos et al., 1999; Fujii et al., 1997; Kudoh et al., 2002), coding for enzymes known to degrade retinoic acid (Fujii et al. 1997; Niederreither et al. 1997; Swindell et al. 1999; White et al., 1996) and are thought to be the sink of the RA gradient. Loss of *cyp26*-related gene function, which should mimic gain of RA function, causes anterior expansion of posterior hindbrain fate with concomitant loss of anterior hindbrain (Hernandez et al., 2007; Kudoh et al., 2002) while overactivation of *cyp*-related genes has the opposite effect, leading to loss of hindbrain and anterior spinal cord (for example, see Kudoh et al., 2002).

As a consequence of their effects on A-P patterning, and based on studies across vertebrate species, Wnts, Fgfs and RA are considered to be the main signalling molecules that specify posterior neural identity and which graded activity is required for A-P patterning of the neural ectoderm. Patterning both overlaps, and is subsequent to, acquisition of posterior identity and the activity of these three pathways confers posterior character to neural ectoderm whilst concurrently activating and regulating the activity of downstream effectors that subsequently pattern the posterior CNS. How Wnt, Fgf and RA are thought to posteriorise neural ectoderm, and the common factors that mediate downstream signalling events, providing an integration point for these three pathways, is discussed below.

1.2.4 Posteriorising neural ectoderm

Although other mechanisms of posteriorisation by these factors likely exist, how these factors promote their posteriorising activity during gastrulation has been analysed in zebrafish (Kudoh et al., 2002). This analysis has served as a paradigm for A-P patterning in vertebrates and epistatic analyses of Wnt, RA and Fgf function in patterning the neural A-P axis showed that broad regionalisation of the neural ectoderm begins during blastula stages. A model was proposed whereby Fgf and Wnt signalling from marginal mesoderm are required to prevent the activation of anterior gene expression in prospective posterior neural ectoderm, in a manner that is independent of RA. This includes suppression of

genes coding for RA-degrading enzymes from the late blastula stage onwards, leading to an RA-free anterior domain whilst permitting accumulation of RA in prospective posterior ectoderm. This is achieved, at least in part, via the activity of RA synthesising enzymes expressed in paraxial mesoderm. RA degradation in the anterior would prevent the expression of posterior neural patterning genes in the anterior neural plate, whilst expanding prospective posterior neural tissue would accumulate levels of RA to a level sufficient to activate expression of posterior neural genes in the early gastrula, such as *hoxb1b* and *meis3*. Further, these results showed that RA is directly necessary and sufficient for the expression of these same markers. And since neither Fgf nor Wnt signalling was sufficient to ectopically activate the expression of posterior neural genes in the absence of signalling of either pathway, this suggests that interactions between the two pathways is essential for posterior neural patterning upstream of RA, directly or indirectly. This is further supported by the observation in this same report that loss of signalling of either pathway led to the suppression of posterior neural markers. In addition, Fgf and Wnt signalling response elements are present in the enhancers of zebrafish *hoxb1b*, as are those for RA, and some of these have been shown to drive gastrula expression of *hoxb1b in vitro* (Ishioka et al., 2010). This emphasises the point that interplay between these three pathways is critical for posterior development and some progress has been made in understanding how Wnt, Fgf and RA signalling is integrated in posterior neural patterning.

Hoxb1b belongs to the Hox gene cluster which, together with the evolutionary related ParaHox (*cad/cdx*) gene cluster (Ferrier and Holland, 2001), are thought to have an evolutionary conserved role as the main effectors of pathways involved in posterior neural development, at least in vertebrates.

Hox gene clusters possess the peculiar trait of colinearity, being expressed in a sequential manner according to their position on the chromosome (Alexander et al., 2009; Garcia-Fernandez, 2005). Furthermore, in general, the order in which they are expressed corresponds to the position on the A-P axis that they affect, from an anterior to posterior direction. Overlapping and combinatorial domains of Hox gene expression, known as the Hox code, are critical for generating regional identity in the posterior neural ectoderm (posterior hindbrain and spinal cord) (Alexander et al., 2009; Gavalas and Krumlauf, 2000; Kiecker & Lumsden 2005; Schilling, 2008) and Wnt, Fgf and RA signalling are all known to directly or indirectly regulate this process (Alexander et al., 2009; Gavalas, 2002; Itasaki et

al., 1996; Kolm et al., 1997; Schilling, 2008). This is true not only during gastrulation, when broad A-P domains are established and regulatory networks for future posterior patterning begin to be defined, but also for subsequent patterning events during the elongation and subsequent formation of the posterior neural tube that occurs during somitogenesis. During this stage, the vertebrate hindbrain is transiently divided into 7-8 segments called rhombomeres (r), with r1 being the most anterior (Gavalas and Krumlauf, 2000; Schilling, 2008). This establishes zones of lineage and gene-restricted axial compartments, governed by the Hox code, and positional information within these compartments is critical for neuronal specification. Rhombomere compartments are established, at least in part, by the activity of Hox genes under the regulation of Fgf and RA: for example, loss-of- *raldh2/aldh1a2* function in both fish and mouse leads to loss of rhombomeres 5-7 identity and concomitant loss of Hox gene expression (Maves and Kimmel., 2005; Niederreither et al., 2000) and this regulation of rhombomere identity is thought to be concentration (posterior to anterior)-dependent. In addition, Fgfs are expressed in rhombomere 4, which has been proposed as a secondary organising centre for hindbrain development (Maves et al., 2002; Walshe et al., 2002) and this Fgf expression is required for establishment and development of rhombomeres r3-r6 in both fish and chick (Marín and Charnay, 2000; Maves et al., 2002; Walshe et al., 2002). Examination of mouse Hox promoters suggests that regulation of rhombomere-specific Hox genes is under the cooperative control of both Fgf and RA and involves a multitude of transcription factors such as Krox20 and Vnhf1 (Alexander et al., 2009; Hernandez et al., 2004; Tümpel et al., 2009).

Unlike the hindbrain, the spinal cord is unsegmented and is a more homogenous tissue, but like the hindbrain is patterned along the A-P axis by the activity of Hox genes, giving rise to diverse populations of neurons in a position-specific manner. In all vertebrates, *cdx* genes have been shown to be critical for posterior development (Ehrman LA, Yutzey, 2001; Epstein et al., 1997; Shimizu et al., 2006; van den Akker et al., 2002), leading to anterior truncations when overexpressed while loss of function experiments produce phenotypes ranging from caudal truncations to anterior homeotic transformations (transformation of one tissue type to another). These phenotypes have been shown to correlate to posterior shift in Hox gene expression (for example, see Subramanian et al., 1995; van der Akker et al., 2002) and are also characteristic of posteriorising activity.

Indeed, it has been proposed that regulation of *cdx*-related genes (*xcad* in *Xenopus*) by Fgf, Wnt and RA signalling may mediate the caudalising activity of these pathways via the regulation of the Hox genes (Alexander et al., 2009; Bel-Vialar et al., 2002; Lohnes, 2003; Schilling, 2008).

In support of this, Cdx proteins have been shown to directly regulate Hox gene expression, as consensus binding sites have been identified on Hox promoters and some were shown to possess important regulatory activity (Charité et al. 1998; Subramanian et al. 1995). Furthermore, in all vertebrates, *cdx* genes mediate the activation of posterior Hox genes by the Fgf, Wnt and RA pathways in a variety of situations (Bel-Vialar et al., 2002; Keenan et al., 2006; Prinos et al., 2001; Shimizu et al., 2005). Both in chick and in zebrafish it has been proposed that *cdx* mediation of Fgf and RA signals is achieved in part by providing competence for activation of Hox genes by Fgf and RA in the spinal cord (Bel-Vialar et al., 2002; Shimizu et al., 2006). Analyses of Cdx1/4 double knockdown phenotypes in zebrafish further showed that *cdx* genes promote spinal cord development by activating posterior Hox gene expression downstream of Fgf signalling, which in turn suppresses the expression of Fgf and RA-dependent anterior Hox gene expression (Shimizu et al., 2006; Skromne et al. 2007).

Shimizu et al (Shimizu et al., 2006) proposed that a Cdx/Hox code may exist that patterns posterior neural ectoderm (posterior hindbrain and spinal cord) downstream of Fgf and RA signalling which is dependent on Fgf and RA gradients. In addition, it has been reported that *cdx1* and *cdx2/4* genes respond differently to Wnt loss-of-function in the mouse (Ikeyaa and Takada, 2001), leading to the suggestion that these gradients may activate a gradient of Cdx transcriptional activity that regulates Hox gene expression, and thus providing an integration point for these signalling pathways in posterior neural development.

1.2.5 Organisers of Anterior Regional Pattern

Although maintenance of anterior neural fate may to a large extent rely on shielding the anterior neural plate from posteriorising activity from marginal mesoderm and perhaps from organiser-derived signalling during late gastrulation (Foley et al., 2000; Foley and Stern, 2001), maintenance of both a neural and an anterior fate must also be consolidated. This may be accomplished by the activity of antagonists of head suppressing molecules as discussed earlier, and together these processes lead to stable expression of genes that are required for proper head

neural development. As gastrulation proceeds, regionalisation of the CNS becomes more apparent and broad divisions of the head neural ectoderm (forebrain, midbrain and anterior hindbrain) can be distinguished by the expression of domain-specific genes (Fig. 2). And by the end of gastrulation a number of local 'organising' centres, with distinct gene expression patterns become established and signalling within, and emanating from, these centres will further regionalise and pattern the brain (Niehrs, 2004; Wilson and Houart., 2004) so that by the end of somitogenesis, the brain consists of most of the primordia that will give rise to a fully patterned head in the adult. Although these signalling centres start becoming apparent from mid-gastrula onwards, the signalling events that lead to their establishment occur earlier in development. Many of the molecular mechanisms are still to be characterised, but evidence suggests that they are the product of either global or more locally established signalling gradients that nonetheless appear to involve the same signalling pathways active in earlier development and are discussed briefly below.

Three secondary organising centres involved in anterior neural patterning have been described in vertebrates: The cells of the anterior neural ridge (ANR) (also known as the ANB) at the border of the anterior neural plate are required for specification and patterning of the telencephalon, the more rostral region of the forebrain, while the zona limitans intrathalamica (ZLI), situated at the boundary between the future dorsal and ventral thalamus, controls patterning in the diencephalon, which is caudal to the telencephalon. More caudally, the midbrain-hindbrain boundary (MHB/isthmus) controls patterning of the midbrain and the anterior hindbrain (cerebellum) (Rhinn et al., 2006). In zebrafish, a small row of cells located at the anterior neural plate border, called row1, has been shown to be essential for patterning the telencephalon (anterior forebrain) (Houart et al., 1998). How this signalling centre is positioned remains unknown, but it has been proposed that the morphogen activity of Bmp signalling may play a role (Wilson and Houart., 2004). What is known, however, is that ablation of these cells at the mid gastrula stage leads to loss of telencephalic fates, and that telencephalic promoting activity of row1 cells requires the activity of Wnt antagonists (Houart et al., 1998; Houart et al., 2002; Rhinn et al., 2006; Wilson and Houart., 2004). The secreted Wnt antagonist, Tlc, can restore telencephalic identity after ablation of row1 cells, as well as being able to illicit telencephalic gene expression in a concentration-dependent manner (Houart et al., 2002). This and other data

obtained with Wnt loss and gain of function experiments has confirmed that patterning of the telencephalon requires low or no Wnt signalling and this requirement may be conserved in vertebrates (Wilson and Houart, 2004). In contrast, patterning of the diencephalon appears to require Wnt signalling, which has led to the suggestion that a gradient of Wnt signalling, from posterior to anterior, patterns the forebrain in a graded manner, although it is not known what this source of Wnts is. In support of this, activation of Wnt signalling has been shown to promote diencephalic fates at the expense of telencephalon in fish, frog and chick (Cavodeassi et al., 2005; Houart et al., 2002; Wilson and Houart, 2004).

One consequence of inhibiting Wnt signalling in the ANR is the activation of Fgf signalling, although whether Fgf signals induce the telencephalon is not yet clear (Rhinn et al., 2006; Wilson and Houart., 2004). However, recent experiments in the mouse have suggested that Fgfs may indeed be inducers of forebrain fate, as shown with the complete loss of telencephalic fates by triple knockdown of three Fgf receptors (Paek et al., 2009). And in *Xenopus*, the neural plate is completely abolished in embryos where Fgf signalling is blocked with the Fgfr inhibitor, SU5402 (Delaune et al., 2004). Whether an inducer or not, Fgf signalling affects a wide variety of processes and is critical for the development and patterning of the telencephalon.

In the ZLI, it is the *sonic hedgehog* (*shh*) gene, another signalling pathway important for embryonic development, that is the critical mediator of ZLI organising activity, possibly in conjunction with Wnt signalling (Kiecker and Lumsden, 2004; Scholpp et al., 2006; Vieira et al., 2005). However, as with the ANR, the mechanisms that position the ZLI are not clear, although they are likely to involve the *irx* and *six* genes, as well as Wnt signalling (Scholpp et al., 2007; Wilson and Houart., 2004): the ZLI is positioned at the border between the mutually repressive expression domain of *six3* anteriorly in the telencephalon and *irx3* posteriorly in diencephalon, and at levels of Wnt signalling that suppress *six3* and induce *irx3* (Braun et al., 2003; Kobayashi et al., 2002).

The MHB is perhaps the best analysed of the secondary organisers that pattern the CNS along its A-P axis and is positioned at the anterior of rhombomere 1 (see later) at the border between the midbrain and anterior hindbrain. In all vertebrates, this is thought to coincide with the posterior border of *otx2* expression in the midbrain and the anterior border of *gbx* gene expression in rhombomere 1, two transcription factors with mutually suppressive properties

(Rhinn and Brand, 2001). And in zebrafish it has been shown that establishment of this boundary is dependent on the global A-P Wnt gradient: Wnt signalling at the margin limits the posterior expression of *otx2* while inducing *gbx1,* leading to the activation of Fgf8 at the MHB (Rhinn et al., 2005). That Fgf8 mediates the organising activity of the MHB has been shown in the chick, where it could induce diencephalon to midbrain transformations when misexpressed (Crossley et al., 1996). In the zebrafish, meanwhile, the MHB and cerebellum do not form in *ace* mutants, which lack a functional Fgf8 protein (Reifers et al., 1998). It is thought that a gradient of Fgf8 created anteriorly from its source at the MHB functions to pattern the midbrain and cerebellum, since it is also required to position the diencephalon-midbrain boundary anteriorly: high levels of Fgf8 would be required for cerebellum (hindbrain) patterning, while lower levels of Fgf8 would pattern the midbrain (Nakamura and Watanabe, 2005, Rhinn et al., 2006 and references therein).

1.3 Aims and Objectives

Many of the major signalling pathways involved in neural development in vertebrates have been identified and include, among others, Bmps, Fgfs, Wnts and RA. These pathways have been shown to be widely conserved and to also have vital functions in other, often overlapping, developmental processes. However, and to a certain extent due to their multiple roles, many of the molecular mechanisms underlying neural induction and patterning events by these and other factors remain to be characterised. A comprehensive knowledge of how the nervous system is induced and patterned is critical for applications of future stem cell and tissue regeneration therapies for neurological diseases and the aims of this PhD were to attempt to clarify some of the molecular mechanisms of neural induction and patterning in the zebrafish embryo and further our understanding of vertebrate neural development. To this end, we embarked on three separate but functionally overlapping projects. In the first project, we carried out a functional analysis of the *eve1* gene, which had been identified as a factor possibly important for neural development. In the second project, we analysed the differential contribution of maternal and zygotic Bmp and Fgf signalling in D/V patterning, for although it is known that Bmp antagonism and Fgf signalling both play critical roles in neural induction, their relative temporal and spatial contributions to this

process have not been clarified. In addition, such an analysis had not been done to date in any vertebrate system. In the third project, we developed an in vitro system to generate neural tissue using a zebrafish organiser-defective *ichabod* mutant. The aims of this project were to develop a system to study neural development in zebrafish in a purely ectodermal background, in order to analyse the direct responses in ectoderm to signalling from major factors known to be important for neural development.

1.4 Methods

1.4.1 General Methods

Whole-Mount *In-Situ* Hybridisation of Zebrafish Embryos and Explants:

- Unless otherwise stated, all steps were carried out in 1.5 ml microcentrifuge tubes.
- When replacing reagents, embryos/explants were always left in some liquid to avoid drying.
- Where washing is referred to, 1ml of liquid was always used.

Fixing embryos and explants

Embryos/explants were fixed in 4% paraformaldehyde (PFA)/Phosphate-buffered saline[1,2] (PBS) overnight at 4°C. The PFA was then removed and the embryos/explants were washed in PBST (PBS + 0.1% Tween20 (Sigma, product number P2287)) for ~10 minutes. The PBST was then removed and was replaced by 1ml of 100% methanol for at least one hour at -20°C prior to the *in-situ* procedure.

Hybridisation

For the hybridisation step, the methanol was first removed then the embryos/explants were rehydrated by washing in PBST for ~10 minutes.
The PBST was then removed and 500 µl of Hybri-buffer[3] was added. This prehybridisation step was done at 65°C for one hour or more. After this, for hybridisation of probe to tissue RNA, the prehybridisation buffer was removed, 200µl of probe solution was added and the embryos/explants were incubated overnight at 65°C. To prepare the probe for this step, labelled anti-sense RNA probe (see *in-situ* probe preparation) was diluted in hybri-buffer. The dilution varied with the strength of the probe, but was typically 100, 200 or 400 X. The diluted probe was placed in an 80-90°C water bath for ten minutes then put on ice for five minutes.

Washing: To remove excess and non-hybridised probe (these steps were all carried out at 65°C)

The probe solution was removed and the embryos/explants were first washed with 2x saline-sodium citrate (SSC[4])/50% formamide (Sigma, product number F9037-100ML)/ 0.1% Tween20 (Sigma, product number P2287) for at least 30 minutes. This solution was then replaced with 2x SSC + 0.1% Tween20 for at least thirty minutes. Subsequently, embryos/explants were washed twice with 0.2x SSC + Tween20 for 30 minutes each wash. In these steps, the first two SSC washes remove some probe, the last two washes remove the remainder of the unhybridised probe. The last two washes are more stringent, and were not done for longer than 30 minutes each.

Antibody detection of labelled probe

After the last SSC wash, SSC solution was removed and replaced by Maleic Acid Buffer (MAB[5]) for ~10 minutes. This was followed by the blocking step, where the MAB was replaced by adding 500 µl of blocking solution[6] for at least one hour at room temperature (r.t.). Then blocking solution was removed and replaced by 200µl of a solution containing stock anti-digoxigenin antibody[7] diluted 1:5000 in blocking solution and left at r.t. for three-four hours or overnight at 4°C.

Staining with BM Purple (done at r.t.)

For staining, antibody solution was removed and embryos/explants were first placed in 1ml Alkaline Phosphatase (AP[8]) buffer for ~10 minutes. The AP buffer was then replaced with 400µl of BM Purple (Roche, product number 11442074001), the embryos transferred to 24-well plates and left at r.t. on a slow shaker until the desired staining was obtained. To stop the staining reaction, the BM Purple was first removed and the embryos washed two-three times with PBST for ten minutes and left in PBST at 4°C for picture taking. Subsequently, embryos/explants were transferred to 4% PFA for longer term storage.

In-situ probe preparation:

DNA/Plasmid Digest – Plasmid linearisation

The plasmid containing the gene of interest was linearised by cutting with the appropriate restriction enzyme at the 5' end of the coding strand of the inserted gene. This is to prevent the RNA polymerase from overrunning the gene coding sequence (see synthesis below). 200 µl reaction mixtures were assembled with the following composition, based on 1 µg/µl of DNA.

dH_2O	168 µl
10x enzyme-specific buffer	20 µl
DNA/Plasmid (1 µg/µl)	10 µl (10 µg)
Restriction enzyme	2 µl
	200 µl

Mixtures were assembled in a 1.5ml microcentrifuge tube and placed in a water bath at 37°C for 2-3 hours followed by a diagnostic gel to determine if the DNA has been cut properly. The linearised DNA was then extracted using phenol/chloroform/isoamyl alcohol (Sigma, product Number 77617-100ML): The same volume of phenol/chloroform was added to the reaction mixture, vortexed for a few seconds until the mixture was milky, then centrifuged at full speed for ~five minutes. The aqueous phase was subsequently removed and placed in a clean tube. To precipitate the DNA, first 10% 3M sodium acetate[9] (NaOAc) was added to the aqueous phase, as well as 2.5x ice cold 100% ethanol, carefully mixed and frozen at -80°C overnight or longer. This mixture was then centrifuged at maximum speed at 4°C for twenty minutes, after which the supernatant was removed and the DNA pellet washed in 160µl of 70% ethanol. After washing, the tube was centrifuged again at maximum temperature at r.t. for 5 minutes, after which the ethanol was removed and the DNA pellet allowed to air-dry then dissolved in in 20 µl of ddH_2O. Once dissoved, diagnostic gels were run to determine the concentration of DNA and the DNA was stored at -20°C.

An alternative to phenol/chloroform/isoamyl extraction and ethanol precipitation which was used on occasions was a column-based PCR purification kit (Qiagen U.K. catalogue number 28104), following the manufacturer's instructions.

Transcription of anti-sense RNA probes

20 µl reaction mixtures were assembled. Below is a working mixture based on Promega (Promega, U.K. catalogue numbers: T3 P2083; T7 P2075; SP6 P1085) reagents which was mostly used, following the manufacturer's instructions:

RNase-free H_2O to 20 µl	? µl
5x transcription buffer	4 µl
100 mM DTT	1 µl
RNA Digoxygenin labelling mix	2 µl
1.5 µg DNA/Plasmid	? µl
RNase inhibitor	1µl
RNA polymerase (T3, T7 or Sp6)	1µl
	20 µl

Fermentas (Fermentas, U.K. catalogue numbers: T3 EP0101; T7 EP0111; SP6 EP0131) reagents were also used (RNA polymerases and buffer), however the quantities and protocol were the same with the exception that DTT is included in the Fermentas buffers. Hence, 1 µl extra of RNase-free water was added.

Mixtures were assembled in a microcentrifuge tube and placed in a water bath at 37°C for 2 hours (1 hour for fermentas enzymes). Diagnostic gels were run to determine if the RNA had been synthesised. To remove plasmid DNA, 2 µl of RNase-free DNase (Promega, U.K. catalogue number M6101) was added and the tube put in a water bath at 37°C for 30 minutes to an hour after which diagnostic gels were run to determine if the DNA has been digested. If required, the mixtures were put through a G50 column (Illustra microspin, GE Healthcare. Product code

27-5330-01) to remove unincorporated nucleotides, sometimes used with 'dirty' probes that can produce a lot of background staining. To precipitate the RNA, RNase-free water was added to make 50 µl, then 5 µl (0.4 M) lithium chloride[10] (LiCl) and 150 µl 100% ice cold ethanol. This mixture was then centrifuged at maximum speed at 4°C for twenty minutes, after which the supernatant was removed and the RNA pellet washed in 500µl of 80% ethanol. After washing, the tube was centrifuged again at maximum temperature at r.t. for 5 minutes, after which the ethanol was removed and the RNA pellet allowed to air-dry then dissolved in in 20 µl of RNase-free water. Once dissolved, diagnostic gels were run to determine if the RNA probe was present and to determine the approximate concentration of RNA, after which 20 µl of Hybri-buffer was added (prevents RNase activity) and the RNA stored at -20°C.

1.4.2 Methods for Research Chapter 1

Reverse Transcription Polymerase Chain Reaction (RT-PCR) – detection of eve1 morpholino-induced alternative transcript

Thirty embryos were injected at the two cell stage with 10 ng of a morpholino (GeneTools, LLC) directed at the *eve1* intron1-exon2 splice site. When the embryos had reached shield stage (50% epiboly) total RNA was extracted from the pooled embryos using TRI reagent (Sigma, U.K. product number T9424-100ML) according to manufacturer's instructions. The same procedure was used with 30 control, uninjected embryos. Genomic DNA was removed by addition of 50µl/ml RNase-free DNase I (Promega, U.K. catalogue number M6101) for 30 minutes at 37°C. The DNase was subsequently heat-deactivated at 70°C for 5 minutes, the RNA concentration quantified and the RT-PCR was then performed in two steps:

1. Reverse Transcription (first strand synthesis of cDNA) was carried out with M-MLV Reverse Transcriptase (Promega, U.K. catalogue number M1701) according to the manufacturer's instructions.

2. Real time PCR (second strand synthesis) with the following specifications:

Component	Volume	Final Concentration
RNase-free H_2O to 200 µl	131 µl	
5x green GoTaq flexi buffer (Promega)	40 µl	1X
25mM $MgCl_2$	8 µl	1mM
dNTP mix, each 10mM	8 µl	0.4mM each
Forward primer* (400pmol)	4 µl	2µM
Reverse primer* (400pmol)	4µl	2µM
Template DNA	4µl	
GoTaq DNA polymerase #	1µl	0.025units/µl
	200 µl	

200 µl reaction mixture was divided into 4 PCR tubes and subsequently mixed after reaction for analyses.

Promega U.K., product number: M8301.

PCR conditions:

Step	Condition	Temperature °C	Time	N° of cycles
1	Denaturation	96	1 min	1
2	"	96	30 secs	Go to step 2, repeat 29 times →
3	Annealing	59	30 secs	
4	Extension	72	1 min	
5	Final Extension	72	1 min	1
6	Stand	4	20 min	1

* For primer sequences and results of PCR reaction see Materials and Methods in Chapter 1

1.4.3 Methods for Research Chapter 3

Immunohistochemistry – GFP detection

Before procedure, animal caps were fixed in 1ml of 4% PFA/PBS in microcentrifuge tubes and incubated overnight at 4°C. The caps were then rinsed in 1ml of 1x PBS prior to incubation overnight in 1ml of 100% methanol at -20°C. Before blocking, caps were again rinsed in 1x PBS. For blocking, PBS was removed and 100 µl of goat serum blocking solution[6] was added to each tube and left on a slow shaker for one hour at r.t.

For antibody detection, blocking solution was removed and 100 µl of anti-GFP primary antibody (rabbit IgG) diluted 1000x in blocking solution was added, mixed well and incubated overnight at 4°C. The caps were then rinsed 3x 10 minutes in 1ml 1xPBS. After rinsing, PBS was removed and

100 µl of biotinylated secondary antibody (anti-rabbit) was added, mixed well and left on a slow shaker for one hour at r.t. Caps were again rinsed 3x 10 minutes in 1ml 1xPBS. After rinsing, 100 µl of streptavidin-horseradish peroxidase (HRP) conjugate solution was added, mixed well and incubated for one hour at r.t. on a slow shaker, followed by 3x 10 minutes rinses in1ml of 1x PBS (streptavidin protein binds to biotin, HRP is a catalyst for the chromagen reaction).

For staining, caps were transferred to 24-well plates, the PBS removed, and 300 µl of the substrate-chromagen mixture consisting of the following dilutions of provided stock solutions was added:

AEC buffer: x20

AEC concentration solution: x25

Hydrogen peroxide concentration solution: x40

Staining was monitored under a dissection microscope, and the reaction was stopped by transferring caps to 1ml 1x PBS, rinsing briefly then transferring to wells containing 4% PFA/PBS. The caps were then stored at at 4°C

1.4.4 Reagents and Buffers

1 4% PFA/PBS

- 2g PFA (Sigma-Aldrich, product number 158127-500G) was dissolved in 50ml PBS at 60°C, frozen at -20'C and used within two weeks after thawing.

2 10X PBS stock solution (1L)

- To 800ml of dH_2O add:
  - 80.0g NaCl (Sigma-Aldrich, product number S7653-1KG)
  - 2.0g KCl (Sigma, product number P9541-500G)
  - 14.4g Na_2HPO_4 (Sigma, product number S3264-500G)
  - 2.4g KH_2PO_4 (Sigma, product number P9791-500G0)
- Adjust pH to 7.3, add dH_2O to 1L

3 Hybridisation Buffer

- 50% Formamide (Sigma, product number F9037-100ML)
- 5X SSC[4]
- 5 mM EDTA[16]
- 0.1% Tween20 (Sigma, product number P2287)
- 50µg/ml Heparin (Sigma, product number H6279-100KU)
- 1mg/ml Torula RNA (Sigma, product number R6625-100G)

4 20X SSC Stock Solution (1L)

To 800ml of dH_2O add:

- 3M Sodium Chloride (Sigma, product number S3014-500G)
- 0.3M Sodium Citrate (Sigma, product number W302600-sample-K)
- Adjust pH to 7.0, add dH_2O to 1L

5 0.1M Maleic Acid Buffer (1L)

To 800ml of dH_2O add:

- 200ml of 0.5M Maleic Acid (Sigma, product number 295876-1L)
- 44g NaCl (150mM) (Sigma, product number S3014-500G)
- Adjust pH to 7.5, add dH_2O to 1L

6 Blocking Solution (50ml)

- Add 1g blocking reagent (Roche, catalogue number 11096176001-50 g) to 47.5ml MAB. Dissolve by heating (prevent boiling). Cool and add 2.5ml of goat serum (Invitrogen, catalogue number 16210072).

7 50X dilution stock of Anti-Digoxygenin (pre-adsorption)

- Add 10µl 1X Dig-antibody (Roche, Catalogue number 11093274910) to 500µl blocking solution[6]
- Add 30 dechorinated embryos, shake for four hours or more at 4°C

8 AP buffer

Prepared just prior to use to avoid precipitation

- **Final concentrations of components:**
  - 0.1M Tris[15], pH 9.5 (Sigma, product number 93349-100G)
  - 0.1M NaCl (Sigma, product number S3014-500G)
  - 50mM $MgCl_2$ (Sigma, product number M8266-100G)
  - 0.1% Tween20 (Sigma, product number P2287)
  - Add dH_2O to desired amount

9 3M Sodium Acetate, pH 5.2 (50 ml)

- Add 20.4g Sodium Acetate (Sigma, product number S8750-250G) to 80ml dH_2O
- Adjust pH to 5.2 with acetic acid
- Add dH_2O to 100ml

10 4M Lithium Chloride, pH (50ml)

- Add 8.5g LiCl (Sigma, product number L9650-100G) to 40ml dH_2O and dissolve
- Add dH_2O to 50ml

11 1000X stock Fish Water (500ml):

- Dissolve 30g artificial sea salt (Sigma, product number S9883-500G) in 500ml dH_2O

12 20X Ringer Stock Solution

- 2.3M NaCl (Sigma, product number S3014-500G)
- 58mM KCl (Sigma, product number P9541-500G)
- 36mM $CaCl_2$ (Sigma, product number C1016-100G)
- 100mM Hepes (Sigma, product number H3375-100G) buffer[16], pH 7.2

13 Pronase (Roche, product number 11459643001-1g) stock solution

- Make 20mg/ml stocks in dH_2O and store at -20°C in microcentrifuge tubes in quantities of 500µl
- Working solution is 1mg/ml pronase (20x dilution in Ringer solution)

14 2% Methylcellulose (Sigma, product number 274429-100G)

- Add 1g methylcellulose to 50ml dH_2O.
- To dissolve, either place on a gentle shaker at 4°C until fully dissolved, which can take many days, or freeze and thaw many times, mixing gently while thawing. This also takes days
- For use with explants, methylcellulose was further diluted to 0.5% in Ringer solution (to 1X Ringer) and 250x dilution gentamycin (Sigma, product number G1272-10ML).

15 Tricaine stock solution

- Make 4mg/ml stock solution by adding tricaine powder (Aldrich, product number A5040-25G) to water and Tris (Sigma, product number 93349-100G), pH 9.0 to 20mM, adjust solution to pH ~7.0 and freeze
- For working solution further dilute to ~8mg/ml in fish water or other working solution, e.g. methylcellulose

16 EDTA 0.5M stock solution, ph 8.0 (100ml)

- Add 18.4g EDTA (Sigma, product number EDS-100G) to 80ml dH_2O
- Add 5M NaOH (Sigma, product number S8045-500G) whilst stirring, until EDTA begins to dissolve and adjust pH to 8.0 when EDTA is dissolved
- Add dH_2O to 100ml

17 1M Tris Buffer, pH 9.5 (100ml)

Add 12.1g Tris base (Sigma, product number 93349-100G) to 80ml dH_2O, adjust pH to 9.5 and add dH_2O to 100ml.

18 1M Hepes buffer, pH 7.2 (100ml) 23.8g to 100ml

- Add 23.8g Hepes (Sigma, product number H3375-100G) to 80ml dH_2O
- Adjust pH to 7.2, add dH_2O to 100 ml

19 0.1M EGTA (50ml)

- Add 1.9g EGTA (Sigma, product number E3889-10G) to 40ml dH_2O
- Adjust pH with NaOH (Sigma, product number S8045-500G) to dissolve (7.5/8.0)
- Add dH_2O to 50ml

20 0.5M Pipes buffer, pH 6.8 (100ml)

- Add 16.1g Pipes (Sigma, product number P6757-25G) to 70ml dH_2O
- Adjust pH to 6.8, add dH_2O to 100ml

Chapter 2. Research Chapter 1

Induction and patterning of trunk and tail neural ectoderm by the homeobox gene *eve1* in zebrafish embryos

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A functional analysis of the *eve1* gene in neural development. Although crucial secreted neural inducers such as Fgf and Bmp-antagonists have been identified, many transcription factors that function upstream and downstream of these signalling molecules have not been identified, and often, even if identified, the processes they regulate are unclear or unknown. This was the case for the zebrafish *eve1* gene, a known target of Bmp, Fgf and Wnt signalling during embryonic development (Griffin et al., 1995; Kudoh et al., 2004; Ramel and Lekven, 2004; Ueno et al., 2007). The zebrafish *eve1* gene is a member of the *eve/evx* family of homeobox genes (Joly et al., 1993), originally characterised in *Drosophila melanogaster* where it was found to have a role in segmentation during larval stages. *Evx* paralogues, *evx1*, *evx2* and *eve1* are thought to have originated by genome duplication events from a single *evx* gene existing in the last common ancestor of the chordates (Avaron et al., 2003). Subsequently, *eve1* was lost in the tetrapod lineage but was maintained in the lineage leading to the teleosts. Comparison of expression patterns of the *evx1* orthologues in zebrafish and *Xenopus laevis* (*Xhox3*), as well as the zebrafish *eve1* gene, suggests that in zebrafish as well as in other teleosts such as medaka and fugu the function of the vertebrate *evx1* gene in embryogenesis has been subdivided between *evx1* and *eve1* (Avaron et al., 2003). Early expression patterns of *Xhox3* and *eve1* in ectoderm and mesoderm overlap during gastrulation, and both genes are expressed in the tail bud (Avaron et al., 2003; Joly et al., 1993; Ruiz i Altaba and Melton, 1989). During somitogenesis, both *evx1* and *Xhox3* are involved, among others, in neurogenesis as well as the development of the hindgut.

In vertebrates, however, the function of *evx* orthologues during gastrulation has not been well characterised at the molecular level. Both *eve1* and *Xhox3* have generally been defined as ventro-posterior markers with a role in tail development. In zebrafish, overexpression of *eve1* was first shown to affect A/P patterning in a concentration-dependent manner, leading to anterior truncation at low doses and loss of posterior patterning at high doses (Barro et al., 1995). It was also found that *eve1* overexpression led to tail duplications (Barro et al., 1995), suggestive of a role for *eve1* in tail development. Subsequently, it was discovered that *eve1* expression was activated by signals with a role in the zebrafish 'tail organiser' (Agathon et al., 2003). These data further reinforced the notion that *eve1* was required for tail development, although no loss-of-function studies were ever carried out to test this.

However, the phenotypes of *eve1* overexpression in zebrafish, together with the early expression patterns of these genes in both ectoderm and mesoderm and the fact they it was downstream of major signalling molecules, was suggestive of a much wider and more important role for *eve1,* possibly in posterior neural development. To address if this was indeed the case, we set out out to perform a functional analyses of zebrafish *eve1*, concentrating on axis formation and posterior (trunk and tail) neural development.

Induction and patterning of trunk and tail neural ectoderm by the homeobox gene *eve1* in zebrafish embryos

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Abstract

In vertebrates, even-skipped related (evx) homeodomain transcription factor encoding genes are expressed in the posterior region during embryonic development, and overexpression experiments have revealed roles in tail development in fish and frogs. Here we analysed the molecular mechanisms of posterior neural development and axis formation regulated by *eve1*. We show that *eve1* is involved in establishing trunk and tail neural ectoderm by two independent mechanisms: First, *eve1* posteriorises neural ectoderm via induction of *aldh1a2*, which encodes an enzyme that synthesises retinoic acid; second, *eve1* is involved in neural induction in the posterior ectoderm by attenuating Bmp expression. Further, *eve1* can restore trunk neural tube formation in the organiser-deficient *ichabod*$^{-/-}$ mutant. We conclude that *eve1* is crucial for the organisation of the antero-posterior and dorso-ventral axis in the gastrula ectoderm, and furthermore has trunk and tail promoting activity.

Introduction

The molecular mechanisms of neural induction and patterning in chordate embryos have been extensively studied in animals such as amphibians, fish, chick and mouse (1, 2). Initial analyses in amphibians revealed that the dorsal organiser (Spemann's organiser) induces neural (CNS) fates in dorsal ectoderm, and subsequently vegetal marginal signals posteriorise proximal neural ectoderm to induce trunk and tail neural cell fates such as spinal cord and caudal hindbrain, while distant animal pole cells give rise to rostral neural tissues including the forebrain, midbrain and part of the hindbrain (3). Molecular analyses of organiser activity has uncovered multiple molecules crucial for neural induction, including the secreted Bmp antagonists Chordin, Noggin and Follistatin, leading to the conclusion that Bmp inhibition is crucial for neural induction (1, 2). Besides Bmp antagonists, Fgf has an important role in neural induction in many species(4-8).

Concomitant with, and subsequent to, neural induction, neural ectoderm is posteriorised by the activity of several factors, among them Fgf (5, 7, 9-11), Wnt (11-14) and retinoic acid (RA) (11, 15-17). RA is essential for posterior neural development in vertebrates, being required for the specification of the future hindbrain and anterior spinal cord (18, 19). In zebrafish Fgf and Wnt signalling posteriorise neural ectoderm by suppressing anterior-specific gene expression independently of RA while inducing posterior genes in an RA-dependent process (11).

Some of the transcription factors acting downstream of posteriorising signals are known and include Homeodomain proteins of the Hox cluster (6, 20), Cdx (21, 22) and Evx (7, 23) families. In the zebrafish gastrula, posterior neural ectoderm and mesoderm are marked by the expression of *eve1,* a member of the *eve/evx* family of homeobox genes that encode transcriptional repressors. *Evx* genes have been implicated in a conserved role in posterior body patterning in a variety of species, including the fly, mouse, worm, frog and zebrafish (24-26). In zebrafish, overexpression of *eve1* disrupts antero-posterior (A/P) patterning in a concentration-dependent manner, leading to loss of head structures and tail duplications at lower doses and mispatterning and loss of posterior tissue at higher doses (25). At the gastrula stage, *eve1* expression is restricted to the ventral side and is maintained by Bmp signalling, a key ventralising molecule. *Eve1* has

been regarded as a ventral marker gene with a presumed role in tail development. However, *eve1* expression begins at blastula stage at around 30% epiboly when it covers most of the margin with the exception of the presumptive organiser (27), suggestive of a potentially wider role for *eve1* in posterior development. Thus it is not clear if *eve1* is involved in trunk development in addition to its accepted role in tail development. In addition, little is known about the mechanism of *eve1* function, and no loss-of-function data in fish have been reported so far. Using loss- and gain-of-function strategies we show here that *eve1* regulates trunk and tail development. We find that *eve1* affects the formation of trunk and tail neural ectoderm via two molecular mechanisms: Induction of the neural ectoderm in both trunk and tail regions at the gastrula stage, at least partly by titration of BMP levels; and posteriorisation of neural ectoderm via an RA signal. Furthermore our data provide evidence that *eve1* exerts its organising activity as a transcriptional repressor.

Results

Overexpression of *eve1* causes anterior truncation, induces posterior neural markers, and suppresses markers for anterior neural and non-neural tissues. To determine the role of *eve1* in antero-posterior (A/P) patterning, we overexpressed *eve1 in vivo* and analysed the expression of *otx2*, an anterior neural marker and *hoxb1b*, a marker for prospective posterior (trunk and tail) neural tissue (7, 20, 28). Phenotypic analysis confirmed previous results (25, 29) such as truncation of head structures (75%, n=32; Fig. 1B), while some embryos showed more severe effects with loss of head and trunk (13%, n=32; Fig. 1C). The only remaining anteriorly-positioned structure was the heart, which continued to beat. Consistent with the lack of anterior structures, *otx2* is suppressed in *eve1* mRNA injected embryos (94%, n = 17; Fig. 1E), whereas *hoxb1b* expression is partially (15%) or circumferentially (85%) expanded (n = 20; Fig. 1G).

Surprisingly, in *eve1* injected embryos *hoxb1b* expression expanded to include the prospective epidermal domain, raising the possibility that *eve1* may have a role in neural induction in addition to its role in A/P patterning. To test this notion, we analysed the expression of three additional neural markers, *sox3*, *zic2a* and *sox31*, and the epidermal marker, *p63*. In *eve1* mRNA injected embryos, the *sox3* (95%, n = 21), *zic2a* (95%, n = 20) and *sox31* (83%, n = 18) positive domain covers most of the embryo including the prospective epidermal domain (Fig.

1I,K,M), with the concomitant suppression of *p63* expression (100%, n = 24; Fig. 1O). Together, these results suggest that *eve1* acts both as a posteriorising and a posterior neural promoting factor.

Zebrafish *eve1* functions as a repressor in posterior neural development. To carry out loss-of-function analyses we first used an *eve1* antisense morpholino (MO) directed at the intron1/exon2 acceptor splice site (eve1MO - see Materials and Methods; and see below). This MO led to the reduction of mature mRNA and the appearance of an alternatively spliced form of mRNA in the embryo (Supplemental Fig. S1A). The phenotypes in embryos injected with eve1MO complement those of *eve1* overexpression, namely a loss of posterior structures with largely unaffected head structures (Fig. 2B,C). In the most severe phenotype, most of the trunk and tail tissue is absent (Fig. 2C) (see supplemental Fig. S1B). As *eve1* is thought to function as a transcriptional repressor (30-32) we reasoned that fusion of the *eve1* homeodomain (DNA binding) to the activator domain of the viral protein VP16 would generate an antimorphic construct (see Materials and Methods). Similar to the effect of eve1MO, injection of eve-VP16 led to a variable reduction of the posterior axis (71%, n = 28; Fig. 2E,F)

We next examined the expression of *otx2* and *hoxb1b* in eve1MO injected embryos. Consistent with the gain-of-function analysis, *hoxb1b* expression was strongly suppressed (96%, n = 28; Fig. 2H), but we found no noticeable difference in *otx2* expression. We further examined the expression of *aldh1a2* (formerly *raldh2*) which codes for an RA synthesising enzyme expressed in posterior paraxial mesoderm (33), as well as *meis3*, another posterior-specific neural gene (34). The expression of *aldh1a2* was much reduced (71%) or absent (29%) in eve1MO injected embryos (n = 24; Fig. 2L); *meis3* showed a similar result (supplemental Fig. S2E). To test the specificity of the eve1MO we co-injected *eve1* mRNA, and found that the expression of both *aldh1a2* (95%, n = 21) and *hoxb1b* (96%, n = 28) was restored and slightly expanded as compared to uninjected embryos (supplemental Fig. S1C1-C6). Furthermore, we found that epiboly defects caused by eve1MO were rescued by coinjection of *eve1* RNA (supplemental Fig. S1D). These results indicate that the morpholino is specific for *eve1*. In further analysis of *eve1* loss-of-function, we found that the expression of *hoxb1b* (75%, n = 16) and *aldh1a2* (88%, n = 16) was suppressed in eve1-VP16 injected embryos (Fig. 2I,M).

Eve1-VP16 and eve1MO show a similar phenotype that is complementary to that of *eve1* overexpression, suggesting that *eve1* exerts its posteriorising influence as a repressor. To explore this possibility, we fused the *eve1* homeodomain to the repressor domain of the *Drosophila* Engrailed (Eng) protein (32, 35) (see Materials and Methods). Injection of eve1-Eng led to expansion of *hoxb1b* (70%, n = 20) and *aldh1a2* (56%, n = 16) expression (Fig. 2J,N), complementary phenotypes to those elicited by eve1MO and eve1-VP16. Since overexpression results suggested a possible role for *eve1* in neural induction, we also looked at *bmp2b* and *bmp4* expression under conditions of *eve1* gain- and loss-of-function. Injection of eve1MO expanded both *bmp2b* (83%, n = 18; Fig. 2P) and *bmp4* (63%, n = 16; Fig. 2T) expression as did eve1-VP16 (76%, n = 17; Fig. 2Q) (70%, n = 23; Fig. 2U) while both were suppressed by eve1-Eng (74%, n = 19; Fig. 2R) (90%, n = 20; Fig. 2V). Together these data suggest that *eve1* acts as a transcriptional repressor in promoting posterior neural development.

***Eve1* induces *hoxb1b* expression via RA signaling.** As *eve1* induces *hoxb1b* expression (Fig. 1G) and eve1MO injection led to loss of *aldh1a2* expression (Fig. 2L), we investigated whether this effect is mediated by the RA pathway by injecting *eve1* and *cyp26a1* mRNAs in different combinations and examining *hoxb1b* as well as *otx2* expression. Cyp26a1 is an RA degrading enzyme and overexpression of Cyp26a1 allows examination of *eve1* function under conditions of suppression of RA signalling (11). *Otx2* expression was suppressed by injection of *eve1* mRNA alone, while *hoxb1b* expression was expanded (Fig. 3B,F). However, when *eve1* and *cyp26a1* mRNAs are co-injected, both *otx2* and *hoxb1b* are suppressed (Fig. 3C, G), suggesting that suppression of *otx2* by *eve1* is RA-independent, while expansion of *hoxb1b* is dependent on RA. As previously reported (11), *cyp26a1* injection alone had no significant effect on *otx2* expression (Fig. 3D), but *hoxb1b* was suppressed (Fig. 3H). To further examine *eve1* function upstream of RA in *hoxb1b* induction, we injected *eve1* mRNA and analysed the expression of *aldh1a2* and *cyp26a1*. *Eve1* causes the expansion of the *aldh1a2* expression domain and suppression of *cyp26a1* in anterior neural ectoderm (Fig, 3J,L). Considering that RA is a long range signalling molecule (36), the induction of *aldh1a2* and suppression of *cyp26a1* may provide the mechanism of *hoxb1b* induction in the animal pole by overexpression of *eve1*.

To complement the *cyp26a1* and eve1MO data, we sought further confirmation that *eve1* functions upstream of *aldh1a2*, and presumably RA, in inducing *hoxb1b*. Injection of an antisense morpholino directed against the *aldh1a2* gene (aldMO – see Materials and Methods) alone resulted in a marked reduction of *hoxb1b* expression (95%, n = 22; Fig. 3N), and this inhibition could not be rescued by co-injection of *eve1* mRNA (100%, n = 26; Fig. 3O). A similar result was obtained with another RA-responsive gene, *meis3* (11) (Supplemental Fig. 2A-D)**.** Further, there is synergism between eve1MO (2ng/nl) and aldMO (low) action in the regulation of *hoxb1b* expression. Low concentrations of either MO alone led to a partial and variable reduction of the *hoxb1b* signal, whereas co-injection of both MOs at the same low doses led to the complete abolition of *hoxb1b* expression in most injected embryos (72%, n = 25), while in the remainder it was variably reduced (Fig. 3P-S). Again, we obtained similar results with *meis3* (Supplemental Fig. 2 E-H). These data provide strong evidence that *eve1* functions upstream of RA in positively regulating the expression of *hoxb1b* and *meis3*, and possibly of other RA-responsive genes as well.

***Eve1* promotes neural ectoderm by antagonising Bmp expression.** Overexpression of *eve1* can suppress epidermal and induce neural marker genes (Fig. 1), suggesting that *eve1* and BMP signalling have antagonistic roles in neural versus epidermal specification in the ectoderm. We used *eve1* and *bmp2b* mRNA injection to determine whether Bmp signalling can suppress *eve1*-mediated induction of the neural markers *hoxb1b* and *sox3*. When *eve1* and *bmp2b* mRNAs are co-injected, both *sox3* (100%, n = 24) and *hoxb1b* (96%, n = 25) expression is suppressed compared to control and *eve1* injected embryos (Fig. 4A-F). Since eve1MO injection leads to the variable expansion of both *bmp2b* and *bmp4* (Fig. 2P,T) we tested for possible inhibition of *bmp4* and *bmp2b* expression in *eve1* overexpressing embryos; *eve1* mRNA injection inhibited *bmp4* (88%, n = 17) and *bmp2b* (89%, n = 19) expression (Fig. 4H,K). Thus *eve1* can regulate expression levels of Bmp in gastrula embryos, suggesting that Bmp signalling is downstream of *eve1*. This suggestion is supported by the fact that injection of *bmp2b* mRNA strongly induced *bmp4* expression in the presence of exogenous *eve1* (Fig. 4I; 100%, n = 20). Likewise, injection of a low concentration of *bmp2b* RNA (7pg/nl) together with *eve1* abolished the ability of *eve1* to induce neural marker or suppress *bmp4* (supplemental Fig. S3).

To further explore the antagonistic nature of *eve1* and Bmp signalling in ectodermal fate specification, we examined their combined effects on the expression of the neural marker *sox3* and the epidermal marker *foxi.1*. At low concentrations, neither *eve1* mRNA (10pg/nl) (100%, n = 25) nor bmp2bMO (100%, n = 26) can induce *sox3* (Fig. 4 M,N) or suppress *foxi.1* (Fig. 4Q,R).However, when *eve1* and bmp2bMO were coinjected at low concentrations, *sox3* was expanded (95%, n = 19; Fig. 4O) while *foxi.1* was suppressed (94%, n = 17; Fig. 4S). These results support the view that *eve1* has a role in posterior neural induction via antagonism of Bmp signalling.

***Eve1* rescues posterior dorsal axis and expression of *hoxb1b* in *ichabod*$^{-/-}$ mutants.** *Ichabod*$^{-/-}$ *(ich*$^{-/-}$*)* mutants have reduced expression of *beta-catenin 2* that leads to loss of the organiser, ventralisation, and a loss of head and trunk structures (37-39) (Fig. 5A). In such embryos, Bmp expression is expanded dorsally and this is thought to account for the observed ventralisation. Since *eve1* antagonises Bmp signalling and has a role in posteriorisation, we injected *ich*$^{-/-}$ embryos with *eve1* mRNA to test whether *eve1* could rescue trunk and tail development. *Eve1* injected *ich*$^{-/-}$ embryos at 24 hours post fertilization (hpf) showed a partial rescue of the posterior dorsal axis in the trunk and tail (100%, n = 38; Fig. 5B) when compared to uninjected embryos (100%, n = 28; Fig.5A). Expression of *hoxb1b* that is absent in *ich*$^{-/-}$ mutants at gastrula (Fig. 5D) (39) is restored in *eve1*-injected embryos (Fig. 5E). Expression of the neural marker *sox3* initially occurs in the trunk/tail domain of gastrula stage *ich*$^{-/-}$ embryos and is gradually reduced and becomes faint by 24 hpf (Fig. 5G), but is retained in the rescued trunk and tail neural tube in *eve1*-injected *ich*$^{-/-}$ embryos (Fig. 5H). Similarly, the neural expression domains of *elavl3* (formerly *huC*), *pax2a* and *egr2b* (formerly *krox20*), which are variably reduced or lost in 24 hpf *ich*$^{-/-}$ embryos (Fig. 5J,M,P) are partially restored in the trunk and tail after *eve1* injection (penetrance = 100%; Fig. 5K,N,Q). These data indicate that *eve1* can induce and maintain some posterior dorsal structures as well as neural gene expression in the trunk and tail of organizer-defective *ich*$^{-/-}$ embryos.

Discussion

***Eve1* promotes posterior development as a transcriptional repressor.** The Even-skipped-related proteins have previously been shown to function as transcriptional repressors in *Drosophila* development (31, 32, 40, 41). Our data suggest that Eve1 also functions as a transcriptional repressor in vertebrates in promoting posterior development and that eve1-VP16 acts as a dominant-negative form. Overexpression of wild-type *eve1* and eve1-VP16 results in opposite phenotypes: *Eve1* suppressed head formation while eve1-VP16 suppressed trunk and tail formation, with consistent effects on the expression of markers genes. Further, inhibition of *eve1* expression by a morpholino phenocopied the eve1-VP16 phenotype, while eve1-Eng phenocopied the effects of *eve1* overexpression on posterior neural markers and Bmp expression. As a result, it is likely that upregulation of *marker* genes by *eve1* is indirect. In neural induction and dorsalisation, suppression of Bmp by *eve1* could explain the induction of neural-specific genes while in posteriorisation, *eve1* may well repress an as yet unidentified repressor of *aldh1a2* (Fig. 6)

***Eve1* induces posterior cell fates via retinoic acid.** Through gain and loss-of-function analyses of *eve1* we explored the mechanisms of *eve1* function in zebrafish trunk and tail development. Overexpression of *eve1* suppressed head structures and in the trunk and tail expanded neural and suppressed epidermal cell fates. These data imply a role for *eve1* in both posteriorisation and neural induction (Fig. 6). The regulation of RA levels via induction of *aldh1a2* and suppression of *cyp26a1* is necessary and sufficient for the induction of the posterior gene *hoxb1b* by *eve1*. This conclusion is supported by the observation that eve1MO inhibits *aldh1a2* expression, and that *eve1* induction of *hoxb1b* and *meis3* is mediated by *aldh1a2* since neither gene could be induced by *eve1* in aldMO-injected embryos. *Eve1* suppresses the anterior gene *otx2* via an RA-independent route, implying two separate mechanisms for *eve1*-mediated posteriorisation: RA-dependent posterior induction and RA-independent anterior suppression. This distinction may assist in creating a border between anterior (RA negative) and posterior (RA positive) gene expression domains. Analogous separable mechanisms have already been observed for two other posteriorising genes, Fgf (9-11) and Wnt (13, 42, 43). Similar to the situation after reduction of RA signalling (11) but unlike the effect of Fgf and Wnt (11), no posterior expansion of anterior gene expression was seen in *eve1* morphants, suggesting

that suppression of anterior genes may not be a primary role of *eve1*. Considering that suppression of RA alone does not expand *otx2* these results further support the idea that *eve1* posteriorises the embryos via the RA pathway. Otherwise we have shown that *eve1* functions in a similar manner to Fgf, RA and Wnt posterior signalling, and as *eve1* is induced by Fgf (11, 23), it is tempting to suggest that *eve1* acts downstream of Fgf in mediating posteriorisation signals.

A role for *eve1* in neural induction. A surprising finding was a role for *eve1* in induction of posterior neural markers. . In embryos where *eve1* is overexpressed, the expression of *sox3, sox31* and other neural markers (see Fig. 1) is expanded through the entire ectoderm, including the animal pole and presumptive epidermis. In these embryos the epidermal marker *p63* is suppressed, implying that prospective epidermal tissue has been re-specified as neural. This conclusion is supported by the finding that *eve1* is necessary for the expression of *hoxb1b* (Fig. 2) and *meis3* (supplemental Fig. S2). In addition, *eve1* suppresses Bmp expression in the gastrula embryo, but *eve1* cannot induce the expression of either *sox3* or *hoxb1b* in the presence of Bmp. Thus it appears that Eve1 does not antagonise Bmp signalling but rather suppresses Bmp expression. As a consequence, a synergistic relationship exists between *eve1* and Bmp2bMO in the induction of *sox3* and suppression of *foxi.1*, a marker for epidermal tissue (Fig. 4). Together these data suggest that *eve1* enhances neural induction by reducing the expression of Bmp in the gastrula ectoderm (Fig. 6).

Further evidence for *eve1*-mediated neural induction and maintenance comes from the experiment using ventralised *ich*$^{-/-}$ embryos. In *ich*$^{-/-}$ embryos, the expression of *hoxb1b, elavl3, pax2a* and *egr2b* is low or absent, *sox3* is only weakly expressed, and neural tissue is greatly reduced at 24 hpf. Injection of *eve1* mRNA into these mutant embryos rescued *hoxb1b* expression and partially restored the expression of *elavl3, pax2* and *egr2b* with a penetrance of 100%. Likewise, overexpression of *eve1* restored a posterior dorsal axis in *ich*$^{-/-}$ mutant embryos. We suggest that *eve1* elicits these effects at least partially by a reduction of Bmp expression, thereby substituting in the posterior domain for the absence of organiser-derived Bmp antagonists.

Taken together the data suggest that *eve1* has dorsalising activity (including neural induction) via regulation of Bmp expression in the gastrula ectoderm. Many genes that regulate Bmp expression and signalling along the dorso-ventral

axis have been reported (for review, see (44)). For example, positive regulators of Bmp, such as Bmp2b and 4 in zebrafish (45) and ADMP (46) in Xenopus are expressed in the organiser and may contribute to fine tuning of Bmp expression and signalling. Besides secreted molecules, many transcription factors have also been shown to suppress Bmp expression and to dorsalise the embryo (e.g. *hex* (47), *iro3* (48)). Here, we propose to add *eve1* as another regulator of Bmp activity that is unique in the sense that *eve1* expression is maintained by the Bmp signal in the ventral side and in turn limits Bmp expression (negative feedback). The variety of mechanisms regulating Bmp expression levels is a manifestation of the importance that precise control of the timing and level of Bmp signalling is crucial in regulating neural versus non-neural patterning, antero-posterior patterning, cell migration and some aspects of gastrulation.

***Eve1* as an effector of the posterior organiser.** *Eve1* has been thought to play an important role in tail development, as overexpression of *eve1* induced ectopic tail structures (25), and induction of ectopic tails by Wnt, Bmp and Nodal induces *eve1* expression (49). Although *eve1* is only expressed in the prospective tail region in the late gastrula, *eve1* expression is much wider in the blastula and early gastrula, being expressed in prospective trunk mesoderm and neural ectoderm at that stage (7, 29). *Eve1* is positively regulated by Fgf (7, 23)and Wnt (50, 51), two signaling pathways that are critical for induction of both trunk and tail structures. Furthermore, it has been proposed both in *Xenopus* and zebrafish that tail formation is a continuation of trunk formation (7, 52) and that both occur as interactions between dorsal and ventral cells. Consindering these ideas and our current data, we propose that *eve1* acts as a posterior organiser in regulating posterior specificity as well as dorso-ventral specificity for trunk and tail tissue. *Eve1* may function as a posterior dorsal gene in the sense that it induces caudal neural tissue. The contrasting function of *eve1* might be understood in the light of the observatiosn that it represses Bmp but enhances RA (through *aldh1a2*). Thus *eve1* would be required for posterior development (RA, and possibly other functions) but limit the ventralizing action of Bmp to facilitate formation of caudal neural tissue

Materials and Methods

RNA probe synthesis and *in-situ* hybridisation. Probes used (except *aldh1a2*), antisense RNA probe synthesis and *in-situ* hybridisation procedures have been previously described (34). RZPD clone IMAGp998B2417171Q1 in pExpress1 was used for synthesis of the *aldh1a2* probe (EcoR1/T7 for probe synthesis).

Constructs, mRNA synthesis and mRNA injection. Capped mRNAs were synthesised by mMessage mMachine SP6 kit (Ambion) according to the manufacturer's instructions. Unless otherwise stated mRNA concentrations (per nl) used for injections were: *bmp2b* 50pg; *eve1* 20pg; eve1-VP16 300pg; eve1-Eng 300pg, *cyp26a1* 500pg. mRNAs were injected through the intact chorion into all blastomeres at the one- to two-cell stage. To make the eve1-VP16 and the eve1-Eng fusion constructs, the *eve1* homeodomain was amplified by PCR (F-primer GCCCTCGAGCAAGAATACTGCAAAGAAAGT and R-primer GCCTCTAGAGTGGATTTGGCCAGTGTAGAC) and subcloned into a pCS2_VP16 and pCS2_Eng vector (53).

Morpholino analysis and injection. *Eve1* mRNA (mildly) and eve1MO (more severely) affected epiboly movements which made it difficult to analyse gene expression in later stages in eve1MO-injected embryos and and in these embryos we concentrated on earlier marker analyses. The eve1MO (GeneTools, LLC) corresponds to the intron1/exon2 acceptor splice site: 5'-CTGTCCTCTGCTACTGAAAAGAATA -3'. The eve1MO was injected at 5ng/nl unless otherwise indicated. The bmp2bMO (GeneTools, LLC) 5'-GCGGACCACGGCGACCATGATC -3' targets the transcription start site; it was used at 0.1ng/nl. The *aldh1a2* MO (Open Biosystems) (54) has the sequence 5'-GTTCAACTTCACTGGAGGTCATCGC - 3', and was used at 1:2 (high) and 1:4 (low) dilution from a 1mM stock. In all cases, 1-2 nl of solution was injected into the yolk as close as possible to the cells of 1-4 cell stage embryos.

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Figures and Figure Legends

Figure 1.

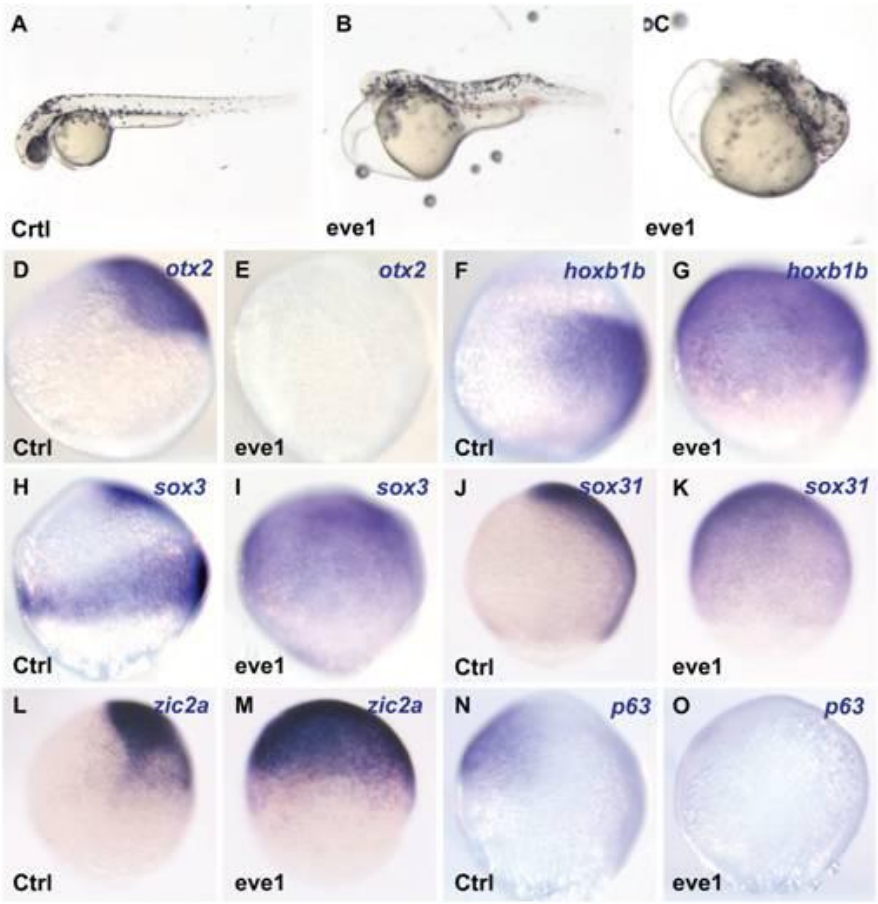


Fig. 1. *Eve1* overexpression causes anterior truncation, suppression of anterior markers and induction of posterior markers. Zebrafish embryos were injected with *eve1* mRNA as indicated at the bottom left corner; Ctrl, uninjected controls. (A-C) Embryos at 48 hpf. (B, C) *Eve1* mRNA injected embryos showing anterior truncation and progressive loss of trunk and tail. (D-O) *In situ* hybridisation of control and *eve1* mRNA-injected embryos at 80% epiboly. Lateral views, dorsal to the right (where discernible). Genes analysed are indicated in the top right hand corner. Expression of the anterior gene *otx2* and epidermal gene *p63* was suppressed (E,O) whereas the expression of *hoxb1b* was expanded by *eve1* overexpression (G). Expansion was also observed for *sox3, zic2a* and *sox31* (H-M).

Figure 2.

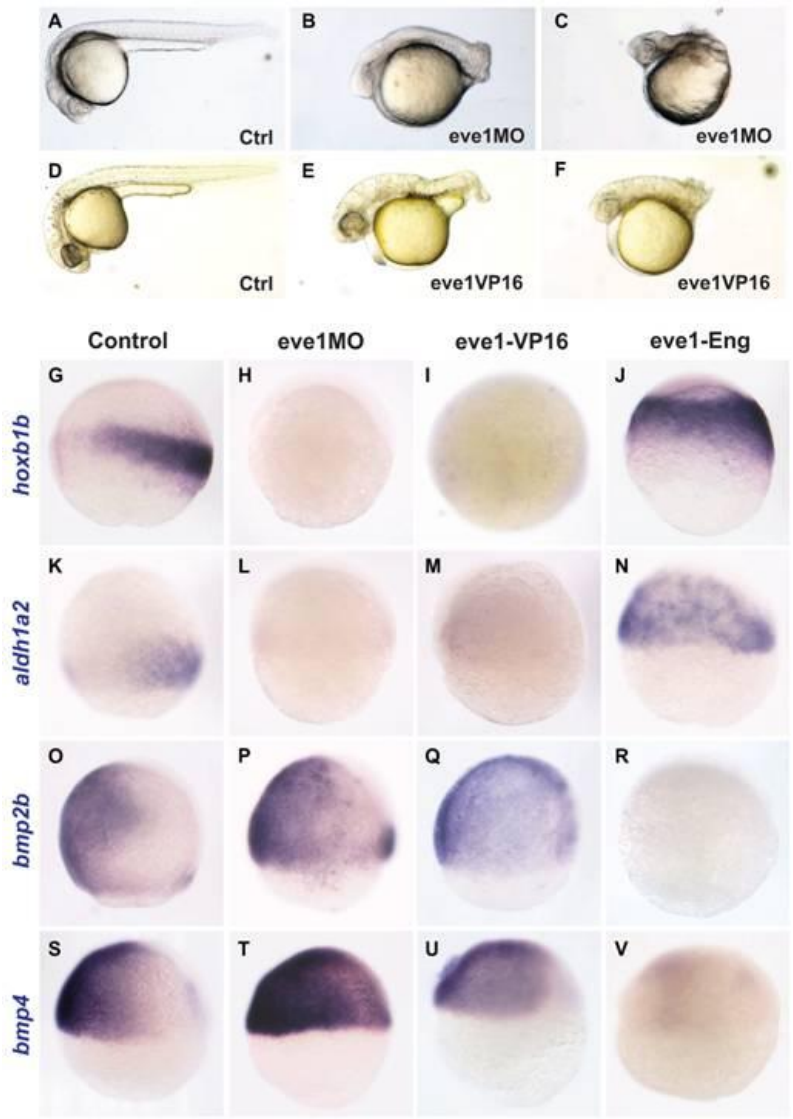


Fig. 2. *Eve1* depletion suppresses trunk and tail and *eve1* acts as a repressor. Zebrafish embryos were injected with eve1MO (B,C,H,L,P,T) or with eve1-VP16 mRNA (E,F, I,M,Q,U) as shown to the left of panels (B,C,E,F) or at the top of the columns for the rest. Embryos at 24 hpf (B,C) and at 28 hpf (E,F), showing variable loss of trunk and tail tissue. (G-V) *In situ* staining of embryos at 70-80% epiboly (G-R) and 60% epiboly (S-V), lateral views, dorsal to the right (where discernible), with probes shown at the left of the rows. *Hoxb1b* and *aldh1a2* are suppressed by eve1MO (H,L) and eve1-VP16 (I,M), while expression of both genes is expanded in eve1-Eng injected embryos (J,N). Conversely, *bmp2b* and *bmp4* expression domains are expanded in eve1MO (P,T) and eve1-VP16 (Q,U) injected embryos, while (R,V) eve1-Eng suppresses expression of both Bmps.

Figure 3.

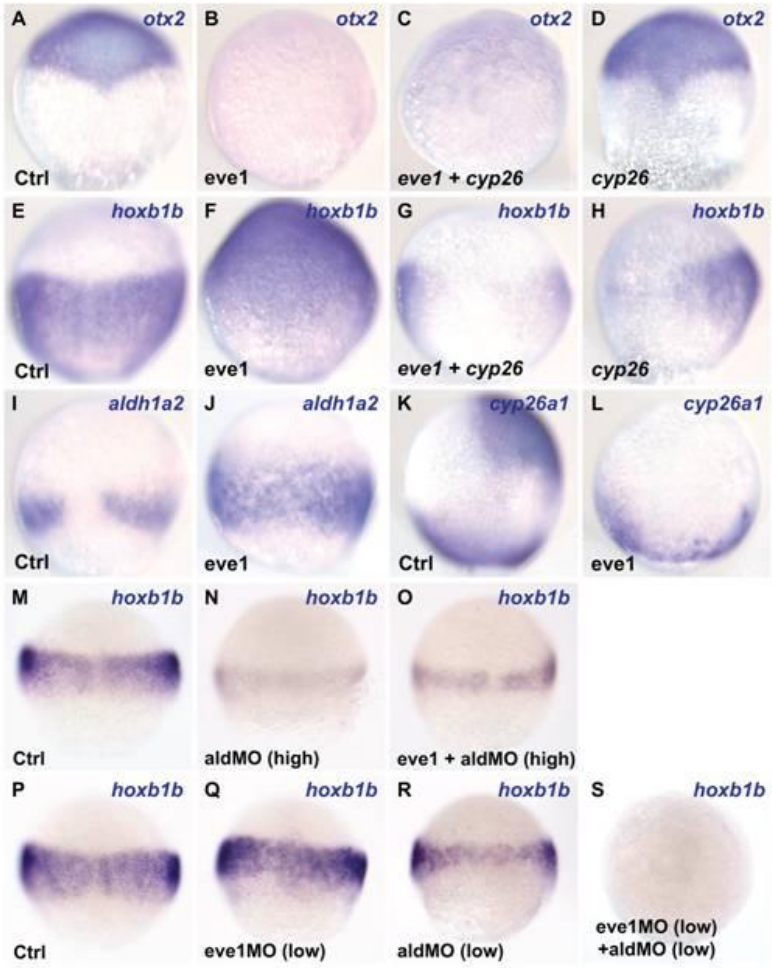


Fig. 3. *Eve1* induces *hoxb1b* expression via an RA signal. (A-J, M-S) Dorsal and (K,L) lateral views (where discernible, dorsal to the right) of zebrafish embryos fixed for *in situ* staining at 80% epiboly (A-L) and 60% epiboly (M-S). Injections are indicated at the bottom left, genes analysed at the top right. (A-D) Suppression of *otx2* by *eve1* does not depend on RA as it resists overexpression of the RA metabolizing enzyme Cyp26a1. (E-H) *Eve1*-mediated induction of *hoxb1b* does not occur when *eve1* and *cyp26a1* mRNAs are co-injected (G), and *cyp26a1* injection alone suppresses *hoxb1b* expression (H; only one of two cells was injected in this embryo). (I,J) *Eve1* induces *aldh1a2* expression. (K,L) Anterior expression of *cyp26a1* is suppressed by *eve1*, but remains unaffected at the margin. (M-O) Injection of high concentrations of aldMO and *eve1* mRNA (see Materials and Methods). *Eve1* cannot rescue *hoxb1b* expression in aldMO injected embryos (O). (P-S) Injection of low concentrations of eve1MO (2ng/nl) and aldMO showed synergism in the suppression of *hoxb1b*.

Figure 4.

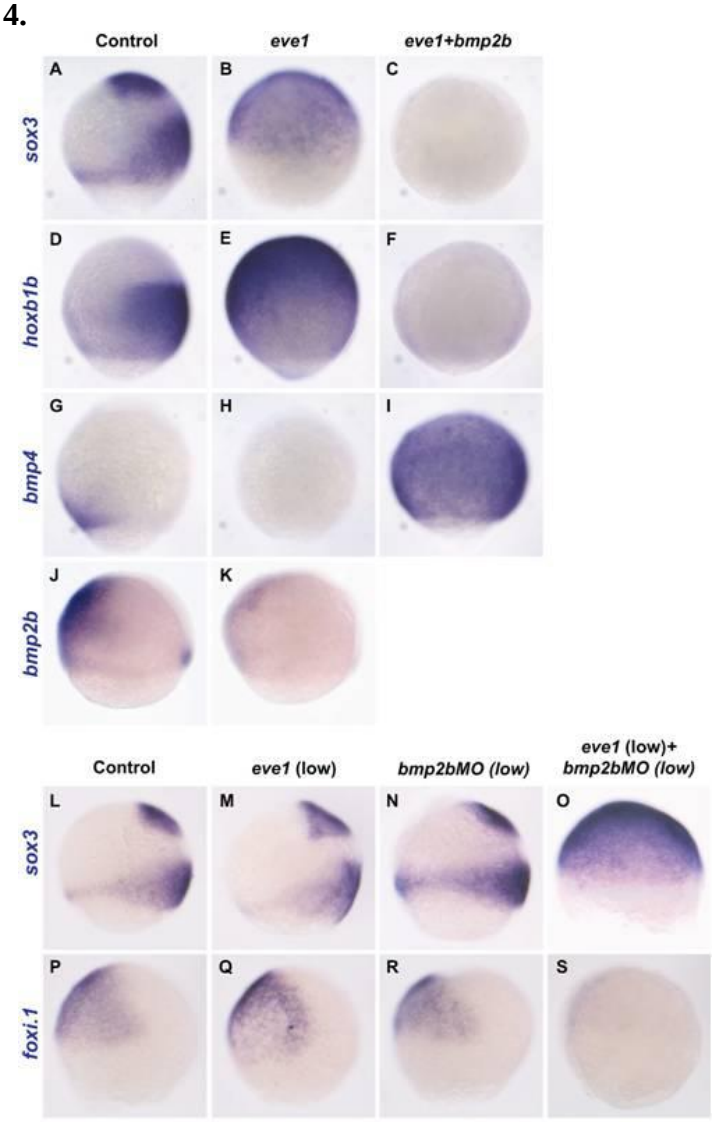


Fig. 4. Interactions between *eve1* and Bmp. Lateral views (where discernible, dorsal to the right) of zebrafish embryos at 70-80% epiboly (A-S). Embryos were injected at the one cell stage (A-K,M,Q) and the 1-4 cell stage (N,R). For coinjection of *eve1* mRNA and bmp2bMO, embryos were first injected with *eve1* at the one cell stage then injected with bmp2bMO at the 4-8 cell stage (O,S). Genes analysed are indicated at the left of the column, injections on top of columns. *Bmp2b* suppresses neural markers *sox3* and *hoxb1b* even in the presence of *eve1* (A-F). (H) *Bmp4* expression is suppressed by *eve1*, but is ubiquitously induced by co-injection of *bmp2b* mRNA (I). *Bmp2b* expression is also suppressed by *eve1* (K). (L-S) *Eve1* and bmp2bMO synergize in ectodermal fate specification. Low levels of *eve1* mRNA (10pg/nl) or bmp2bMO (100pg/nl) injected individually do not affect *sox3* or *foxi.1* expression (M,N,Q,R), but co-injection at the same concentrations induced *sox3* (O) and suppressed *foxi.1* (S).

Figure 5.

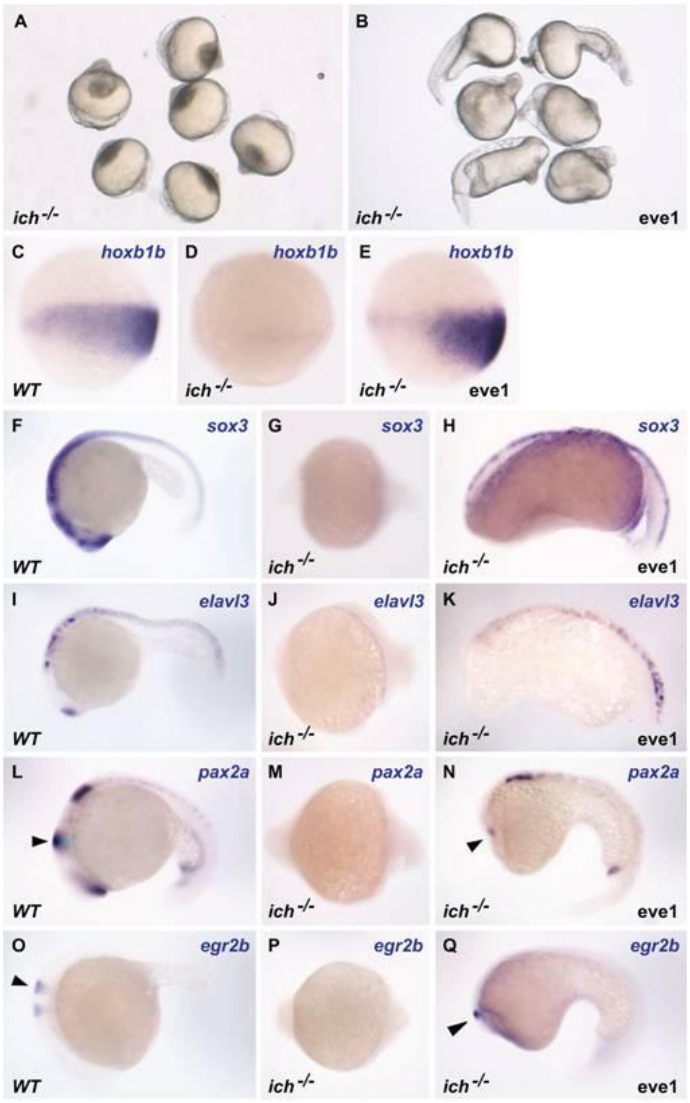


Fig. 5. *Eve1* rescues posterior neural development in *ich*$^{-/-}$ mutants. Homozygous *ich*$^{-/-}$ mutant embryos were injected with *eve1* mRNA. Genotype is indicated at bottom left, injections at the bottom right, and *in situ* probes at the top right. (A) Uninjected *ich*$^{-/-}$ embryos at 24 hpf. (B) *Eve1* mRNA injection leads to varying levels of rescue of posterior dorsal axis. (C-E) Embryos stained for *hoxb1b* at 80% epiboly, presumed lateral view, dorsal to the right. (D) Expression of *hoxb1b* is absent in uninjected *ich*$^{-/-}$ embryos but is rescued by injection of *eve1* mRNA (E). (F-Q) *In situ* hybridisation of wt and *ich*$^{-/-}$ embryos at 24 hpf (anterior to the left). Neural gene expression and posterior dorsal axis formation was partially rescued by the injection of *eve1* mRNA. Rescue of *pax2a* (L-N) appears to extend to the midbrain-hindbrain boundary (black arrowheads), while *egr2b* expression appears to extend to rhombomere 5 (O-Q; arrowheads).

Figure 6.

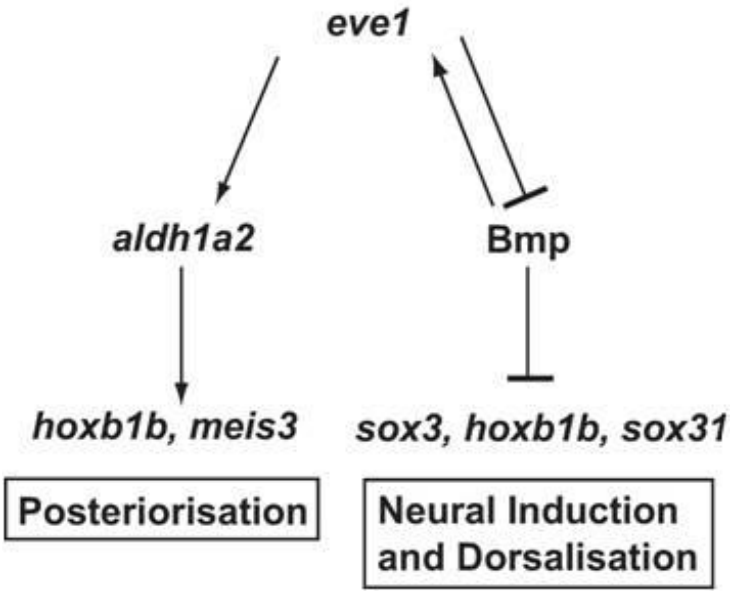
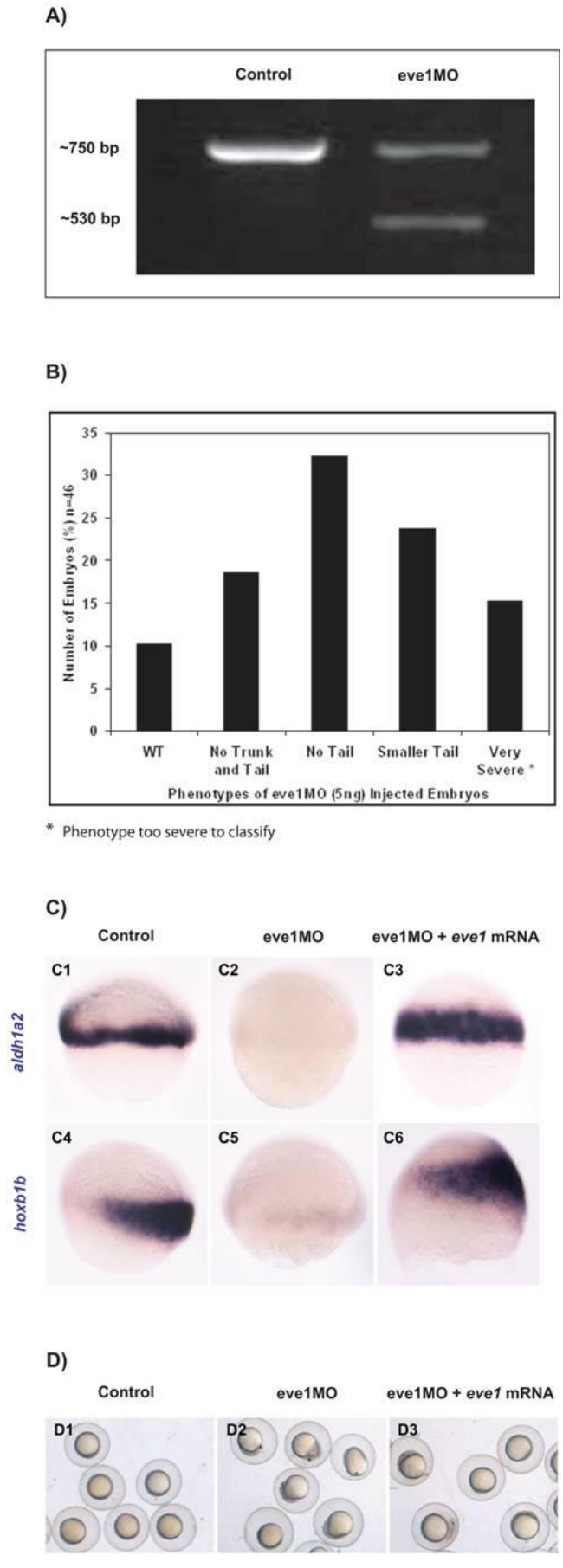


Fig. 6. Role of *eve1* in posteriorisation and neural induction. See text for discussion.

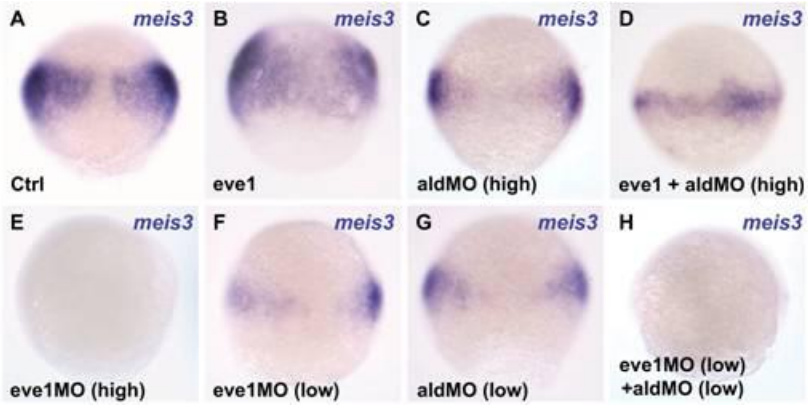
Supplemental Data

Figure S1.



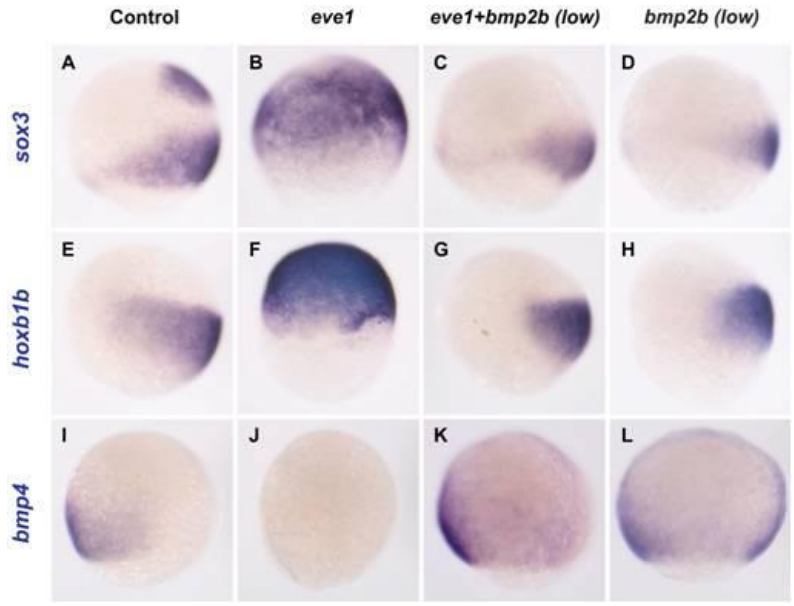
Supplemental Fig. 1. Eve1MO phenotypic classes and specificity of eve1MO. (A) PCR of *eve1* transcripts in uninjected and eve1MO injected embryos. Note the two bands in the eve1MO lane compared to the single band in control embryos, which corresponds to the correct size for *eve1* mature mRNA. Sizes are indicated to the left. (B) **R**ange of phenotypes observed in eve1MO injected embryos. (C) *Eve1* mRNA injection rescues the eve1MO-mediated suppression of *aldh1a2* (C3) and of *hoxb1b* (C6), indicating that the MO is specific. (D) *Eve1* mRNA injection rescues eve1MO-induced epiboly defects; photos taken when control embryos reached 80% epiboly.

Figure S2.



Supplemental Fig. 2. *Eve1* induces *meis3* expression via an RA signal. Dorsal views (where discernible) of zebrafish embryos fixed for *in situ* staining at 70% epiboly. Injections are indicated at the bottom left, genes analysed at the top right. (A-D) Injection of *eve1* mRNA and high concentrations of aldMO (see Materials and Methods). *Eve1* cannot rescue *meis3* expression in aldMO injected embryos (D). High levels of eve1MO (5ng/nl – see Materials and Methods) suppresses *meis3* expression (E). (F-H) Injection of low concentrations of eve1MO (2ng/nl) and aldMO showing synergism between *eve1* and *aldh1a2* in the regulation of *meis3*.

Figure S3.



Supplemental Fig. 3. *Eve1* regulates Bmp expression but not Bmp signalling. Lateral views (where discernible, dorsal to the right) of zebrafish embryos fixed for *in situ* staining at 80% epiboly. Genes analysed are indicated at the left of the columns, injections on top of columns. Induction of the neural markers *sox3* (B) and *hoxb1b* (F) by *eve1* is antagonised even by low doses (7pg/nl) of *bmp2b* co-injected with *eve1* (100%; n = 25; C) (100%, n = 34; G). (D,H,L) Embryos of *sox3* (D), *hoxb1b* (H) and *bmp4* (L) expression following low doses (7pg/nl) of *bmp2b* injection.

Supplemental Materials and Methods

RNA isolation and PCR analysis (Fig. S1 B). Controls and eve1MO-injected embryos were collected at the 50% epiboly stage, and mRNA was isolated using Tri Reagent (Sigma) as instructed by the manufacturer. PCR was performed with the following primers designed from exons one and three (5'-ACTCCTGATACTCTTTAATCA -3'. and 5'- CGTCACGAAGTCCTACTATCC -3')

Chapter 3. Research Chapter 2

Differential Roles of Maternal and Zygotic Fgf and Bmp in Neural and Epidermal Induction in Zebrafish Embryos.

To be submitted to Development within two weeks of Thesis submission.

Analyses of differential maternal and zygotic Bmp and Fgf signalling in D/V patterning. Both Bmp antagonism and Fgf signalling have been shown to be critical for neural induction. However, their relative contributions to this process have not been clarified. In zebrafish, in has been shown that there is a differential requirement for Bmp antagonism and Fgf signalling in anterior and posterior neural induction, respectively (Dee et al., 2007; Kudoh et al., 2004; Rentzsch et al., 2004). In zebrafish, it has also been shown that germ ring (zygotic posterior) Fgf signalling is necessary for induction of posterior (trunk and tail) neural tissue and that this is independent of Bmp and organiser signalling, including organiser-derived Fgfs (Dee et al.,2007; Kudoh et al., 2004; Rentzsch et al., 2004). However, Fgf transcripts are also detected maternally in zebrafish and it is not known what roles, if any, maternal and organiser-derived Fgf signals play in neural induction. Data from both chick and frog have also suggested that marginal Fgf signalling is important for the development of neural tissue in posterior ectoderm but have further implicated pre-gastrula (blastula) Fgf signalling in neural induction (Delaune et al., 2004; Streit et al., 2000; Wilson et al., 2000). However, it is not clear if maternal or zygotic Fgf signalling, including that emanating from the organiser, is involved. In the chick, maternal determinants are not well characterised making it difficult to assign any roles in neural development to maternal and early zygotic Fgf signalling.

Although in the chick this is still unresolved, in both Xenopus and zebrafish inhibition of the Bmp pathway expands both anterior and posterior neural tissue, and suppresses epidermal tissue. Ectopic activation of Bmp signalling, meanwhile, leads to the opposite phenotype. As is the case with Fgf signalling, Bmps are also expressed maternally in fish and frog. However, it has been difficult to analyse maternal-specific roles of Bmp signalling due to lack of maternal-effect mutants and a lack of small-molecule chemical inhibitors that can be washed away at any particular stage. In *Xenopus*, although maternal Bmp transcripts are present, Smad proteins (effectors of Bmp signalling) are not phosphorylated and thus it is assumed that maternal Bmp signalling is dispensable for development (Faure et al., 2000). In zebrafish, although maternal-effect *smad5/sbn* mutants have compromised zygotic Bmp signalling and are dorsalised (Hild et al., 1999; Kramer et al., 2002), nevertheless it is not known if this is downstream of any Bmp ligand or receptor. So again like with Fgf, no in depth molecular analyses of the effects of maternal Bmp signalling on D/V patterning

have been reported for any vertebrate model and the differential contributions of maternal and zygotic Bmp signalling to both neural and epidermal induction remains unknown.

To determine the respective contributions of maternal and zygotic Bmp and Fgf to neural induction, we carried out stage-specific analyses of Bmp and Fgf signalling using three main strategies: Use of small-molecule Bmp and Fgf receptor inhibitors to separate maternal and zygotic functions; use of organiser-deficient *ichabod* mutants to ascertain whether organiser-specific Fgf signalling was required for neural induction; and finally, use of an improved zebrafish ectodermal explant assay, based on the *ichabod* mutant, to identify the absolute requirement for Bmp and/or Fgf signalling in the initiation of the process of neural induction.

Differential Roles of Maternal and Zygotic Fgf and Bmp in Neural and Epidermal Induction in Zebrafish Embryos.

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Keywords: neural induction, neural competence, *ichabod* animal cap assay

Summary

Neural induction is the process that drives ectoderm to form neural tissue in vertebrates. Both Bmp antagonism and Fgf signalling are implicated in this process, but the relative roles associated with the timing of the contribution of these pathways remains largely unclear. To address this issues, we exposed wildtype and organiser-deficient *ichabod* embryos to inhibitors of the Bmp and Fgf signalling pathways in a stage-specific manner and analysed the differential contribution of maternal and zygotic Bmp and Fgf signalling to neural induction. We show that inhibition of maternal Fgf signalling blocks both anterior and posterior neural induction while inhibition of zygotic Fgf signalling only suppresses posterior neural induction. We also show that both maternal and zygotic Bmp signalling are necessary for epidermal specification as well as suppression of neural induction. Finally, by using for the first time ectodermal explants excised from the *ichabod* mutant line, which lacks the organiser, we show that although suppression of Bmp signalling induces neural tissue in *ichabod* explants, this induction is blocked by co-suppression of maternal Fgf, implying that maternal Fgf signalling is necessary for neural induction independently of Bmp antagonism. From these results, we propose that maternal

Fgf and Bmp give competence to ectoderm to differentiate to neural plate and epidermis, respectively.

Introduction

During neural induction, cells of the ectoderm make a choice between neural or epidermal fates. Analysis of the molecular mechanisms underlying neural induction have resulted in the emergence of two classes of model to explain this process. The default model of neural induction, based mainly on work with *Xenopus*, proposes that suppression of Bone morphogenic protein (Bmp) signalling is necessary and sufficient for cells to acquire a default, neural fate. Bmp signalling, restricted ventrally, specifies epidermal fates (Hemmati-Brivanlou and Melton, 1997; Weinstein and Hemmati-Brivanlou, 1999). Furthermore, many members of the Bmp signalling pathway were potent epidermal inducers including Bmp4 and Bmp7 (Hawley et al., 1995; Wilson and Hemmati-Brivanlou, 1995), and analyses of organiser-derived signals that promote neural induction showed many of these to be Bmp antagonists. Among these, the secreted molecules Chordin (Sasai et al., 1994; Sasai et al., 1995), Noggin (Smith and Harland, 1992; Lamb et al., 1993; Smith et al., 1993) and Follistatin (Hemmati-Brivanlou et al., 1994) inhibit Bmp activity by modulating Bmp protein- receptor interactions. Abrogation of activity of these genes leads to ventralisation with concomitant loss of neural tissue (e.g. Schulte-Merker et al., 1997; Bachiller et al., 2000; Kuroda et al., 2004) showing that suppression of Bmp signalling is a crucial step in neural induction.

Besides Bmp antagonism, Fibroblast growth factors (Fgfs) have an important instructive role in neural induction, independently of Bmp inhibition (see Wilson and Edlund, 2001; Stern, 2005). In Ascidians, for example, it is Fgfs that are the neural inducers whereas it is not clear what role Bmp antagonism plays in neural induction (Bertrand et al., 2003; Hudson et al., 2003). In vertebrates, there is also data that cannot be explained by the default model. In zebrafish, neural induction in the posterior neural ectoderm occurs via Fgf signalling emanating from the vegetal marginal zone (germ ring) even in the ventro-vegetal ectoderm where Bmp signalling is most active (Dee et al., 2007; Kudoh et al., 2004; Rentzsch et al., 2004). In the chick, neural markers are not induced in competent epiblast in response to the Bmp antagonists Noggin and Chordin (Streit et al., 1998; Streit and Stern, 1999a; Streit and Stern, 1999b) and in certain situations in *Xenopus*, Bmp antagonists do not induce neural markers when Fgf signalling is compromised (Launay et al., 96; Sasai et al., 1996; Hongo et al., 1999; Pera et al.,

2003; Delaune et al., 2005). Importantly, these data also suggest a requirement for Fgf signalling in anterior, as well as posterior, neural induction.

Although there is now consensus that both Bmp antagonists and Fgf play essential roles in neural induction, clarifying their relative roles has been difficult, in part due to the complex patterns and timing of Bmp and Fgf expression: genes of both families are ubiquitously expressed maternally, subsequently becoming localised to ventral gastrula cells for Bmp and the blastoderm margin and organiser for Fgf (Hemmati-Brivanlou and Thomsen, 1995; Kishimoto et al., 1997; Lee et al., 2010; Shawi and Serluca, 2008; Thisse et al., 2001; Yamauchi et al., 2009). However, it has been suggested that Fgf signalling is required for neural induction prior to gastrulation in both chick and frog (Delaune et al., 2005; Streit et al., 2000; Wilson et al., 2000). In addition, zebrafish maternal-effect *smad5/sbn* mutants are dorsalised and this dorsalised phenotype is independent of zygotic Bmp signalling (Hild et al., 1999; Kramer et al., 2002), suggesting that maternal Bmp is essential for dorsoventral (D/V) patterning in late blastula to gastrula stages. However, it is still not known if maternal Smad5 functions downstream of any Bmp ligand or receptor, and it remains unclear if Bmp and Fgf signalling have differential maternal and zygotic roles in ectodermal patterning.

To address the relative roles and temporal requirements of Bmp antagonism and Fgf signalling in neural induction in zebrafish, we made use of pharmacological inhibitors of the Bmp and Fgf pathways, as well as other loss- and gain-of-function approaches. The availability of mutant lines is one of the great strengths of using zebrafish as a model organism, and for the first time, we have also used an animal cap assay system using a genetic mutant, *ichabod*. The *ichabod* mutant lacks an organiser, is ventralised and does not develop any anterior structures, including neural tissue (Bellipanni et al., 2006; Kelly et al., 2000; Maegawa et al., 2006). Using these various approaches, we find that both Fgf and Bmp signalling have differential maternal and zygotic roles in ectodermal patterning. We show that maternal Fgf signalling is crucial for both anterior and posterior neural induction, since loss of maternal Fgf leads to suppression of both anterior and posterior neural markers. We also show that embryos with compromised maternal Bmp receptor signalling are dorsalised, suggesting that continuous Bmp receptor signalling is required for epidermal specification and that maternal Bmp signalling must be suppressed for future neural specification events. Furthermore, we show that loss of Bmp signalling rescues both the

anterior and posterior expression of neural markers in *ichabod* mutants but that this rescue is lost when Fgf receptor signalling is blocked. Our data suggest that it is maternal, and not zygotic, Fgf receptor signalling that is crucial for the initial neural induction process, independently of suppression of the Bmp pathway. Our data further suggest that organiser-derived Fgf signalling is not required for the induction of anterior neural tissue and that the primary function of the organiser in neural induction in zebrafish may be to suppress the Bmp signalling pathway. We propose a model whereby differential maternal and zygotic Bmp and Fgf signalling first confer on ectodermal cells the capacity to acquire epidermal and neural fates, respectively. Specification of cell fates subsequently occurs via domain-specific zygotic Bmp and Fgf signalling.

Results

Formation of the CNS shows an inverse sensitivity to gain of Bmp and loss of Fgf signalling along the A-P axis

Previous research has shown that weakly enhanced Bmp activity reduces rostral neural induction and that progressively higher Bmp activity reduces trunk and tail neural tissues (Dosch et al., 1997; Kishimoto et al., 1997; Nguyen et al., 1998**;** Bachiller et al., 2000; Anderson et al., 2002). To examine more precisely the dose dependence of head and trunk neural induction on Bmp signalling, we first overexpressed *bmp2b* mRNA, observed the phenotype at 22 hrs and compared it to neural (*sox3*) and epidermal (*p63*) marker gene expression at the same stage (Fig. 1). Low Bmp mostly suppresses only head formation (95%) (Fig. 1B), with a few embryos also showing some suppression of trunk structures (5%) (n=38). Meanwhile, high Bmp suppresses both head and trunk (91%) (Fig. 1C), although trunk structures are still apparent in some embryos (9%) (n=33). However, the tail always remains in the high Bmp embryos as well as in the most severely ventralised mutants (e.g. zebrafish *ichabod.* Kelly et al., 2000; Bellipanni et al., 2006; Maegawa et al., 2006). At 22 hrs, *sox3* is expressed in the neural tube (Fig. 1D) but compared to uninjected controls, expression is reduced in a manner that complements the phenotypic analyses of *bmp2b* overexpression (Fig. 1E,F). In the most ventralised embryos, expression of *sox3* is either absent (64%) (Fig. 1F) or very faint (36%) (n=22), and is limited to the posterior (tail). *p63* expression is seen weakly all over the epidermis at 22 hrs, but is activated in a

concentration-dependent manner by overexpression of *bmp2b* mRNA (Fig. 1H-I). In embryos showing the severest phenotype, *p63* expression marks all the visible tissue (100%) (n=26) (Fig. 1I). These data agree with earlier results and show that suppression of Bmp signalling is essential for specification of neural tissue especially in the anterior neural domain.

Previous research has shown that progressively increasing suppression of Fgf gradually reduces neural tissue in a posterior to anterior direction (opposite to Bmp) (e.g. Kudoh 2004; Delaune et al., 2005). To examine the detailed differential dose response to reduction of Fgf signalling, we made use of the Fgf receptor inhibitor PD173074 (Mohammadi et al., 1998) (see Materials and Methods). Our results confirmed previous data with the pharmacological Fgf inhibitor SU5402, which showed a dose-dependent reduction in the A/P axis, from posterior to anterior, with concomitant loss of neural gene marker expression (Delaune et al., 2005, Kudoh 2004). Opposite to the effect seen with *bmp2b* injection, phenotypic analyses showed that mild inhibition of Fgf signalling only suppresses tail (100%, n=40) (Fig. 1J), while increased doses of Fgf inhibition suppresses trunk (89%, n=37) (Fig. 1K) and eventually most (73%) or all (27%) (n=30) of the head (Fig. 1L). Analyses of *sox3* expression agree with the phenotypic data, showing a dose-dependent loss of the neural tube from posterior to anterior in a dose –dependent manner (Fig. 1M-O). At the highest concentration of PD173074, the expression of *sox3* at 22 hrs is completely absent in some of the embryos (Fig. 1O), while in the remainder only a dot of anterior expression is visible. We obtained a similar result with *otx2* (data not shown), although we found that with at highest concentrations of PD173074 *otx2* expression was lost in all the embryos we examined. In agreement with previous observations in various organisms (see Böttcher and Niehrs, 2005; Stern, 2005), this shows that Fgf signalling is essential for neural development. Furthermore, we show that where *sox3* expression is absent, the visible tissue is now marked by the expression of the epidermal marker, *p63*, which is very weak in the 22 hr control, uninjected embryos (Fig. 1P-R). However unlike with Bmp overexpression, loss of Fgf signalling leads to an accumulation of epidermal tissue posteriorly (vegetal pole). These data suggest that loss of Fgf signalling leads not only to loss of neural tissue but also to induction of an epidermal gene, *p63*.

Neural induction shows an inverse sensitivity to gain of Bmp and loss of Fgf signalling along the A-P axis

At 22 hrs, rostral and caudal neural tissue shows inverse sensitivity to Bmp overexpression and loss of Fgf signalling. However, both conditions show a loss of neural tissue, as evident by the concentration-dependent reduction of *sox3* expression and activation of *p63*. To determine whether epidermis is induced at the expense of neural plate, we looked at the expression of *sox3* and *p63* in embryos at the late gastrula stage under the same dose-dependent conditions of Bmp activation and loss of Fgf function. In agreement with the 22 hr data, and as we previously showed (Cruz et al., 2010), low doses of *bmp2b* injection suppressed anterior *sox3* expression while at higher doses posterior expression is much reduced (Fig. 2, compare A-D). However, some *sox3* expression remains near the margin (posterior domain) even with 100pg mRNA injection (Kudoh et al., 2004). Analysis of the epidermal marker, *p63,* complements the *sox3* data and shows a concentration-dependent expansion (Fig. 2E-H) that eventually covers the whole anterior region (Fig. 2H). Thus it appears that induction of epidermis has occurred at the expense of anterior neural plate, but since *p63* expression does not expand posteriorly and posterior *sox3* expression is still evident (Fig. 2D : Kudoh et al. 2004), it suggests that posterior neural tissue has not been respecified as epidermal. This is in agreement with previous observations that suppression of Bmp signalling is not a prerequisite for posterior neural induction in zebrafish (Dee et al., 2007; Kudoh et al 2004; Rentzsch et al., 2004).

Also consistent with the 22 hr data, graded loss of Fgf expression leads to gradual reduction (Fig. 2I-J) and eventual loss (Fig. 2I,K) of *sox3* expression from a posterior to anterior direction. At 80 µM concentrations of PD173074, posterior *sox3* expression is reduced (83%) or absent and there is a posterior shift in the *sox3* anterior domain (100%) (n=29) (Fig. 2I). At 120 µM posterior *sox3* expression is completely suppressed (95%) and anterior expression is now reduced and shifted more posteriorly (90%) (n=20) (Fig. 2J). At the highest concentrations of PD173074 we could use without overly affecting embryonic mortality and epiboly movements, 160 µM, *sox3* expression is totally suppressed both in the anterior and the posterior in some embryos (24%, n=38) (Fig. 2K), whilst in the remainder (76%) only a small dot of presumed anterior *sox3* expression remains, implying that the neural plate has failed to be specified. Our

p63 data shows that this is not caused by cell death or epiboly failure since *p63* expands to eventually cover all (8%) or most (92%) (n=24) of the ectoderm at the late gastrula stage (Fig. 2, compare L-N) and we observed little overt difference in epiboly movements between exposed and control (unexposed) embryos. The complementary expansion of *p63* and reduction of *sox3* marker genes implies that neural ectoderm has been respecified as epidermal. The *sox3* analyses following exposure to PD173074 both at late gastrula and 22 hrs shows that Fgf signalling is required for neural induction in the whole neural plate, but that posterior neural tissue is more sensitive to loss of Fgf receptor signalling.

Maternal and zygotic Bmp are both important in epidermis formation and suppression of neural induction.

Although much literature exists highlighting the need for regulation of Bmp signalling for efficient neural induction, it has not been resolved if maternal Bmp signalling plays a role in this process. To address this question, we have used the small-molecule Bmp type I receptor inhibitor, Dorsomorphin (DM) (see Materials and Methods) to specifically knock down Bmp signalling in a temporal fashion. Washing off the inhibitor at the midblastula transition (MBT) allowed us to examine the effect of blocking maternal Bmp signalling downstream of the receptor and exposure from the MBT to fixation allowed us to compare the effects of blocking zygotic versus maternal Bmp signalling (Fig. 3). We analysed the expression of three neural genes: *sox3*, *otx2* (anterior neural) and *hoxb1b* (posterior neural) and the epidermal marker *p63* in embryonic ectoderm at the late gastrula stage.

When compared to control (unexposed) embryos (Fig. 3A,E,I,M), we found no difference in the expression patterns of the four genes in the three conditions used: Exposure to DM from 1 cell to MBT (Fig. 3B,F,J,N); from MBT to fixation (Fig. 3C,G,K,O) and exposure from 1 cell to fixation (Fig. 3D,H,L,P). Anterior *sox3* (our data) and *otx2* (as previously observed, along with other anterior neural genes, with *bmp2b/swirl*$^{-/-}$ mutants (Nguyen et al., 1998; Barth et al., 1999; Imai et al., 2001)) expression domains were expanded ventrally but not vegetally in all three conditions and in all embryos (Fig. 3E,F,I,J,M,N). Equally, posterior *sox3* expression is expanded ventrally (Fig. 4E,I,M), as has also been observed with *swirl*$^{-/-}$ mutants and we further show the same ventral expansion of the posterior

neural marker, *hoxb1b* (Fig. 3G,K,O). Again, all the embryos were similarly affected. In all conditions, the prospective epidermal marker, *p63*, normally expressed in ventral anterior ectoderm under the control of Bmp signalling (Bakkers et al., 2002) is always completely abolished (Fig. 3H,L,P) when compared to control embryos (Fig. 3D). Our data imply that both maternal and zygotic Bmp signalling is essential for epidermal induction.

Blocking maternal Fgf suppresses both anterior and posterior neural induction

We have shown that PD173074 efficiently suppresses the expression of the neural marker, *sox3*, and induces the epidermal Bmp-responsive gene, *p63*, in a dose-dependent manner. This demonstrates a requirement for Fgf receptor signalling in neural induction and agrees with the current consensus, which also appears to demonstrate that Fgf signalling is necessary for neural induction prior to the onset of gastrulation (Delaune et al., 2005; Streit et al., 2000; Wilson et al., 2000). However, the exact timing for this requirement remains unclear, as does whether Fgf signalling, rather than Bmp inhibition (or both), is required at all for anterior neural induction. To address this question in zebrafish, we again made use of PD173074 to compare the maternal versus zygotic requirements for FgfR signalling in zebrafish neural induction.

Surprisingly, we find that blocking maternal Fgf receptor signalling (exposure to PD173074 to the MBT) suppresses both anterior and posterior prospective neural plate markers (Fig. 4). Although a little anterior *sox3* expression remains (100%) (Fig. 4B), posterior expression is completely lost in most of the embryos (82%) (n=33). Similarly, the expression of both *otx2* (93%) and *hoxb1b* (100%) (n=29) (Fig. 4F) is suppressed. At the same time, the *p63* expression domain expands to cover most of the embryo (100%, n=29) (Fig. 4J) with the exception of the presumed organiser domain and nearby tissue, which is consistent with the remaining *sox3* expression we observed. In comparison, in embryos exposed to PD173074 from the MBT to fixation at the late gastrula stage, anterior *sox3* expression is only slightly reduced (81%) and posterior expression shows variable reduction (expression is never observed in ventro-lateral domains) (78%) (Fig. 4C) and is lost completely in a few of the embryos (22%) (n=32). Similar to the maternal data, the anterior expression domain is always shifted posteriorly which

is consistent with Fgf signalling being critical for posteriorisation (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Kudoh et al., 2002; Kudoh et al., 2004). As for anterior *sox3* expression, loss of zygotic Fgf signalling also leads to posterior shift of the *otx2* expression domain in all the embryos stained (n=28) (Fig. 4G). *Hoxb1b* is also lost (61%) or much reduced (39%), with any remaining expression being limited to its dorsal most expression domain (Fig. 4G), similar to what we show for posterior *sox3* expression. As was observed in *Xenopus* with SU5402, epidermis expands dorsally (Delaune et al., 2005) as marked by the expression of *p63* (Fig. 4K) but expansion is not as pronounced as with exposure prior to MBT (compare Fig. 4J and K). As we have already shown (see Fig. 2K), exposure to PD173074 from the two cell stage to fixation led to the suppression of *sox3* expression (Fig. 4D). As for anterior *sox3* anterior expression, *otx2* was also completely (77%) or partially (23%) (n=22) suppressed although we did find that *otx2* was more sensitive to loss of Fgf signalling than *sox3*, as was observed at 22 hpf. *Hoxb1b* expression meanwhile was lost in all the embryos (Fig. 4H) while *p63* expanded to cover the whole or most of the embryo as previously shown (Fig. 4L). Taken together the data we present here implies a crucial role for maternal Fgf receptor signalling in both anterior and posterior neural induction in the zebrafish embryo.

Bmp antagonism rescues anterior neural tissue in *ichabod* mutant embryos

Organiser-derived signals negatively regulate Bmp signalling on the dorsal side of developing embryos and this is a prerequisite for anterior neural induction to occur. However, it is still not clear whether other signals are required in addition to suppression of Bmp. The zebrafish *ichabod* mutant is a maternal-effect mutant deficient in *β-catenin2* signalling. Embryos lack the organiser and are ventralised, leading to loss of head and trunk structures, including neural tissue (Bellipanni et al., 2006; Kelly et al., 2006; Maegawa et al., 2006). We decided to expose *ichabod* embryos to DM to test whether suppression of Bmp signalling is sufficient to induce neural plate markers in the absence of organiser signals, including organiser-expressed Fgfs, which have been shown to mediate *β-catenin2* signals in establishing the organiser (Maegawa et al, 2006).

In *ichabod* embryos at the late gastrula stage, *sox3* is expressed only posteriorly in a thin ring near the margin (Fig. 5A) while *otx2* and *hoxb1b*

expression is absent (Fig. 5E, I). The epidermal marker *p63* meanwhile expands to cover dorso-anterior parts of the embryo (Fig. 5M), consistent with the embryos being ventralised. When exposed to low concentrations (10µM) of DM, posterior *sox3* expression expands slightly (Fig. 5B) and *hoxb1b* expression is induced radially in the posterior (Fig. 5J) similar to the posterior expression of *sox3*. However, both anterior *sox3* and *otx2* expression (Fig. 5F) is still not activated. Complementing these neural markers, at the same low dose of DM, *p63* expression is reduced from the vegetal ectoderm but is still expressed in the animal pole ectoderm (Fig. 5N).

When *ichabod* embryos were exposed to a high dose of DM, both anterior *sox3* and *otx2* (Fig. 5C,G) are induced when compared to both unexposed controls (Fig. 5A,E) and *ichabod* embryos exposed to low doses of DM (Fig. 5B,F). Consistent with rescue of neural induction, *p63* expression is now completely suppressed in all the embryos (Fig. 5O). (D,H,L,P) At a very high dose, rescue of neural markers is further enhanced. Both anterior *sox3* (Fig. 5D) and *otx2* (Fig. 5H) expression expands to cover the animal pole while both posterior *sox3* (Fig. 5D) and *hoxb1b* expression expands anteriorly. This dose-dependent effect of Bmp suppression by DM in the *ichabod* mutant implies that the main role of the organiser in anterior neural induction is the suppression of Bmp signalling and that organiser-derived Fgf signalling is not necessary for the induction of anterior neural tissue. It also indicates that simply rescuing Bmp antagonism is sufficient to restore not only neural induction but also antero-posterior patterning that is indicated by *sox3* (anterior and posterior), *otx2* (anterior) and *hoxb1b* (posterior) expression.

Maternal Fgf is crucial for anterior neural induction independently of Bmp inhibition

Our data suggest that maternal Fgf signalling is critical for all neural induction and that organiser-derived Fgf signalling is not needed for the expression of anterior neural genes in the organiser-deficient *ichabod* embryo. However, our data does not show whether or not maternal Fgf neuralising activity occurs via the inhibition of the Bmp pathway. To address this question, we co-treated both wildtype and *ichabod* embryos with DM + PD173074. However, these embryos had severe epiboly defects and died before the mid-gastrula stage

(data not shown) so instead we made use of caps excised from the animal poles of wild type and *ichabod* embryos and fixed at the late gastrula stage. This experiment would also allow us to examine if, by simply modifying these two signals, neural fates could be induced in the ectodermal explants without influence from other cell layers such as mesendoderm and the yolk syncytial layer. Both wildtype and *ichabod* caps should lack all mesoderm derivatives and consist purely of tissue of an anterior character. In agreement with this observation, *ntl* expression, a marker for presumptive mesoderm, is completely absent in all the animal caps we tested (Fig. 6O,P). Furthermore, we speculated that if there was a direct requirement for maternal Fgf independent of suppression of Bmp then exposing *ichabod* animal caps, which lack both organiser and marginal signals, to PD173074, should suppress the DM-mediated induction of anterior *sox3* and induce *p63* expression.

We first analysed the expression of *sox3* and *p63* under three different conditions of loss of Bmp signalling in *ichabod* caps and compared the results to (untreated/uninjected) wildtype and *ichabod* controls. In wildtype controls, both *sox3* and *p63* expression is patchy, which is possibly due to different amounts of either ventral or dorsal tissue being cut in the caps (Fig. 6A,H). In the *ichabod* controls, however, *sox3* expression is absent in all the caps (Fig. 6B) while the opposite result is observed for *p63* (Fig. 6I) which is strongly induced, consistent with the results observed in *ichabod* embryos (compare Fig. 5A,M). However, injection of either a start-site-directed *bmp2b* morpholino (see Materials and Methods) or *noggin* mRNA, or exposure to DM, reverses these effects. *Sox3* is now induced in all the caps (Fig. 6C-E) while *p63* expression is suppressed (Fig. 6J-L). But when we co-exposed the *ichabod* caps to PD173074 until the MBT plus DM (until fixation, see Materials and Methods), *sox3* expression is now suppressed in all the caps (Fig. 6F). Interestingly, *p63* expression is also absent (Fig. 6M) suggesting that *p63* expression is not directly regulated by maternal Fgf signalling. However when we co-exposed *ichabod* caps to PD173074 from the MBT plus DM (from 1-2 cell stage, see Materials and Methods), we found that *sox3* was still expressed in all the caps (Fig. 6G) although not to the same extent visible with DM exposure alone. Again, there was no difference with *p63* expression (Fig. 6N) when compared to DM exposure alone. Taken together, our data show that in the absence of organiser and marginal signals, loss of Bmp signalling still promotes anterior neural induction and suppresses epidermis.

However, induction of anterior neural markers is likely to occur because of the presence of maternal Fgf signalling.

Discussion

Neural induction shows an inverse sensitivity to gain of Bmp and loss of Fgf signalling along the A-P axis.

We have re-examined the relative requirements for Bmp antagonism and Fgf signalling in neural induction with a dose analyses. Comparison of the gastrula and 22 hr stage data shows that when neural tissue is not specified, epidermal fate is expanded. In the case of Bmp overexpression, head neural tissue is preferentially suppressed while with suppression of Fgf trunk/tail neural tissue is preferentially suppressed. However, it is noteworthy that even though Bmp antagonism and Fgf signalling are differentially involved in head and trunk/tail neural induction, respectively, at the highest doses of Bmp overexpression posterior neural ectoderm can be largely reduced. This suggests that suppression of Bmp signalling is necessary for posterior neural induction but to a lesser extent than in the anterior. Equally, when Fgf signalling is blocked with the highest dose of the Fgf receptor inhibitor, PD173074, all anterior neural tissue can be suppressed, suggesting that Fgf is also important for anterior neural induction but is required at lower levels than in the posterior. Although this dose response, as well as previous reports (Delaune et al., 2005; Kudoh et al., 2004; Marchal et al., 2009; Wills et al., 2010) shows a differential requirement for Bmp-antagonism and Fgf signalling in anterior and posterior neural induction, the timing and extent for this requirement remains unclear. To answer this question, we carried out analyses of stage-specific suppression of Bmp and Fgf signalling and we discuss the results in the next paragraphs.

Maternal Bmp maintains competence for epidermal cell fates in the ectoderm.

We analysed stage-specific roles of Bmp signalling and Bmp antagonism in neural induction by treating embryos with a Bmp receptor inhibitor, DM. We particularly focused on comparison of the stages before and after the mid-blastula transition (MBT), when zygotic transcription starts. We found that blocking Bmp

signalling both before (maternal) or after (zygotic) the MBT expanded neural tissue and suppressed epidermal fate, suggesting that both maternal and zygotic Bmp signalling is crucial for suppressing neural and promoting epidermal fates. However, maternal Bmp is expressed regardless of dorso-ventral axis and seen even in prospective anterior neural cells. Furthermore, epidermal fate is not specified before the MBT (Hild et al., 1999) although maternal Bmp is essential for cells to later acquire epidermal fate (our data). The function of maternal Bmp could therefore be called "epidermal capacitation" (giving the cells a capacity or competence to become epidermis). On the other hand, zygotic Bmp (.e.g. *bmp2b*, *4* and *7*) expression is highly tissue-specific and overlaps with the epidermal marker, *p63*, (e.g. bmp4) (Kishimoto et al., 1997; Kudoh et al., 2004), and when gastrula cells are exposed to Bmp they express *p63* and differentiate to epidermis (Bakkers et al., 2002). Zygotic Bmp function at gastrula stage could therefore be called "epidermal specification".

Maternal Fgf maintains competence for neural cell fates in the ectoderm.

We also examined the stage-specific contribution of Fgf signalling to neural induction by treatment with the Fgf receptor inhibitor, PD173074 and found a very interesting difference between maternal and zygotic Fgf signalling: PD173074 treatment until the MBT strongly suppressed both head and trunk/tail neural induction whereas treatment from the MBT mainly suppressed trunk/tail neural induction, and head neural tissue was still formed. We previously showed that treatment after the MBT with another Fgf receptor inhibitor, SU5402, specifically suppressed posterior neural induction and a similar result was obtained with overexpression of a dominant-negative form of the Fgf receptor (XFD) (Kudoh et al., 2004). This data further support the crucial role of zygotic Fgf in posterior neural induction. The non-suppression of anterior *sox3* by XFD in the previous report is possibly due to the dose-dependent, partial suppression of Fgf signalling in the embryos, and the phenotype should mainly reflect the loss of function of zygotic Fgf. As seen in this report, a low dose of PD173074 mimics the zygotic, stage-specific treatment of the drug: In both cases, anterior *sox3* expression remains but posterior expression is highly suppressed. In contrast, a higher dose of PD173074 is needed for loss of anterior *sox3* expression to occur, and treatment must also include the maternal stage (fertilisation to MBT).

We can again interpret this data with the "capacitation and specification" concept. Before the MBT, several maternal Fgfs (e.g. Fgf2 and Fgf4) are expressed ubiquitously (data not shown). As seen in our data, maternal Fgfs are essential for both anterior and posterior neural induction, but still not sufficient to specify neural cell fates since the expressing cells include prospective epidermis. The function of maternal Fgf could then be interpreted as "neural capacitation", i.e., sensitising cells to future neuralising signals. On the other hand, zygotic Fgfs are expressed in the blastoderm margin (e.g. Fgf3, Fgf8 and Fgf24) and are crucial for the induction of posterior neural tissue in the adjacent vegetal ectoderm with concomitant suppression of epidermal marker genes (Dee et al., 2007; Kudoh 2004; Rentzsch et al, 2004). Therefore the role of zygotic (marginal) Fgf is in neural specification of posterior (trunk and tail) neural tissue. We have shown that in embryos where zygotic (including organiser) Fgf signalling is suppressed, anterior neural markers are still expressed. Furthermore, when we blocked Bmp signalling in organiser-deficient *ichabod* embryos by treatment with DM, we found that both anterior and posterior neural markers are restored in the correct position along the animal-vegetal axis. These data indicate that Bmp antagonism is sufficient to restore both anterior and posterior neural induction independently of Fgf function in the organiser and confirm that the importance of Fgf in anterior neural induction (independent of Bmp-antagonism) is not due to organiser-specific Fgfs, but rather due to the ubiquitous, maternal Fgfs at the level of neural capacitation.

The *ichabod* animal cap assay is a useful assay system to study neural induction and patterning.

One of the limitations of chemical exposure experiments in embryos is the dose effect of the chemicals. It is often challenging to sufficiently block a signal in large embryos and we often tend to observe partial effects in chemical inhibitor experiments (e.g. Fgf inhibitor, SU5402 in Kudoh et al., 2004). Most chemical inhibitors require more than 10 times higher doses in zebrafish embryos when compared to mammalian cell culture (e.g. DM). This is possibly because chemicals cannot effectively penetrate through surface cells to reach deeper cells in the embryo. Another difficulty for embryonic experiments is associated with morphogenetic defects and embryonic death. When we combined the highest

effective doses of PD173074 and DM in the same embryo, most embryos died with gastrulation defects and we could not examine neural and epidermal gene markers that appear in the late gastrula. To overcome this problem, we decided to examine neural induction using zebrafish animal caps which we found have two advantages: Firstly, the animal caps do not die in the combined dose of chemicals that we used for the embryos, possibly because they do not undergo gastrulation defects and associated yolk breakdown. Secondly, the marker response seems more sensitive in the caps than in the embryos possibly because of the smaller size. However, to develop a useful animal cap assay in zebrafish, we had to overcome the problem of the influence of the organiser in the animal cap. Previously, Sagerström and others (Sagerström et al., 1996) developed a zebrafish animal cap assay in which they dissected 10 small animal caps which were combined to make one cap. As this can be limiting to the development of a high throughput assay system, we tried to dissect larger animal caps. But we found that animal caps dissected from wildtype embryos are often partly induced for neural cell fates possibly because of the influence of the early organiser. This idea has been shown in *Xenopus* animal caps by Linker and others (Linker and Stern, 2004; Linker et al., 2009) who discuss that the animal cap is not a clean enough system to study neural induction since it is also partly induced for a neural fate. To overcome these limitations, we prepared organiser-free animal caps from *ichabod* embryos and found that the *ichabod* cap is more uniformly devoid of neural induction signals and develops into epidermis. When the Bmp signal is suppressed in the *ichabod* cap, it effectively undergoes neural differentiation. We therefore decided to use this *ichabod* cap to analyse the combined effects of DM and PD173074, and we believe that the *ichabod* animal cap assay would be useful to study other neural induction and patterning signals. We also believe that application of the animal cap assay to other zebrafish mutant lines would be useful for analyses of gene function in neural induction, patterning and ensuing differentiation.

Bmp antagonism is not sufficient for anterior neural induction in the absence of maternal Fgf signalling.

It has been shown that Fgf signalling can inhibit the Bmp pathway (Pera et al., 2003; De Robertis and Kuroda, 2004) and we conducted a combined chemical exposure with DM and PD173074 to determine if the effects in anterior neural

induction that we observed with loss of maternal Fgf function were not simply due to loss of Bmp antagonism. As seen in our *ichabod* animal cap data, neural marker expression was suppressed in the double treatment caps when compared with caps exposed only to DM, suggesting that Fgf signalling is indeed active in the *ichabod* caps (prospective anterior neural ectoderm) in the absence of an organiser and marginal mesoderm which are known sources of zygotic Fgfs. This data also supports the idea that maternal Fgf signalling has a crucial function in anterior neural induction (neural capacitation), independently of Bmp inhibition. Interestingly, when the caps were double treated with DM and PD173074, both neural and epidermal markers are suppressed suggesting that epidermal induction requires Bmp signalling not simply to suppress neural cell fates but also to actively promote epidermal cell fates. According to our stage-specific suppression of Bmp data, continuous Bmp signalling is required from early cleavage and throughout gastrulation for ectoderm to become epidermis.

The question of when neural induction actually begins is a complex one, and the onset probably involves the activity and crosstalk of a variety of signalling pathways (reviewed in Stern 2005). Our stage-specific analyses have allowed us to determine that, at least in zebrafish, initiation of neural development occurs in the early blastula before the onset of zygotic gene expression and requires maternal Fgf signalling and tight regulation of the Bmp pathway. Our data indicate a differential role between zygotic Fgf, expressed in the blastoderm margin and maternal Fgf. We have defined this early, maternal role of Fgf signalling as neural capacitation, and, applying a similar concept, we have defined the role of maternal Bmp as epidermal capacitation. Both maternal transcripts are essential for the acquisition of neural (Fgf) and epidermal (Bmp) fates but these fates are only specified in the ensuing zygotic transcript period by the domain specific Fgfs and Bmps. We have also for the first time used a genetic mutant in an animal cap assay and we strongly believe similar assays, using zebrafish, could provide strong and viable tools to study other important developmental processes apart from neural induction.

Materials and Methods

RNA probe synthesis and *in-situ* hybridisation. Probes used, antisense RNA probe synthesis and *in-situ* hybridisation procedures have been previously described. (Kudoh et al., 2001).

mRNA synthesis and injection. Capped mRNAs were synthesised by mMessage mMachine SP6 kit (Ambion) according to the manufacturer's instructions. mRNA concentrations (per nl) used for injections are indicated in the Figures or Figure legends, except for *noggin* mRNA which was injected at a concentration of 25pg/µl. mRNAs (1-2nl of solution) were injected through the intact chorion into all blastomeres at the one- to two-cell stages.

PD173074 and Dorsomorphin treatments. For knockdown of Fgf signalling, we used the specific Fgf receptor inhibitor, PD173074 (Sigma-Aldrich) (Mohammadi et al., 1998), which prevents binding of ATP to the tyrosine kinase domain of the Fgf-RS. PD173074 was dissolved in DMSO at a concentration of 40mM and embryos were incubated in PD173074 further diluted in fish water. Dorsomorphin (DM), a selective inhibitor of the Bmp type I receptors, which prevents Smad phosphorylation (Sigma-Aldrich) (Yu et al., 2008), was used for Bmp signalling knockdown. DM was dissolved in DMSO at a concentration of 10mM and embryos were incubated in dorsomorphin further diluted in fish water. Unless otherwise indicated, PD173074 was used at a concentration of 160µM while for DM it was 100µM.

Morpholino analysis and injection. The bmp2bMO (GeneTools, LLC) 5'-GCGGACCACGGCGACCATGATC -3' targets the transcription start site; it was used at 5ng/nl and 1-2 nl of solution was injected into the yolk as close as possible to the cell(s) at the one to two cell stage.

Animal cap dissection and culture. Prior to dissection, embryos were dechorinated with Pronase (1mg/ml, Sigma-Aldrich) in 1x Ringer's solution (zebrafish book) on an agarose bed. Subsequent to Pronase treatment, embryos were washed four times with 1x Ringer's solution and then put in fish water (treated or untreated), still on agarose. Control (untreated/uninjected) embryos, embryos which were injected only and embryos whose caps were exposed from the MBT were dechorinated at the eight cell stage. For exposures from the 2 cell

stage, embryos were dechorinated at the 1cell stage and immediately exposed either to PD173074, DM, or to both. All embryos were allowed to develop at 28°C until the 256/512 cell stage when the animal caps were dissected (prior to the MBT). Except where treatment was from the 2 cell stage to fixation, all dissection was done in 0.5% methyl cellulose/1xRinger with great care being taken to avoid cells close to the margin. For treatments from the 2 cell stage to fixation, dissection took place in the treated fish water. The caps were then transferred to a different dish with the same concentration of chemical prepared together with that of the first dish. For treatments from the 2 cell stage to the MBT, embryos were first washed four times with fish water prior to dissection. For treatments from the MBT, the animal caps were first dissected then placed in treated water. All caps were transferred to 24 well plates (in treated or untreated fish water) after a ten minute recovery period, with a maximum of 20 caps per well, and allowed to develop to fixation.

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Figures and Figure Legends

Figure 1.

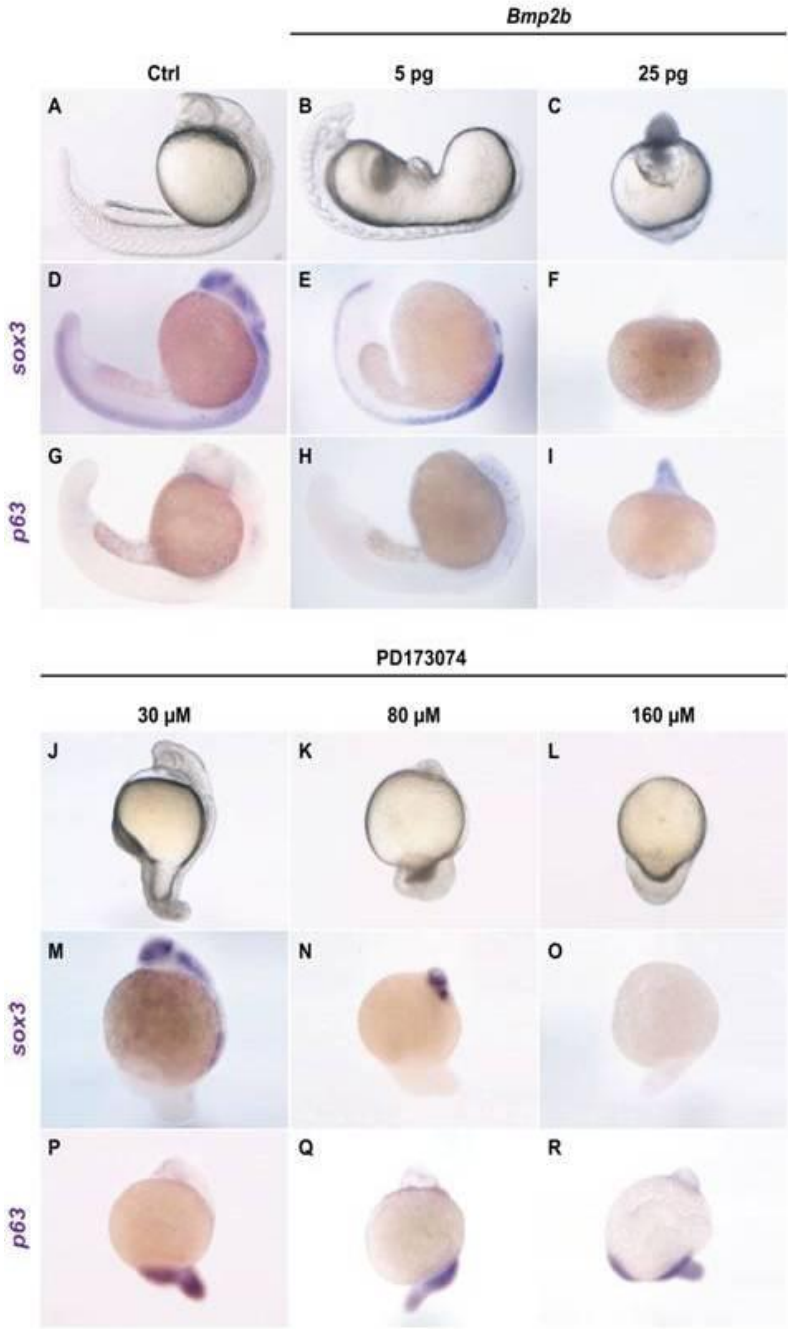


Figure 1. Opposite sensitivities to Bmp signalling and loss of Fgf along the A-P axis for neural ectoderm (22hpf embryos). Zebrafish embryos were injected with different concentrations of *bmp2b* mRNA (B,C,E,F,H,I) or exposed to different concentrations of PD173074 (J-R) as indicated at the top of the rows. All the embryos shown are at 22 hpf, [lateral views, anterior to the top (Ctrl, uninjected/unexposed controls). Where the embryos have been stained, probes used are indicated to the left of the columns. (B,C) Concentration-dependent

effects of *bmp2b* overexpression showing preferential loss of anterior structures. At high concentrations of *bmp2b* mRNA most visible tissue is concentrated in the anterior and forms a bump structure (C). (D-I) *In situ* hybridisation of control and *bmp2b* mRNA-injected embryos. (D-F) Anterior *sox3* expression is preferentially suppressed in response to graded Bmp activity, being completely abolished at the highest concentrations of *bmp2b* mRNA (F). (G-I) Concomitantly, *p63* expression is induced in the anterior with low concentrations of *bmp2b* mRNA (H). Excess epidermis forms in the prospective anterior domain (I). (J-L) Concentration-dependent effects of exposure to PD173074 showing preferential loss of posterior structures At the highest concentrations of PD173074, all head and trunk structures are lost (L), with all the tissue concentrated in the posterior, opposite to what was observed with injection of *bmp2b* (compare to C). (M-R) *In situ* hybridisation of PD173074-exposed embryos. (M-O) Posterior *sox3* expression is preferentially lost, being completely abolished or extremely faint at the highest concentrations of PD173074 exposure (O). (P-R) Complementing the *sox3* data, *p63* expression is induced most strongly in the posterior of the embryos, and marks tissue that does not express the neural marker, *sox3*.

Figure 2.

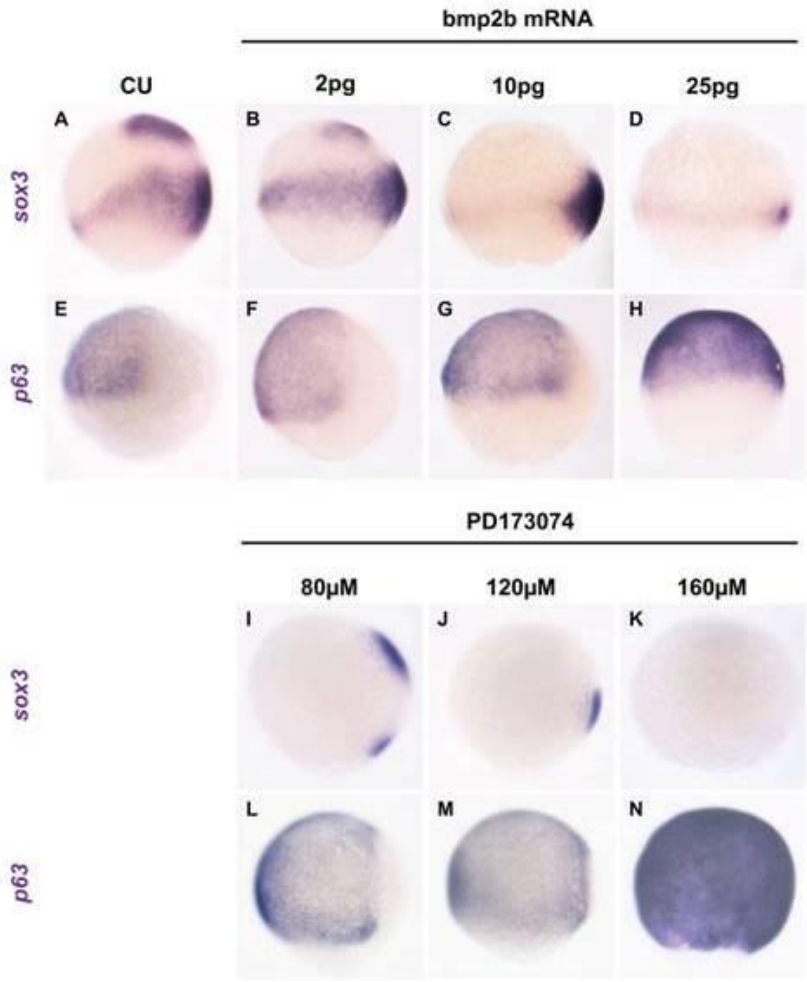


Figure 2. Opposite sensitivities to Bmp signalling and loss of Fgf along the A-P axis for neural ectoderm (gastrula embryos). *In situ* staining at the late gastrula stage of zebrafish embryos either injected with different concentrations of *bmp2b* mRNA (B-D, F-H) or exposed to different concentrations of PD173074 (I-N) as indicated at the top of the columns. (CU, uninjected/unexposed embryos). Lateral views, dorsal to the right (where discernible), probes used shown to the left of the rows. (B-D) Anterior *sox3* expression is preferentially suppressed in embryos injected with *bmp2b* mRNA. Even with the highest dose used (D) posterior *sox3* expression is still visible as a ring near the margin. (F-H) Concentration-dependent induction of the epidermal marker *p63* by *bmp2b* overexpression which complements the *sox3* data. At the highest concentration p63 expression expands to cover the whole of the anterior, but does not expand posteriorly (H). (I-K) PD173074 preferentially suppresses posterior *sox3* expression. At low concentrations, posterior sox3 expression is restricted to a dorsal-most domain while anterior expression remains largely unaffected except

for a small posterior shift (I). At intermediate concentrations, posterior expression is abolished and anterior expression is also much reduced and is shifted posteriorly to the margin (J) consistent with the loss of all mesoderm tissue. With the highest dose (K) all *sox3* expression is lost. (L-N) *P63* expression in PD173074 exposed embryos, like with Bmp overexpression, shows an expansion that complements that of *sox3*. But unlike with Bmp, *p63* expression expands posteriorly to the margin (M,N compare with H).

Figure 3.

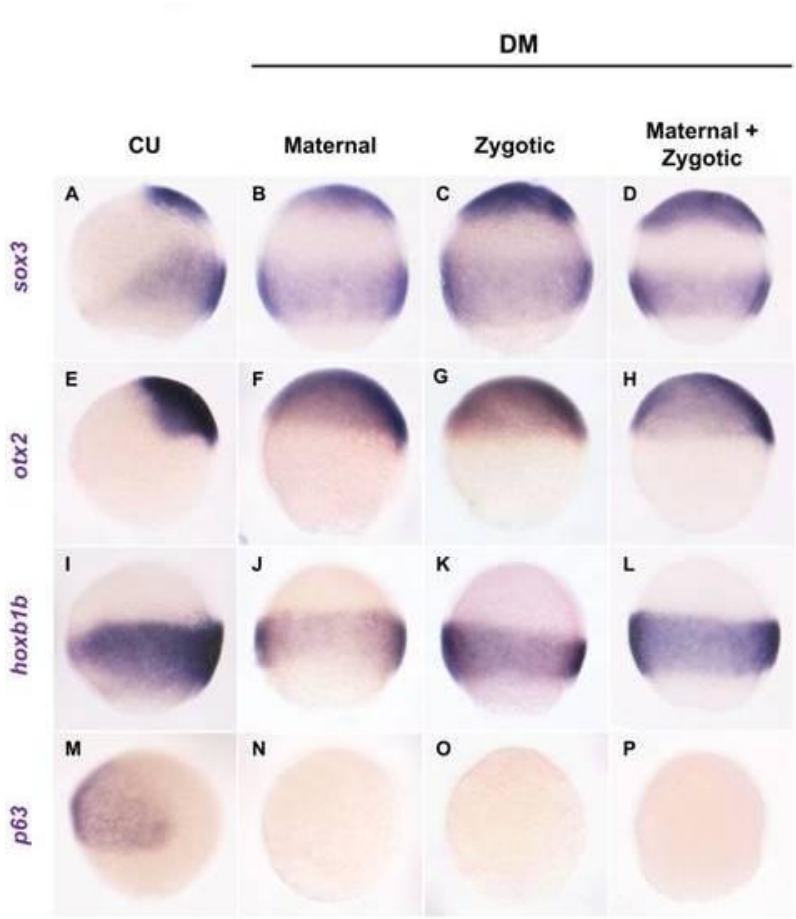


Figure 3. Roles of maternal and zygotic Bmp in epidermal and neural specification. *In situ* staining at the late gastrula stage of zebrafish embryos exposed to DM in a stage-specific manner as shown at the top of the columns. (CU, unexposed controls). Lateral views, dorsal to the right (where discernible). Probes are shown to the left of the rows. (B-D) Both anterior and posterior *sox3 expression* domains are expanded in all conditions of DM exposure, as are the expression domains of the anterior neural marker *otx2* (F-H) and the posterior neural marker *hoxb1b* (J-L). In all conditions the epidermal marker, *p63*, is

completely suppressed (N-P). We did note, however, that the penetrance of anterior neural marker expansion in embryos exposed to DM from the two cell stage to the MBT (*sox3* (B), *otx2* (F) was lower than in the other two conditions. Posterior expansion was similar in all three exposures (see results). Nevertheless, the expression of *p63* was completely suppressed in all exposures with 100% penetrance.

Figure 4.

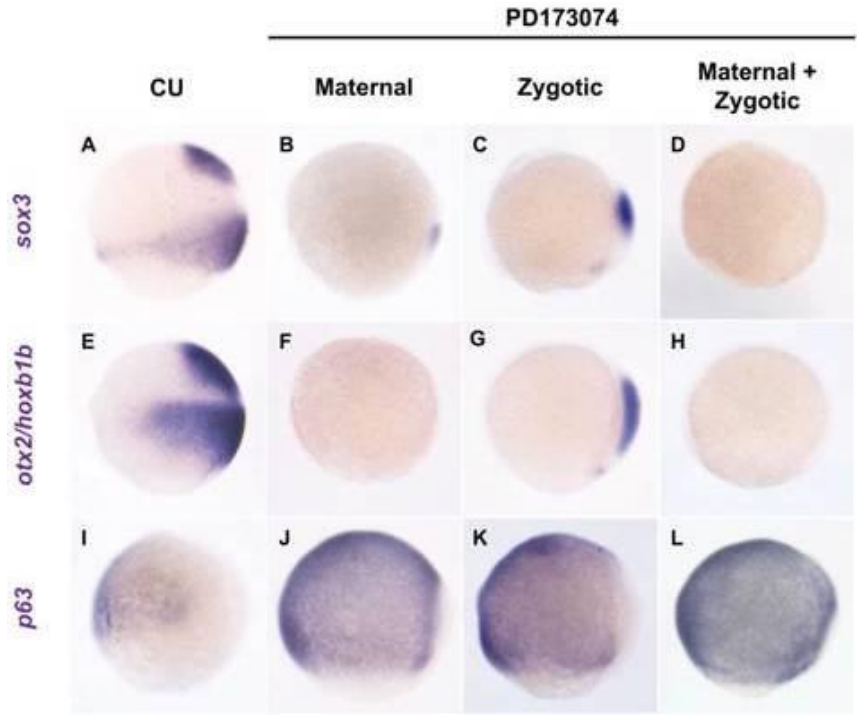


Figure 4. FgfR signalling is necessary for both anterior and posterior neural induction. *In situ* staining at the late gastrula stage of zebrafish embryos exposed to PD173074 in a stage-specific manner as shown at the top of the columns. (CU, unexposed controls). Lateral views, dorsal to the right (where discernible). Probes used are shown to the left of the rows. (B,F,J) Embryos exposed to PD173074 from the 2 cell stage to the MBT show an almost total suppression of neural tissue at the expense of epidermis. *Sox3* posterior expression domain is abolished while the anterior domain is much reduced and shifted posteriorly (B) while expression of *otx2* and *hoxb1b* is also lost (F) with concomitant expansion of epidermis as observed with *p63* staining (J). (C,G,K) Exposure from the MBT leads to a marked reduction of posterior neural marker expression and dorsal and posterior expansion of epidermal tissue. (C) Posterior *sox3* expression is restricted dorsally, as is the expression of *hob1b* (G) but anterior *sox3* expression remains largely

unaffected although shifted posteriorly (C), similar to the effects on the expression of *otx2* (G). Expression of the epidermal marker, *p63*, is expanded posteriorly and dorsally but dorsal expansion is not as extensive as observed with exposure to the MBT (K, compare to J). (D,H,L) Exposing embryos from the 2 cell stage to fixation leads to complete loss of all neural marker expression tested while *p63* expression expands to cover most of the embryonic ectoderm.

Figure 5.

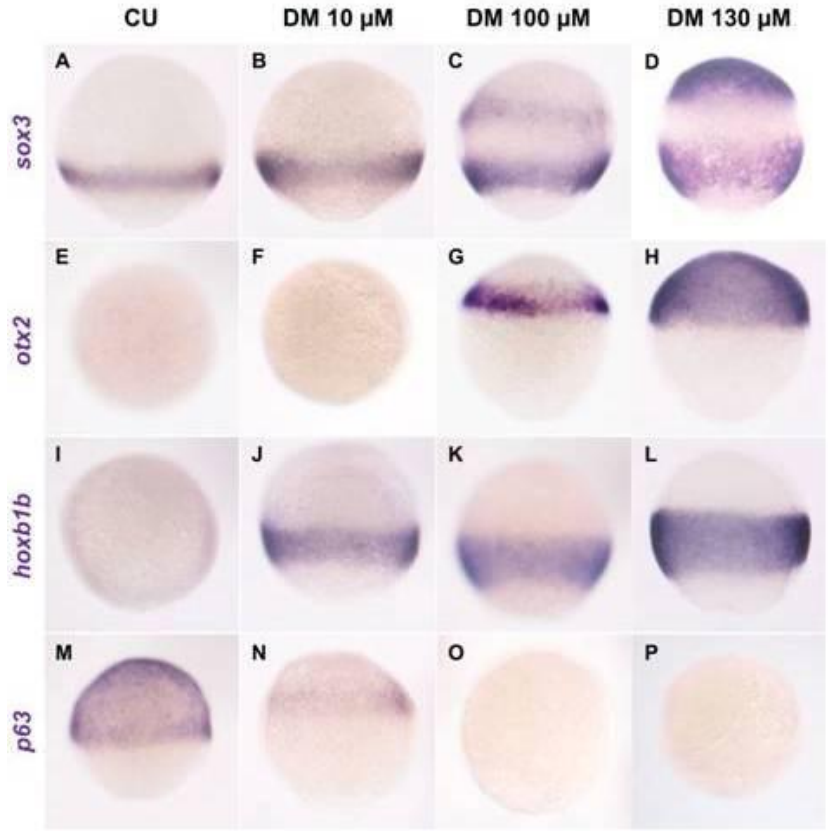
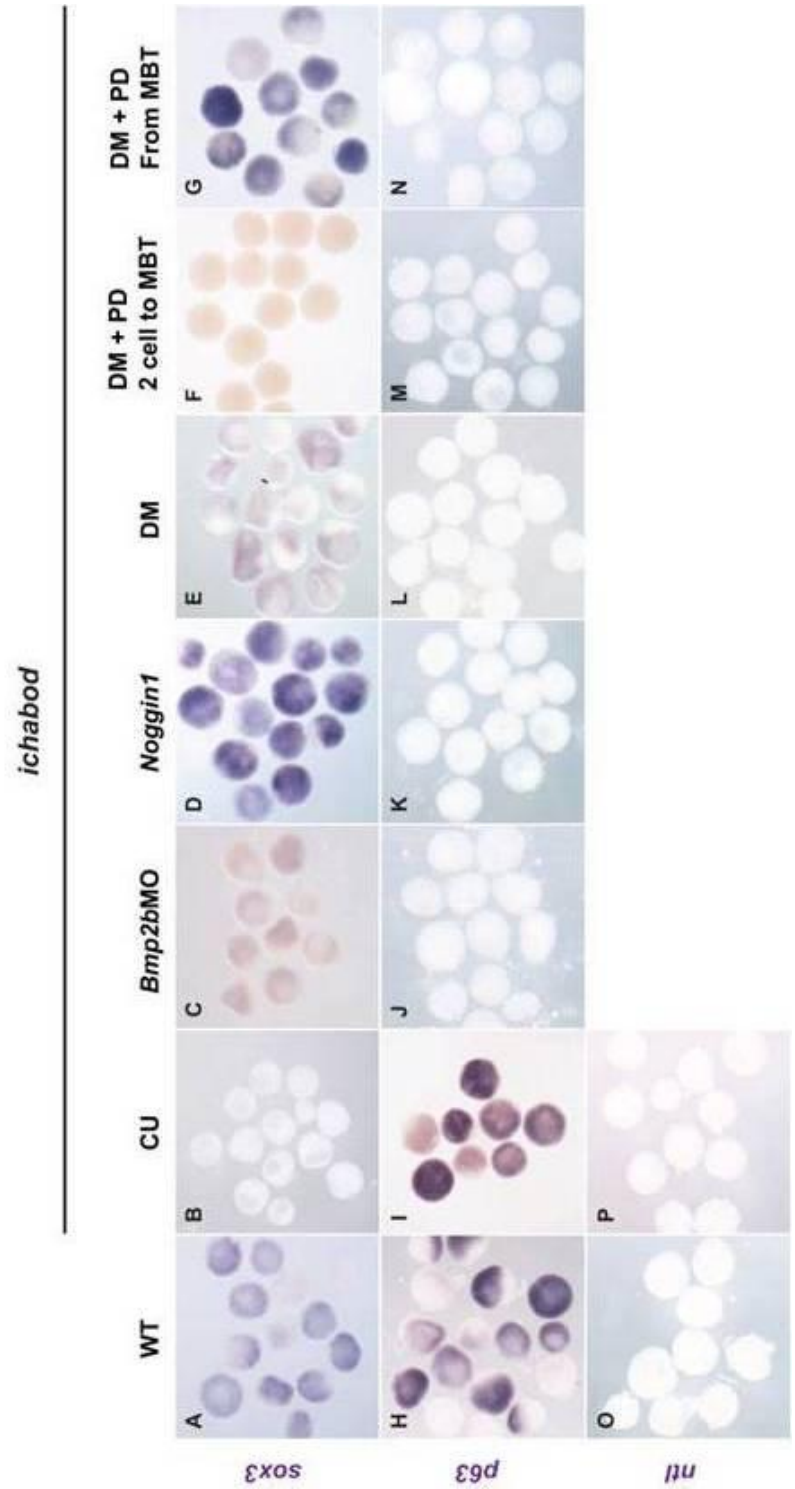


Figure 5. Anterior and posterior neural ectoderm is restored by DM in a dose-dependent manner in *ichabod* embryos. *In situ* staining of *ichabod* embryos at the late gastrula stage exposed to different concentrations of DM from the 2 cell stage as indicated at the top of the columns. Probes used are shown to the left of the rows. All (100%) of the embryos responded in a similar manner to graded DM exposure and >20 embryos were scored per gene/per condition. (A-D) Staining of unexposed *ichabod* embryos. Posterior *sox3* expression is always present as a ring near the margin (A) but no anterior expression is visible.

Likewise, there is no expression of the anterior neural marker, *otx2* (B) or, in most cases, the posterior neural marker gene, *hoxb1b* (C) although some patchy expression is seen with a low penetrance as a very thin ring near the margin, similar to posterior *sox3* expression domain (see A). (D) *P63* expression covers all anterior tissue in *ichabod* embryos, consistent with ventralisation. (E-H) Posterior neural ectoderm is fully restored in ichabod embryos exposed to low concentrations of DM. (E) *Sox3* posterior domain is slightly expanded, but the anterior domain is still not induced, as is also the case with *otx2* (F). *Hoxb1b* has now been activated and is visible radially with a penetrance of 100% (G). Even with low concentrations of DM, *p63* expression is visibly reduced (H). (I-L) Anterior neural ectoderm is partially restored in *ichabod* embryos exposed to intermediate concentrations of DM. More severe loss of Bmp signalling leads to induction of both anterior *sox3* (I) and *otx2* (J) expression, with correct A/P polarity, and is visible as a thin ring which appears to mark the A/P boundary. (K) *Hoxb1b* expression is further expanded, and consistent with rescue of neural tissue, *p63* expression has been lost (L). (M-P) Exposure to high concentrations of DM completely restores anterior neural ectoderm in *ichabod* embryos. Both anterior *sox3* (M) and *otx2* (N) expression now mark the entire anterior ectoderm, and both posterior *sox3* (M) and *hoxb1b* (O) expression domains are further expanded suggesting that organiser-derived Fgfs are not necessary for neural induction in zebrafish. (P) *P63* expression is again absent.

Figure 6.


ichabod
WT
CU
Bmp2bMO
Noggin1
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DM + PD
2 cell to MBT
DM + PD
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sox3
p63
nti

Figure 6. Anterior neural induction does not occur in *ichabod* caps exposed to both DM and PD173074. Novel ectodermal explant assay which for the first time makes use of a genetic mutant. (A-P) *In situ* staining of wt and *ichabod* animal caps fixed when embryo controls were at the late gastrula stage. (WT, wild type uninjected/unexposed caps; CU, control uninjected/unexposed *ichabod* caps. Probes used are shown to the left of the rows, injection and exposure conditions are shown at the top of the columns. (A,H) In WT animal caps, expression of *sox3* and p63 respectively is patchy, revealing their unsuitability for this assay. However, in control *ichabod* caps *sox3* expression is absent (B) while *p63* is expressed ubiquitously, consistent with the embryo data (Fig. 5A,J). (O,P) *ntl* expression is absent in both WT and *ichabod* control caps, suggesting there is no mesoderm/posterior contamination in our experiments. (C-E, J-L) *Ichabod* caps exposed to, or injected with, three different inhibitors of the Bmp pathway. (C-E) *Sox3* expression is induced in all the caps when compared to controls (see B), while as expected with loss of Bmp signalling, expression of the epidermal marker, *p63,* is lost (J-L) in all the caps. (F,G,M,N) *Ichabod* caps exposed in a stage-specific manner to PD173074, while also being exposed to DM from the 2 cell stage to fixation. (F) When *ichabod* caps are exposed to PD until the MBT, *sox3* expression is absent in most of the caps, and only mildly visible in the two caps that have *sox3* expression. However, when the caps are exposed to PD from the MBT, most of the caps show *sox3* expression (G) although weakly in some of the caps. In both conditions, there is no *p63* expression (M,N).

Chapter 4. Research Chapter 3

Development of an Ectodermal Explant Assay for the Analysis of Neural Development in the Zebrafish Embryo.

Development of a zebrafish ectodermal explant assay. The major signalling pathways important for neural development may have multiple and often overlapping functions during embryogenesis, affecting processes such as cell fate specification and morphogenesis in the different germ layers. This makes it difficult to analyse the direct effects that signalling by these molecules have on ectoderm, as responses may be mediated by signals from different cell layers. In order to be able to analyse the direct responses induced in ectoderm by signals important for neural development, a system was needed that was free of mesendoderm tissue contamination. To this end, we decided on the development of a zebrafish *in vitro* ectodermal explant assay which involves excising a group of cells from the animal pole of embryos at or near the MBT, i.e. before the onset of mesodendoderm specification, thus creating a cell mass purely of ectodermal character. And since the explants should lack an organiser and consequently Bmp antagonists, this should produce masses of ventralised naïve cells of an anterior character that are homogenous for expression of genes under the control of Bmp signalling. Modification of gene signalling could then be used to generate neural tissue and to analyse the molecular mechanisms involved in neural development. The basis for an explant assay comes from a similar system that has been used in Xenopus research for over twenty years, as well as a previous attempt to generate such a system in zebrafish. However, the epidermal nature of the *Xenopus* explants has been questioned (Linker et al., 2009) and the zebrafish protocol was time consuming and not appropriate for high throughput analyses, as only one explant was obtained for every ten animal caps that were cut. We aimed to improve this system by obtaining one explant for every embryo cut and additionally to extend its use to analyses of all stages neural development. And since such an *in vitro* cell mass culture (cell aggregates) can be generated using mammalian stem cells, data could be generated and compared between the two systems.

Development of an Ectodermal Explant Assay for the Analysis of Neural Development in the Zebrafish Embryo.

Abstract

We set out to develop a zebrafish ectodermal explant assay that would be suitable for analyses of all stages of early zebrafish neural development. Based on a novel one embryo-one explant system, we show that out system generates explants of an anterior, ectodermal character free of mesendoderm contamination and in large enough numbers. Possibly due to early organiser signalling, we find that explants generated from wild type embryos are not suitable for analyses of anterior neural development. Instead, we have used for the first time ectodermal explants excised from a mutant line, the organiser-deficient *ichabod*. We show that the *ichabod* system is clean and considerably more homogenous in terms of gene expression than the wild type. Further, we show that our mesendoderm and organiser-free explants provide a viable system to analyse all early neural development processes in zebrafish, using a multitude of embryological and molecular methods.

Introduction

Development of the early vertebrate nervous system is and has been the subject of intense research for obvious reasons. It is imperative that the molecular mechanisms involved in neural development, from the initial neural inducing signals up to the formation of functional neuronal networks (Fig. 3), are understood so that therapies may be developed for debilitating neurodegenerative diseases. However, unravelling the molecular mechanisms involved in neural development has not been easy. Many of the signalling pathways involved in neural development are also involved in other, often overlapping, developmental processes. For example, it has been shown that interactions between Bmp and Fgf signalling pathways can affect both neural and regional cell fate specification (Kudoh et al., 2004), indicating that responses to Bmp and Fgf signals can be context-dependent. Furthermore, both Fgf and Bmp signalling play critical roles in neural induction, the initial mechanisms that lead to the patterning of the ectoderm into neural and non-neural domains (see Stern, 2005). Evidence from zebrafish suggests that Fgf signalling, active in marginal prospective mesoderm, induces posterior (trunk and tail) neural tissue (Koshida et al., 2002; Kudoh et al., 2004; Rentzsch et al., 2004). Meanwhile, research done mostly with *Xenopus* animal caps has historically suggested that Bmp antagonism, provided by signals emanating from the dorsal organiser, specifies anterior (head) neural tissue (Munoz-Sanjuan and Hemmati-Brivanlou, 2002; Weinstein and Hemmati-Brivanlou, 1999) in overlying ectoderm. In addition, more recent data has implicated Fgf signalling in anterior, as well as posterior, neural induction in vertebrates (Stern, 2005). But both Bmp and Fgf signalling also have seperable, overlapping roles in processes such as morphogenic cell movements and stem cell differentiation as well as in cell fate specification in both endoderm and mesoderm (Böttcher and Niehrs, 2005; Dale and Jones, 1999; Dorey and Amaya, 2010; Hogan, 1996; Kishigami and Mishina, 2005). Dorsal mesoderm is where the vertebrate organiser forms, and mesoderm is also the source of posteriorising signals, such as retinoic acid (RA) and *eve1* (refs), which can ectopically convey posterior identity on neural ectoderm.

Overlapping functions, context-specific interactions and non-ectoderm sources of signalling can complicate efforts to analyse neural-specific roles of these signalling pathways and how they interact at a molecular level to specify neural and non-neural fates in the ectoderm. Responses induced in the ectoderm

may be indirect, as effects on neural specification may be partly mediated by signals from the other germ layers. To overcome the problems associated with germ layer interference, it is desirable to develop a system that is not only free of mesendoderm contamination but also homogenous in terms of gene expression to allow for consistent, unambiguous results. To this end, *Xenopus* researchers have for over twenty years used ectodermal explant assays. Animal pole cells, which reliably give rise to ectoderm in the frog, are excised from the animal pole of *Xenopus* embryos (animal caps) before the onset of mesendoderm specification and the appearance of the organiser (Ogata and Cho, 2007). This results in explants of an anterior, ectodermal nature where cell division continues and the time scale of gene expression largely correlates with that observed in normal development. Data from *Xenopus* animal caps originally led to the proposal of the 'Default' model of neural induction, whereby neural was the 'default' state of the ectoderm which was revealed with suppression of Bmp signalling by organiser-derived Bmp antagonists, and that this was necessary and sufficient for neural induction (Munoz-Sanjuan and Hemmati-Brivanlou, 2002; Weinstein and Hemmati-Brivanlou, 1999). For example, untreated *Xenopus* animal caps normally have epidermal character, presumably because of a lack of an organiser and its derivatives. This epidermal character can, however, be converted to neural by the ectopic addition of Bmp antagonists normally expressed in the organiser (Lamb et al., 1993; Sasai et al., 1995). However, the epidermal character of the *Xenopus* explants has been questioned, with the suggestion that *Xenopus* animal pole cells have already received some pro-neural signals which predisposes them to a neural fate (Linker et al., 2009; Linker and Stern, 2004). Further, it is alleged that pure epidermis, induced by Bmp, cannot be converted to neural tissue by the suppression of Bmp alone. The implications are that *Xenopus* animal caps are not homogenous in terms of gene expression, possibly because of early organiser signalling or some residual organiser activity that leads to some patterning events in the caps.

To address the questions raised above, we decided to develop a zebrafish ectodermal explant assay that would be suitable for the study of all stages of early neural development. Sagerström and others (Sagerström et al., 1996) previously developed a zebrafish animal cap assay, but to avoid mesendoderm contamination, they combined ten very small caps excised from ten embryos to make one cap . This made the system very laborious and not suitable for high

throughput analyses. To improve on this, we designed a one embryo-one cap system similar to *Xenopus* and looked at its suitability for neural development studies. We show that our assay is viable in terms of survival and throughput and that the caps are of anterior character and almost 100% free of mesoderm contamination. However, we initially found that gene expression was not homogenous in explants excised from wild type (wt) embryos and thus not suitable for neural induction assays. But one of the great attributes in zebrafish genetics is the wide availability of mutants lines, such as the *ichabod* mutant. This mutant lacks an organiser, exhibits a ventralised phenotype where most of the tissue is epidermal and lacks all anterior structures, including neural (Bellipanni et al., 2006; Kelly et al., 2000; Maegawa et al., 2006). Using for the first time a mutant line to generate ectodermal explants, we show that gene expression is now considerably more homogenous in the *ichabod* caps when compared to wildtype and thus viable for neural induction analyses. Furthermore, we show that our *ichabod* ectodermal explant assay, free of mesoderm and organiser tissues, is viable for analyses of the other stages of early neural development: Patterning, neurulation (neural tube formation) and neurogenesis, and that aanalyses can be carried out using standard embryological tools.

Materials and Methods

RNA probe synthesis, *in-situ* hybridisation and immunochemistry. Probes used, antisense RNA probe synthesis and *in-situ* hybridisation procedures have been previously described (Kudoh et al., 2001; see Methods section). Detection of transplanted GFP-expressing cells was done with the Immunohistochemistry Staining Kit (Peninsula Laboratories, BACHEM S-4011.0001) according to manufactorer's instructions (see Methods section), with minor modifications. Animal caps were fixed in 4%PFA/PBS and incubated overnight at 4º C. The caps were then rinsed in 1xPBS for 10 minutes and incubated overnight in 100% Methanol at -20º C. Before blocking, the caps were rinsed again in 1xPBS. Caps were treated with the anti-GFP primary antibody (Ams Biotechonology, rabbit IgG anti GFP) in 1:1000 dilution. The substrate-chromogen mixture contained the following dilutions in deionised water: AEC buffer, x20; AEC concentration solution, x25; hydrogen peroxide concentration solution, x40. Staining was visualised using a Nikon dissection microscope. The reaction was stopped by

rinsing briefly in 1xPBS then transferring the caps to 4%PFA/PBS. Acetylated α-tubulin staining was carried out using the following protocol, adapted from Gard, D.L., 1991: Caps were fixed in FGT (85mM PIPES (pH6.95), 1mM EGTA, 1mM $MgCl_2$, 0.4% Triton X-100, 4% Formaldehyde, 0.25% Glutaraldehyde and 0.5µM Taxol) pre-warmed to 28° C. The caps were incubated at 28° C for 4 hours then overnight at 4° C. They were then rinsed in 1xPBST (PBS + 0.4% Triton) for ten minutes and subsequently incubated overnight at -20° C in 100% Methanol (MeOH). The caps were rehydrated with 25% PBST/75% MeOH, then 50% PBST/50% MeOH, then 75% PBST/25% MeOH and finally PBST. Caps were then treated with $NaBH_4$ in PBST for 2x30 min at room temperature (RT), then rinsed with PBST for 3x30 min at RT. After blocking for >3 hours in blocking solution (1% milk, 1% DMSO/PBST), caps were incubated with primary antibody (mouse monoclonal acetylated tubulin antibody, Sigma #T7541 (6-11B-1)) diluted 1:200 in blocking solution overnight at 4° C. After washing 4x30 min in PBST, caps were incubated in secondary antibody (anti-mouse IgG-Alexa488 (Invitrogen cat# A11001), diluted 1:300 in blocking solution, overnight at 4° C. They were then washed again in PBST 3x30 min. For nuclear staining, the caps were then incubated with Hoechst (0.5µg/ml in PBST) then again washed 3x20 min in PBST. The caps were then kept in the dark at 4°C for confocal analyses, which was done on a Zeiss LSM 510 META Laser Scanning Microscope.

mRNA synthesis and injection. Capped mRNAs were synthesised by mMessage mMachine SP6 kit (Ambion) according to the manufacturer's instructions. mRNA concentrations (per nl) used for injctions are: *Noggin1*, 25pg; GFP, 300pg. For *noggin*-GFP coinjection for transplantation, *noggin1* and GFP mRNAs were mixed and then diluted to the appropriate concentrations. GFP fluorescence in injected embryos (donor) and caps (host) was observed using a GFP filter (Nikon dissection microscope). mRNAs (1-2nl of solution) were injected through the intact chorion into all blastomeres at the one- to two-cell stage.

Morpholino analysis and injection. The *bmp2b*MO (GeneTools, LLC) 5'-GCGGACCACGGCGACCATGATC -3' targets the transcription start site; it was used at 5ng/nl and 1-2 nl of solution was injected into the yolk as close as possible to the cell(s) at the one to two cell stage.

Dorsomorphin and RA treatments. Dorsomorphin (DM), a selective inhibitor of Bmp type I receptors (Yu et al., 2007) (Sigma-Aldrich) was used for Bmp signalling knockdown. DM was dissolved in DMSO at a stock concentration of 10mM and embryos were incubated in dorsomorphin further diluted in fish water. For exposures, DM was further diluted in fish water to 100µM. RA (Sigma-Aldrich) was dissolved in ethanol at a stock concentration of $1x10^{-3}$ and further diluted in fish water to $1x10^{-6}$ for exposurers.

Animal cap dissection and culture. Prior to dissection, embryos were dechorinated with Pronase (2.5mg/ml, Sigma cat# P8811) in 1x Ringer's solution (zebrafish book) on an agarose bed. Subsequent to Pronase treatment, embryos were washed four times with 1x Ringer's solution and then put in fish water (treated or untreated), still on agarose. Control (untreated/uninjected) embryos and embryos which were injected only were dechorinated at the eight cell stage. For exposures to DM, embryos were dechorinated at the 1cell stage and exposed to DM from the 2 cell stage. For exposure to RA, embryos were first injected with *bmp2b*MO, allowed to recover in 28°C for twenty minutes, dechorinated, then exposed to RA. All embryos were allowed to develop at 28°C until the 256/512 cell stage when the animal caps were dissected (prior to the MBT). Dissection was done with a tungsten needle in 0.5% methyl cellulose/1xRinger + gentamicin (see Methods) with great care being taken to avoid cells close to the margin. All caps were transferred to 24 well plates (in treated or untreated fish water) after a ten minute recovery period, with a maximum of 20 caps per well, and allowed to develop to fixation. The caps were monitored approximately every hour.

Injury to *ichabod* caps. At the late gastrula stage (~90% epiboly) the caps were washed four times with fish water to remove all the DM as the caps disintegrate after that if left in DM. The individual caps were then pierced with the joined sharp ends of a pair of tweezers and allowed to develop in 28°C to 24hpf.

Results

Developing a one embryo-one explant system, free of mesoderm contamination.

Previous attempts to generate an ectodermal explant assay in zebrafish relied on a time-consuming process which was not suitable for large scale analyses

(Sangerstrom et al., 1996). Ten animal caps were excised from ten different embryos and then combined to make one explant. To make the process faster, we decided to employ a one embryo-one explant system (see Materials and Methods) (Fig. 1(I)) and found it to be viable. With our system, more than 60 explants can be generated per person per hour. The caps are excised at the 256/512 cell stage, before the mid-blastula transition (MBT), which corresponds to the onset of zygotic gene expression in zebrafish, and also before mesendodermal specification . After excision, the animal caps quickly heal and form a ball which continues to grow and are healthy, as lethality is minimal for the first two days. Thereafter, cell death sets in and the caps eventually die, although some can survive for up to four days. We next tested for mesoderm contamination in our caps since it was critical that the explants were purely of ectodermal character. Although mesoderm-specific markers, such as *notail* (*ntl*), are only expressed from about 30% epiboly, after the MBT, the process of mesoderm specification may begin in the early blastula, before the MBT as genes required for mesoderm development are expressed then (Gore et al., 2005; Leung et al., 2003) . It was still possible, therefore, that the explants were contaminated with some mesodermal cells, so we looked for contamination by looking for *ntl* expression in the explants with *in situ* hybridisation (Fig. 1(II)). We did find some contamination among the caps, but we also observed that all the caps that expressed *ntl* also had protrusions, the end of which were marked by *ntl* expression, suggestive of some patterning occurring (Fig. 1(II)A). These protrusions were never present in the *ntl*-negative explants (Fig. 1(II)B) and by removing any caps with protrusions as soon as they became visible, we produced caps that were almost always 100% *ntl*-free and in large enough numbers.

Ichabod explants provide a cleaner system for analyses of neural development.

In order to determine the suitability of the assay for neural development studies, we next performed a gene expression analysis by comparing the expression of neural and non-neural marker genes between wt embryos and ectodermal explants dissected from wt embryos (Fig. 2(I)A-L). We first sought to determine the mesoderm-free and anterior nature of our explants by looking for expression of *ntl* as well as *hoxb1b*, an early marker for prospective posterior neural tissue (Alexandre et al., 1996). Both genes are normally activated by

signals emanating from the margin (posterior) (e.g. Griffin et al., 1995; Rodaway et al., 1999), but since the ectodermal explants are excised before the onset of posterior/mesendoderm-specific gene expression, the expression of both *ntl* and *hoxb1b* is not expected to be activated in the animal caps. As expected, both *ntl* and *hoxb1b* expression is absent in the wt animal caps (Fig. 2(I)A,B) but is evident in the posterior of wt embryos (Fig. 2(I)G,H). These data are in agreement with the excised explants being of an anterior ectodermal character, which is further confirmed by presence of staining for *otx2* (an anterior neural marker)(Fig. 2(I)C), *sox3* (marks anterior and posterior neural tissue) (Fig. 2(I)D) and the two epidermal (ventral anterior) marker genes, *p63* (Fig. 2(I)E) and *foxi.1* (Fig. 2(I)F).

However, expression of both neural and non-neural genes activated in wt animal caps is patchy and thus unreliable for use in the study of early neural development. The reasons for the patchy gene expression we observed are not clear, but there is a ventral to dorsal gradient of Bmp signalling in the gastrula ectoderm. High levels of Bmp specify epidermis on the ventral side, while dorsally, low levels of Bmp signalling, mediated by organiser signals, specifies anterior neural tissue. Since cutting the caps is not an exact procedure, we reasoned that the patchyness we observed was due to lack of homogeneity in gene expression in the explants due to the presence of early organiser signals (Fig. 2(II)). To overcome this problem, we decided to use the zebrafish *ichabod* mutant line for our ectodermal explant assay. Since the *ichabod* mutant is ventralised and lacks an organiser, caps cut from mutant embryos should consist purely of epidermis induced by Bmp signalling (see kelly et al, 2000). We again compared the expression of the same neural and non-neural genes as before, but this time between *ichabod* embryos and the *ichabod*-derived animal caps. We again checked for posterior/mesoderm contamination and found that both *ntl* (Fig. 2(III)A) and *hoxb1b* (Fig. 2(III)B) expression was absent in the *ichabod* caps. However, the expression of both *otx2* (Fig. 2(III)C) and *sox3* (Fig. 2(III)D) are now abolished in the *ichabod* caps and, consistent with the *ichabod* mutant being ventralised, the expression of both epidermal markers, *p63* (Fig. 2(III)E) and *foxi.1* (Fig. 2(III)F), are now expressed ubiquitously, suggesting that the ectodermal explants are purely of an epidermal and anterior character (Fig. 2(IV). Our data show that our assay system for the study of neural development, derived from the *ichabod* mutant, is a viable one: Firstly, it is more homogenous in terms of gene expression than the caps derived from wt embryos and therefore more

reliable; and secondly, anterior gene expression in the *ichabod* embryos (Fig. 2(III)G-L) is recapitulated in the *ichabod* caps, suggesting that gene expression in the *ichabod* caps follows a similar pattern to that seen in the *ichabod* embryos.

***Ichabod* Explants and Neural Induction.**

To further evaluate the viability of our ectodermal explant assay, we analysed how the caps would respond to previously characterised neuralising signals during the various phases of embryonic neural development (see Fig. 3), starting with neural induction. As has been reported, suppression of the Bmp pathway is necessary for neural induction to occur. In a previous report, we have also shown that exposure to high levels of the small-molecule Bmp receptor inhibitor, dorsomorphin (DM), induces the expression of both anterior and posterior *sox3* in the ventralised *ichabod* embryo and suppresses the expression of the epidermal marker, *p63* (Research Chapter 2, Fig. 6D). Therefore, suppressing Bmp signalling by various means should phenocopy these results in the *ichabod* explants. We targeted the knockdown of the Bmp pathway by three different means: Injection of a morpholino oligonucleotide directed against the transcriptional start site of the *bmp2b* gene (see Materials and Methods) which should knockdown not only *bmp2b* expression but should also disrupt the regulatory Bmp positive feedback loop and knockdown all Bmp signalling (Biehs et al., 1996; Schulte-Merker et al., 1997); exposure to DM, which inhibits Bmp type I receptors, preventing phosphorylation of the Bmp pathway signalling effectors, the SMADS (Yu et al., 2007); and injection of mRNA coding for the soluble Bmp antagonist, Noggin1, which functions by binding to Bmp ligands and interfering with their binding to their cognate receptors (Groppe et al., 2002). In caps excised from *ichabod* embryos injected with *bmp2b*MO, expression of the neural genes *sox3* (Fig. 4E, compare to A) and *otx2* (Fig. 4G, compare to C) is induced in *ichabod* embryos. At the same time, epidermis is suppressed as shown by the absence of *p63* (Fig. 4F, compare to B) and *foxi.1* (Fig. 4H, compare to D) staining. *Sox3* is also induced by exposure to DM (Fig. 4I) and *noggin1* injection (Fig. 4K), while *p63* is also suppressed in these conditions of Bmp knockdown (Fig. 4J,L). Thus the *ichabod* caps not only respond to neuralising signals in a manner similar to that observed inthe embryos as previously reported, but they do so in a clean and reliable manner as observed by the ubiquitous expression of neural markers and the complete suppression of epidermal genes.

***Ichabod* Explants and Neural Patterning.**

We next looked at the usefulness of using the *ichabod* animal cap assay for neural patterning processes. Concomitant with and subsequent to neural induction, neural ectoderm is patterned by a variety of factors, including posteriorising signals that convey to cells posterior (hindbrain/spinal cord) positional and informational identity along the A/P axis (e.g., see Kudoh et al., 2002). Among proposed posteriorising factors is retinoic acid (RA) (Blumberg et al., 1997; Conlon, 1995) which has been shown to be required for hindbrain and anterior spinal cord development mainly via the regulation of expression of *hox* genes, including *hoxb1b* (Gavalas and Krumlauf, 2000; Kudoh et al., 2002; Maden, 2002). Exposure to RA ectopically induces *hoxb1b* as well as other posterior-specific gene expression and suppresses anterior-specific genes such as *otx2* in wt embryos (Kudoh et al., 2002), conveying posterior character to anterior ectoderm in exposed embryos. To analyse if exposure to RA would show the same effects in the explants, we exposed animal caps excised from both wt and *ichabod* embryos since both caps have anterior character and don't normally express *hoxb1b*. We first tested the anterior nature of the caps by suppressing Bmp signalling, since Bmp inhibition leads to expansion of *hoxb1b* expression domain in the posterior of both wt and *ichabod* embryos (Research Chapter two). As expected, neither *bmp2b*MO in wt caps (Fig. 5B) nor exposing *ichabod* caps to DM (Fig. 5E) induced *hoxb1b* expression. However, when suppression of Bmp is combined with exposure to RA, *hoxb1b* is strongly and ubiquitously induced in both wt and *ichabod* explants (Fig. 5C,F, compare to A,D) suggesting that the caps have been posteriorised. It also shows that animal caps can be used for experiments to analyse direct changes in gene expression patterns in response to RA signalling independently of mesendodermal influences and, in the case of the *ichabod* mutant, independently of organiser influences.

Transplantation of cells with altered expression of a gene of interest from one embryo (donor) to another with unaltered expression of the same gene (host) is a powerful tool for use in embryology. Cell-autonomous mechanisms, as well as cell-cell interactions that mediate local patterning and inductive/repressive events, can be revealed and analysed. As mentioned above, the *noggin* gene is a well studied suppressor of the Bmp pathway. *Noggin* loss of function ventralises embryos while overexpression dorsalises embryos as well as *ichabod* animal caps

(our data) with concomitant induction of neural, and suppression of epidermal, genes (Furthauer et al., 1999; Lamb et al., 1993; Smith and Harland, 1992; Smith et al., 1993). To test the transplantation method on ectodermal patterning (neural/non-neural), we injected *noggin1* mRNA into *ichabod* donor embryos, together with GFP mRNA as a label for the donor cells (see Materials and Methods) and transplanted cells to uninjected *ichabod* caps (host). We looked for local induction of a neural gene, *otx2*, and suppression of an epidermal marker, *foxi.1*, and show that *noggin*/GFP-expressing donor cells suppressed the expression of *foxi.1*(Fig. 5H) and induced the expression of *otx2* (Fig. 5J) in the host caps when compared to GFP-expressing cells alone (Fig. 5G,I). Again, the uniformity of gene expression in the *ichabod* mutant is a very obvious advantage, making it easier to visualise and distinguish local gene induction and suppression events. Together, our data show that the *ichabod* ectodermal explant assay is viable for the analyses of neural patterning events, using a variety of embryological methods.

Neurulation (neural tube formation).

The neural tube is the structure that will give rise to the central nervous system (CNS) and in zebrafish neurulation begins at the late gastrula to bud stage and is a continuous process throughout somitogenesis (Kimmel et al., 1990; Blader and Strahle, 2000; Figure 3). At 24 hours post fertilisation (hpf) the neural tube in wt embryos expresses many of the same genes that mark the neural plate, such as *sox3* (Thisse et al., 2001), while epidermis still expresses *p63* at this stage (Thisse and Thisse, 2005). In *ichabod* embryos the expression of *sox3* at 24 hpf is absent or very faint (Cruz et al., 2010) while *otx2* expression is never observed (Maegawa et al., 2006). *P63* expression, meanwhile, is induced in most of the visible tissue (data not shown). We have already shown that, at the late gastrula stage, suppressing the Bmp pathway in *ichabod* caps rescues the expression of neural genes while epidermis-specific genes are suppressed (Fig. 4). To test whether our explant assay is applicable to the study of later neural development, we again looked at the effect of suppressing the Bmp pathway in *ichabod* caps, but this time at 24 hpf when the neural tube has been specified. As can be seen, *ichabod* explants not exposed to DM do not express either *sox3* (Fig. 6A) or *otx2* (Fig. 6E) but do express the epidermal marker, *p63* (Fig. 6C) in a similar manner to caps analysed at the late gastrula stage (compare Fig. 4). However, and also in a

manner consistent with the late gastrula data, both neural genes are induced in the caps at 24 hpf when exposed to DM (Fig. 6B,F) while *p63* is now suppressed (Fig. 6D). These results are consistent with *ichabod* embryo data that we show in a previous report (Cruz et al., 2010; Research chapter 2) and our data show that the explant assay can be useful for studies of later, as well as early, neural developmental processes.

A by product of our research with the explants at later stages was the intriguing observation that DM-exposed *ichabod* explants show presumed A/P polarity and develop a structure morphologically similar to a neural tube when injured at the late gastrula stage (Fig. 6H). This structure never appears in unexposed caps (Fig. 6G), suggesting that the injury (piercing with tweezers) is the cause. We do not know why this happens and to date we have not investigated this further, thus we don't know the nature of the structure. It is fascinating, however, to think that this may be a neural tube-like structure, offering even further possibilities to analyse later neural development processes such as neurulation and neurogenesis.

Neurogenesis.

Concurrent with neural tube formation is the first stages of neurogenesis, when prospective neurons first start to differentiate. This process continues long after the neural tube has formed (Blader and Strahle, 2000; Fig. 3), but the early onset of neurogenesis in zebrafish, when compared to most other vertebrates, offers important possibilities to investigate the molecular signals underlying this process. The Hu/Elav family of proteins are expressed exclusively in post-mitotic neurons (Park et al., 2000) and with *in situ* hybridisation we have used the *huC/elavl3* gene to identify the presence of differentiating neurons in our *ichabod* animal caps at 24 hpf. In wt embryos, the presence of differentiating neurons can be seen all along the neural tube (Fig. 7A), while in *ichabod* embryos *elavl3* staining is lost (Fig. 7B), as is the case with *ichabod* explants at 24 hpf (Fig. 7C), suggesting that neurogenesis has not occurred in the *ichabod* mutant and is consistent with lack of neural tissue. When Bmp signalling is suppressed with DM in the *ichabod* caps, however, the expression of *elavl3* is rescued and the presence of differentiating neurons is apparent as seen by the spotted appearance of *elavl3* staining (Fig. 7D). These data imply that neurogenesis has occurred in these caps due to suppression of the Bmp pathway, and to further confirm these results, we

used an antibody against acetylated α-tubulin, a microtubule element found mainly in neurons, to detect the presence of neurons in our animal caps. Confocal microscopy analysis of a fluorescent conjugate (Alexa488, see Materials and Methods) further confirmed that neurons, with projecting axons, are indeed present in the *ichabod* caps exposed to DM (Fig. 7F), while the fluorescent signal in the unexposed caps is almost completely absent (Fig. 7E). With these results, we feel that this explant assay system offers a great opportunity to study the signals that promote neurogenesis in vertebrates, not only in general terms but also at the level of individual neurons.

Discussion

Animal cap assay as a tool to study neural development in zebrafish.

We have developed a zebrafish ectodermal explant assay that has high-throughput capabilities and is clean. Previous attempts to develop an explant assay in zebrafish were time consuming and laborious, where ten caps had to be dissected from ten embryos and then combined to make one single explant (Sagerstrom et al., 1996). Our assay involves obtaining one explant per embryo (Fig. 1) and >60 caps can be dissected per person per hour, making it viable in terms of numbers. We have also for the first time used a mutant line, *ichabod*, for an ectodermal explant assay and the wide availability of mutant lines in zebrafish is one of the great advantages of our assay over pioneering *Xenopus* explant assays. Using the organiser-deficient mutant, *ichabod*, has enabled us to develop a system that is clean, being relatively free of both mesendoderm (posterior) and organiser contamination, providing an aggregate of cells consisting purely of ectoderm and homogenous in terms of gene expression, thus lacking any discernible patterning.

We have shown that our explants are amenable to manipulation by standard embryological methods. The same *in situ* hybridisation protocol that we use for the embryos worked well with our explants, allowing for analyses of changes in gene expression both at the level of the whole cap (e.g. see Fig. 4D) or at a local level (e.g. see Fig. 5H) in response to ectopic manipulation of major signalling pathways of interest. We have also shown that our caps are amenable to changes in, signalling induced by microinjection of morpholino oligonucleotides and mRNAs, as well as changes induced by exposure to chemicals that induce or

inhibit gene expression, such as DM. Indeed, we showed in a previous report (see research chapter 2) that ectodermal explants may be more suited to chemical exposures than embryos. There is considerably less lethality among the caps than embryos, possibly because they are not affected by gastrulation defects and associated lethality, allowing us to use higher doses of the chemicals. Also, marker gene response in the caps seems to be more sensitive to the chemicals, likely due to a smaller cell mass that the chemicals must penetrate.

We demonstrated that cells from donor embryos can be transplanted into the explants and affect the expression of marker genes in a fashion that complements known effects in vertebrate embryos as well as explant cultures. This shows that transplantation of cells (or tissue) with signalling modifications, such as those induced by microinjection and chemical exposure, may be used to analyse early neural developmental processes.

The *ichabod* ectodermal explant assay can be used to analyse all the stages of early zebrafish neural development.

Critically, we have demonstrated that our ectodermal explant assay is suitable for analyses of all aspects of zebrafish early neural development, up to 24hpf and beyond (see Fig. 3). We have shown that our assay can be used for analyses of neural induction, as suppressing the Bmp signalling pathway in *ichabod* caps leads to the induction of neural genes and suppression of epidermal markers in a manner similar to that observed in *ichabod* embryos (see research chapter 2). These data are consistent with a requirement for inhibition of Bmp signalling for neural plate specification in both zebrafish and *Xenopus*. Furthermore, in a previous report (research chapter 2) we used the *ichabod* explant assay to demonstrate an absolute requirement for maternal Fgf signalling in neural induction. We showed that suppressing Bmp alone is not sufficient for neural induction to occur in *ichabod* caps if maternal Fgf signalling is compromised, which would have been difficult to demonstrate in the embryo due to lethality associated with chemical exposures.

We have also demonstrated that our system is suitable for neural patterning assays. Caps derived from both wt and the *ichabod* embryos are suitable for analyses of posterior neural plate patterning events, such as those regulated by the posteriorising factor, RA, as the caps all have anterior character. Furthermore, transplantation of cells to the caps showed that local ectodermal patterning can be

achieved, as we generated chimeras that possess both neural and epidermal markers in response to local Bmp inhibition.

With our system, neural patterning studies can also be extended to later stages, such as neurulation and neurogenesis. We have shown that suppression of Bmp in *ichabod* caps not only induces neural markers at the late gastrula stage, but that expression of these markers, which also mark the neural tube, is maintained up to the 24hpf stage. We have also shown that neuronal-specific markers are induced in the *ichabod* cap in response to inhibition of Bmp signalling, and shows that differentiating neurons with projecting axons can be generated with our system. Modulating key signalling pathways may enable us to analyse the mechanisms involved in neural tube formation as well as patterning. and allow us to identify signals that generate specific neuronal types.

Future directions.

The properties of our zebrafish ectodermal explant assay provide a firm foundation for future analyses of early zebrafish neural development, without interference from other germ layers. We aim to study the direct responses in the ectoderm to the major signalling pathways involved in neural development, as well as how the signalling pathways interact at the molecular level in the specification of the nervous system. By modifying key signalling pathways, we aim to generate explants that show some semblance of physiological patterning, possessing the many cell lines that normally pattern neural ectoderm. With our assay, both anterior and posterior neural cell types can be analysed, such as those that give rise to forebrain, midbrain, hindbrain, spinal cord, placodes and neural crest. Together, they provide the opportunity to study the molecular mechanisms underlying the differentiation of each cell lineage. Although many of the signals involved in cell lineage commitment have been characterised, many are still to be found. Our *ichabod* explants, consisting of naïve cells, provide a great opportunity for the identification of new factors.

We have shown that neurons with axon projections, as well as neural tube markers, can be induced in *ichabod* caps in response to Bmp knockdown. It is feasable, therefore, that by modifying signals (known and new) we can generate specific neuronal types (motor, inter and sensory neurons) within the explants in conjunction with relatively ‘patterned’ central and peripheral nervous system tissues. If achievable, this would allow us to analyse the generation of functional

interconnected neuronal networks which is currently the subject of much research for tissue regeneration therapy.

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Figures and Figure Legends

Figure 1.

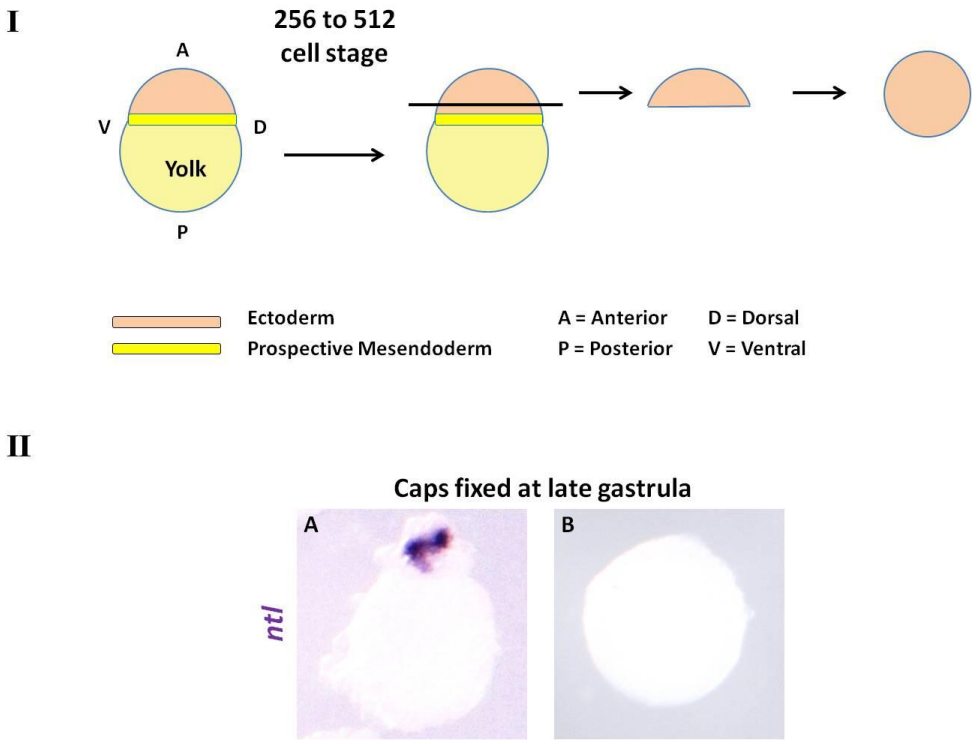

Fig. 1 Zebrafish ectodermal explant assay. (I) Caps are excised at the 256/512cell stage, before the MBT. The explants consist only of ectoderm and after cutting quickly heal and form a ball. One explant is obtained per embryo. (II) Caps with protrusions usually express the mesoderm marker *ntl* (A) and are removed before analyses, generating explants that are almost almost 100% mesoderm-free (B).

Figure 2.

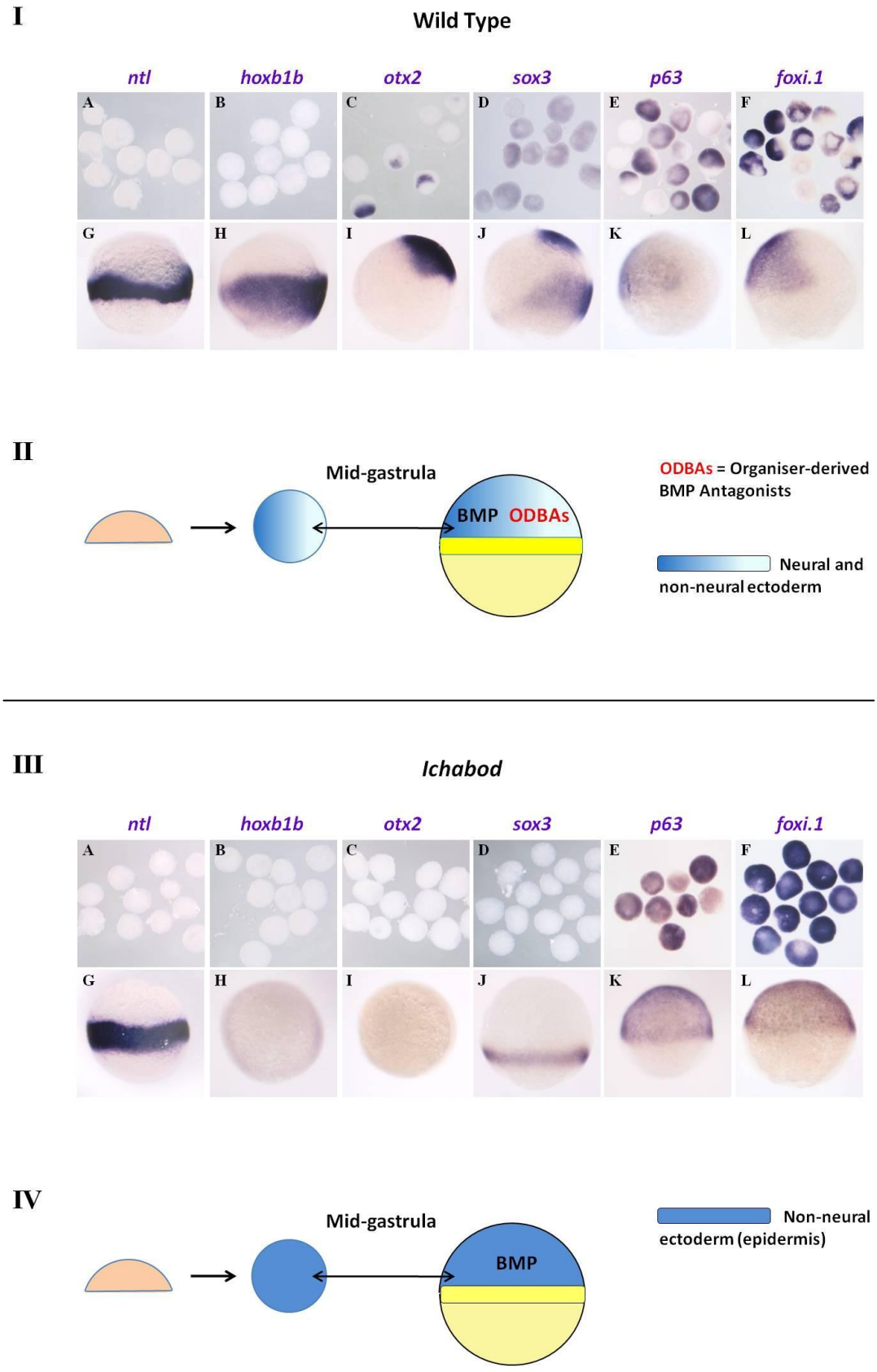
I
Wild Type
ntl
hoxb1b
otx2
sox3
p63
foxi.1
A
B
C
D
E
F
G
H
I
J
K
L
II
Mid-gastrula
BMP
ODBAs
ODBAs = Organiser-derived BMP Antagonists
Neural and non-neural ectoderm
III
Ichabod
ntl
hoxb1b
otx2
sox3
p63
foxi.1
A
B
C
D
E
F
G
H
I
J
K
L
IV
Mid-gastrula
BMP
Non-neural ectoderm (epidermis)

Fig. 2. *Ichabod* embryos provide 'cleaner' explants than wt embryos. (I,II) Wt explants are free of posterior tissue but anterior gene expression is patchy. (I) *In situ* hybridisation of wt explants (A-F) and embryos (G-L) fixed at late gastrula. The expression of both *ntl* (I(A)) and *hoxb1b* (I(B)) is absent in wt caps, while both genes are expressed in the posterior of wt embryos (I(G,H)). This is in agreement with the caps being anteriorised. However, the anterior neural markers, *otx2* (I(C)) and *sox3* (I(D)), as well as the epidermal markers *p63* (I(E)) and *foxi.1* (I(F)), are expressed in wt caps, as they also are in the embryos (I(I-L)). In the explants, this is possibly due to early prospective-organiser activity, which imparts on the ectoderm both neural and epidermal properties (II). (III,IV) *Ichabod* explants are also free of posterior tissue, but anterior neural markers are not expressed. (III) *In situ* hybridisation of *ichabod* explants (A-F) and embryos (G-L) fixed at late gastrula. The expression of both *ntl* (III(A)) nor *hoxb1b* (III(B)) is again absent, but although *ntl* is expressed in the *ichabod* embryo (III(G)) *hoxb1b* is not (III(H)). This is also the case for *otx2* and *sox3,* which are not expressed either in the caps (III(C,D)) or embryos (III(I,J)), consistent with a ventralised phenotype. In agreement with this, the expression of both epidermal markers, *p63* and *foxi.1,* is now ubiquitous in the explants (III(E,F)) and in the anterior of *ichabod* embryos (III(K,L)). (IV) Due to lack of organiser activity, the ectodermal explants are ventralised and no neural markers are activated. This makes for a considerably more homogenous system than the wt.

Figure 3.

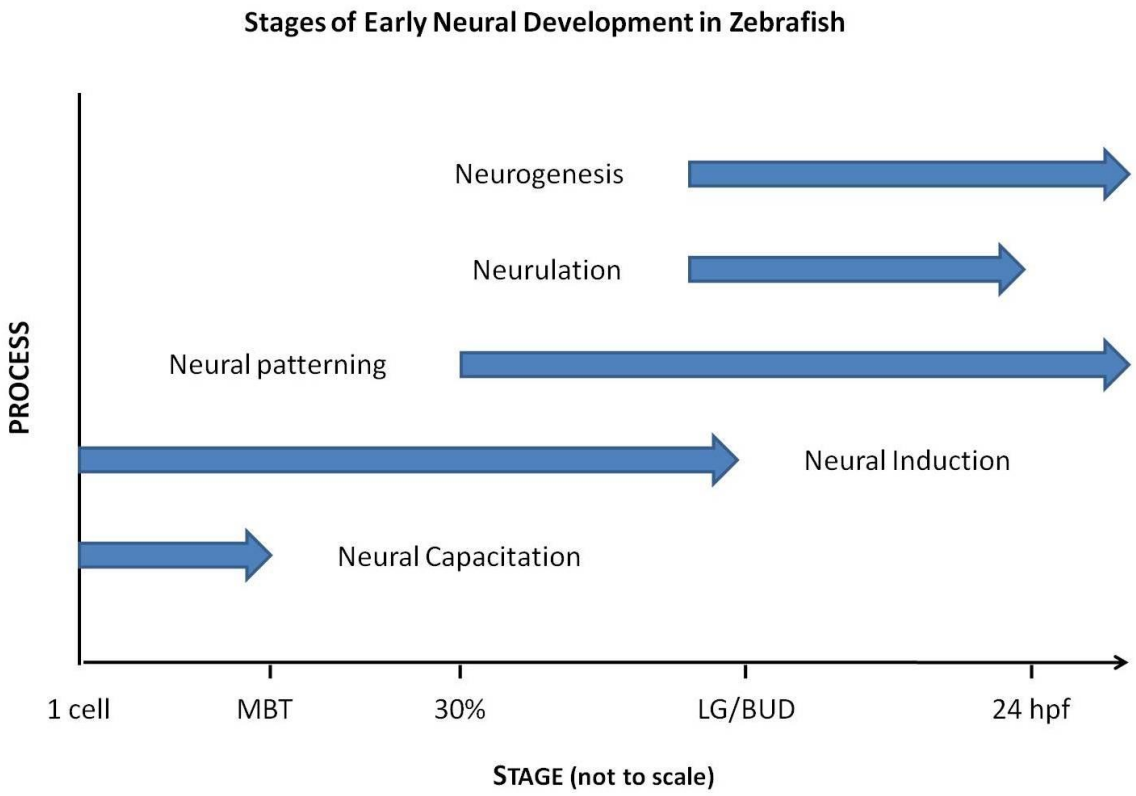

Fig.3 Stages (approximate) of early zebrafish neural development. Stage timing is not to scale.

Figure 4.

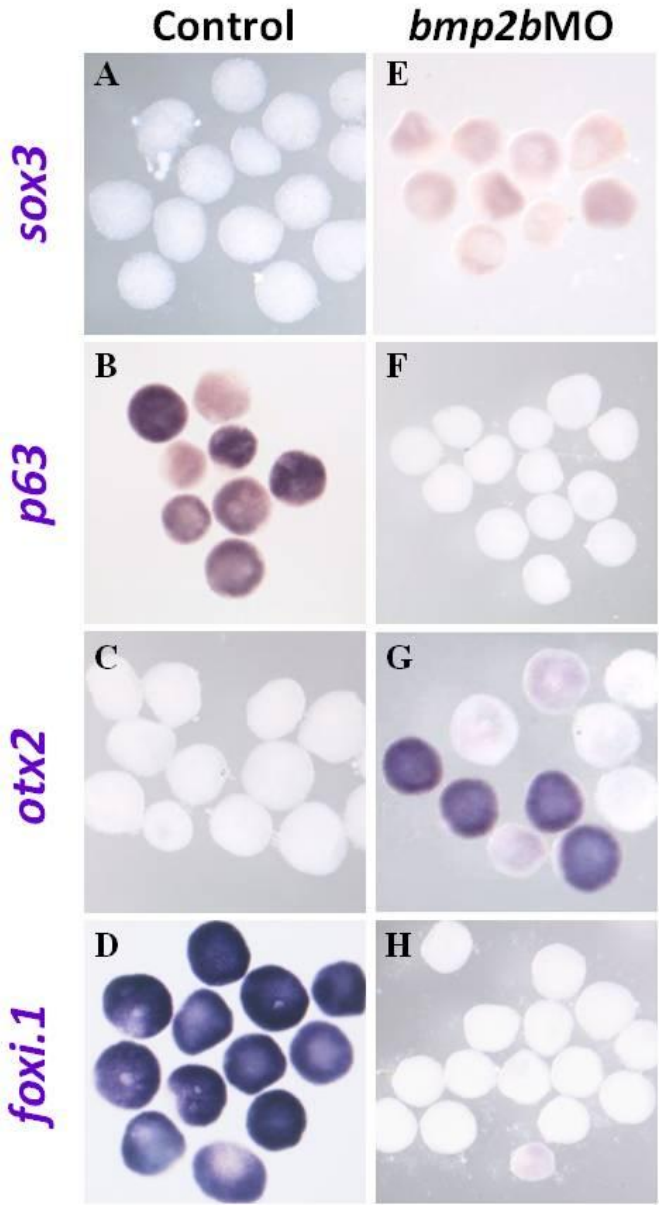

Fig. 4. Neural induction in *ichabod* explants. (A-L) *In situ* hybridisation of explants fixed when control embryos reached the late gastrula stage. (A-D) Control, control uninjected/unexposed *ichabod* caps. Expression of both neural markers, *sox3* (A) and *otx2* (C), is absent in all the caps observed, while the expression of the epidermal markers *p63* (B) and *foxi.1* (D) is ubiquitous. This is consistent with the explants being ventralised. (E-H) Knockdown of Bmp signalling by *bmp2b*MO induces the expression of both *sox3* (E) and *otx2* (G) with concommitant suppression of *p63* (F) and *foxi.1* (H). Inhibition of the Bmp pathway by DM also induces *sox3* (I) and suppresses *p63* (J) expression and a

similar result was obtained with injection of *noggin1* mRNA (K,L). These data are consistent with a requirement for Bmp inhibition in neural induction that has been shown in both wt and ichabod embryos.

Figure 5.

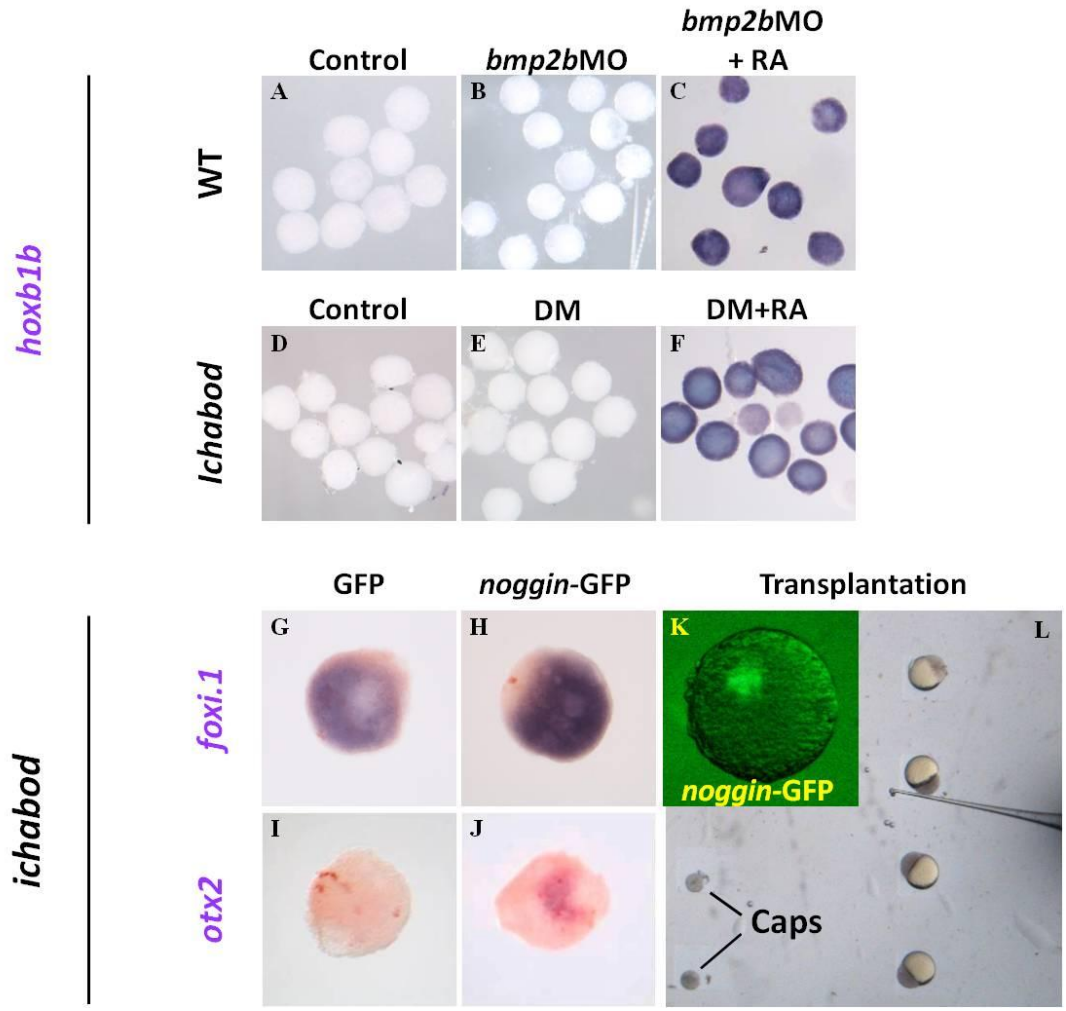

Fig. 5. Neural patterning in wt and *ichabod* explants. (A-J) Explants were fixed when control embryos had reached the late gastrula stage. Control, uninjected/unexposed controls. (A-F) *Hoxb1b* expression is not induced either in wt caps by *bmp2b*MO (B) or in *ichabod* caps by DM (E) when compared to Controls (A,D), consistent with the caps being anteriorised. However, *hoxb1b* expression is induced by exposure to RA in addition to Bmp knockdown in both wt (C) and *ichabod* (F) caps. This suggests that the explants have now aquired posterior character. (G-L) Transplantation of *noggin1*-expressing cells. AEC staining shows that GFP-expressing cells had no effect on the expression of either *foxi.1* (G) or *otx2* (I). However, when *noggin1* is coinjected with GFP, local

suppression of *foxi.1* (H) and induction of *otx2* (J) occurs, generating explants with two different tissue types. (K) *noggin*-GFP-expressing cells transplanted to an *ichabod* cap. (L) At around 30% epiboly cells are removed from injected embryos (right) and transplanted to uninjected caps which are then allowed to develop normally.

Figure 6.

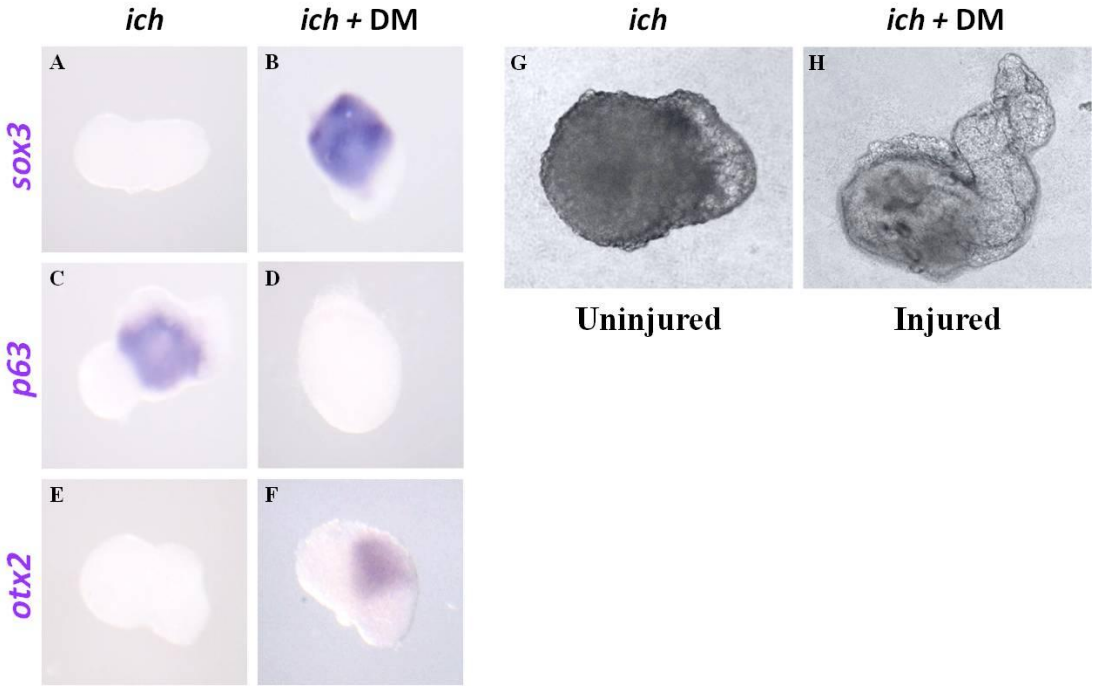

Fig. 6. Neurulation in *ichabod* explants. (A-F) *In situ* hybridisation of *ichabod* caps fixed at 24hpf. As in *ichabod* embryos, at this stage neither *sox3* (A) nor *otx2* (E) is expressed suggesting neural tissue has not been specified, while *p63* expression can be observed (C). When the caps are exposed to DM, however, *p63* expression is abolished (D) as observed at earlier stages. Furthermore, both *sox3* and *otx2*, normally expressed in the neural tube in wt embryos, is now induced. (G-H) In response to injury, *ichabod* caps exposed to DM develop a neural tube-like structure at 24hpf (H) which is not seen in unexposed caps (G).

Figure 7.

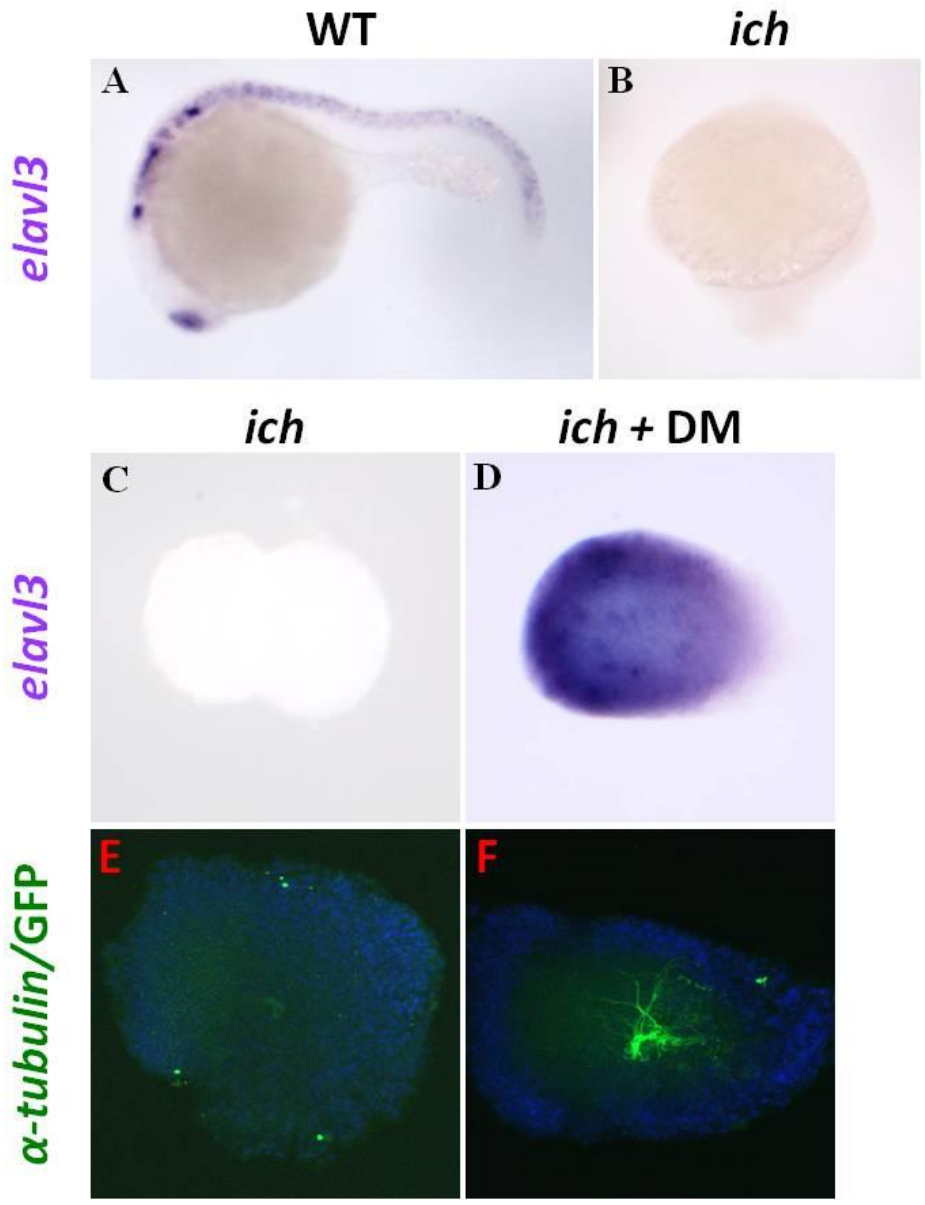

Fig. 7. Neurogenesis in the *ichabod* explant. (A-D) *In situ* hybridisation of the neuronal marker, *elavl3*, in embryos and caps fixed at 24hpf. (A) In wt embryos at 24hpf, *elavl3* stains many differentiating neurons along the neural tube, while staining is absent in *ichabod* embryos at the same stage (B). As in the embryo, *ichabod* caps do not express *elavl3* (C) which is meanwhile strongly induced by exposure to DM (D), suggesting that suppression of Bmp signalling induced neurogenesis in our *ichabod* explants. (E-F) Confocal imaging of α-tubulin staining (green), counterstained with Hoechst (blue), of *ichabod* embryos unexposed (E) and exposed (F) to DM. Again, this shows that neurogenesis has been induced in response to Bmp knockdown.

Chapter 5. GENERAL DISCUSSION

Many of the major signalling pathways involved in neural development in vertebrates have been identified and include, among others, Bmps, Fgfs, Wnts and RA. These pathways have been shown to be widely conserved and to also have vital functions in other, often overlapping, developmental processes. However, and to a certain extent due to their multiple roles, many of the molecular mechanisms underlying neural induction and patterning events by these and other factors remain to be characterised. The main objective of this PhD was the elucidation of some of these molecular mechanisms as well as the development of a zebrafish ectodermal explant assay that could be used to study neural development. To these ends, three different, but overlapping, projects were carried out. In the first project a functional analysis of the *eve1* gene was undertaken, a gene which had been identified as being potentially important for axis formation and posterior (trunk and tail) neural development. The conclusions were that *eve1*, as a transcriptional repressor, functions as an important posterior organiser with essential roles in both neural patterning and neural induction.

In the second project, possible differential contributions of maternal and zygotic Bmp and Fgf signalling to neural induction were analysed, as although Bmp antagonism and Fgf signalling were known to play critical roles in this process, their relative contributions have not been clarified. The results showed that maternal Bmp is essential for epidermal induction, while maternal Fgf signalling is critical for both anterior and posterior neural induction. They sensitise cells to future zygotic Bmp and Fgf specification signals and these properties of maternal Bmp and Fgf were described as capacitation. In addition, it was shown that neural induction by maternal Fgf in anterior ectoderm is independent of Bmp antagonism. Finally, and although not complete, a zebrafish ectodermal explant assay based on the *ichabod* mutant was developed, which will allow future analyses of many aspects of neural development in an environment free of other germ layer, as well as organiser, signalling. The implications of the major findings of these projects are discussed below, with reference to other model organisms where appropriate.

5.1 *Eve1* is critical for posterior neural development.

Eve1 was characterised as a key posterior organiser and in this project it was shown for the first time that *eve1* has essential posteriorising activity. Strong evidence was provided that this activity is mediated by RA in a manner that is similar to what has previously been demonstrated with Fgf and Wnt. Surprisingly, it was also demonstrated that *eve1* has dorsalising activity, including neural induction, and that this activity is likely due to titration of the Bmp signal in posterior ectoderm. However, the phenotypes observed in *eve1* morphants, as well as its position downstream of three major secreted signalling pathways (Bmp, Fgf and Wnt) and upstream of RA suggest that the importance of *eve1* extends beyond that which we investigated.

Data from this project is the first to provide *in vivo* evidence that *eve1* functions as a transcriptional repressor in vertebrates, as this had only been previously shown for its *Drosophila melanogaster* homologue, *even-skipped*. This is crucial for analyses of developmentally important gene networks that involve *even-skipped* homologues, specially so since genes coding for Eve family members are present in the genomes of animals even across phyla, including Ascidians and worms (Ahringer, 1996; Barro et al., 1995; Ferrier et al., 2001; Moran-Rivard et al., 2001; Ruiz i Altaba and Melton, 1989; Schroder et al., 2000). For example, it allows for the assumption that *eve1* directly suppresses Bmp transcription via a negative feedback mechanism that limits the ventralising activity of Bmp and facilitates posterior neural induction, while activation of *aldh1a2* expression is likely via suppression of an as yet unidentified suppressor of *aldh1a2*. It was also shown that epibolic movements were affected when *eve1* expression, especially in loss-of-function analyses. This was important as few genes have been characterised that affect this process, which has been linked to gastrulation and morphogenic movements (Solnica-Krezel, 2005; Warga and Kimmel, 1990). Future analyses should clarify the role of *eve1* in this process.

Apart from functioning downstream of Bmp signalling in D/V patterning, *eve1* is also positively regulated by both Fgf and Wnt signalling, at least in the zebrafish gastrula (Griffin et al., 1995; Kudoh et al., 2004; Ramel and Lekven, 2004; Ueno et al., 2007). Previous findings in zebrafish had shown that Fgf and Wnt posteriorise neural ectoderm in a manner similar to *eve1*: Neither Fgf nor Wnt can activate posterior neural genes during gastrulation in the absence of RA,

and as is also the case with *eve1*, inhibition of the Fgf or Wnt pathways leads to suppression of posterior neural patterning genes. However, RA alone can induce the same genes when Fgf or Wnt signalling is compromised (for example, see Kudoh et al., 2002). Further, and although this has not been reported for Fgf inhibition, *aldh1a2* expression is partially lost in Wnt loss-of-function analysis in a manner that complements loss of *eve1* expression in Wnt8 morphants in zebrafish (Kudoh et al., 2002). Together, these data suggest that, like *eve1*, both Fgf and Wnt directly or indirectly regulate RA synthesis in gastrula embryos, probably upstream of *aldh1a2* as this is the only reported *aldh* gene that is expressed at this stage of development in zebrafish. This, in addition to results in this report, makes it tempting to speculate that *eve1* may well be a crucial effector of posterior neural patterning activity of both Fgf and Wnt. And interestingly, in both chick and zebrafish, it has been proposed that one mechanism by which Fgf suppresses Bmp activity in D/V patterning at gastrula stages is by downregulating Bmp transcription, and consequently signalling (Londin et al., 2005; Wilson et al., 2000). This is similar to what was observed with *eve1*, and raises the possibility that *eve1* may also act downstream of Fgf signalling as an enhancer of posterior neural induction.

Only *eve1* function in neural development was investigated here, and there is little data for Evx protein molecular function in vertebrates. *Evx* genes have proven to be particularly difficult to analyse, as shown by the pre-implantation lethal phenotype of *evx1* null mice and the high mortality of our *eve1* morphants. But some evidence still suggests that *eve1* may also be important for mesoderm development. In the worm *C. elegans*, for example, mispatterned posterior body muscles were reported in mutants of the *eve1* homologue, *vab-7* (Ahringer, 1996). Similarly, a role in patterning axial mesoderm has been suggested for the *Xenopus eve1* homologue, *Xhox3* (Barro et al., 1995; Ruiz i Altaba and Melton, 1989). And in zebrafish, phenotypic loss- and gain-of-function analyses by ourselves and others (Barro et al., 1995) have shown that the whole posterior is affected after *eve1* manipulation, including misshaped somites. Since Fgf, Wnt and Bmp signalling have all been shown to be important for mesoderm development (Winslow et al., 2007), it is again tempting to speculate that *eve1* may mediate some aspects of signalling by these pathways in mesoderm development. Thus *eve1* may be critical not only for A-P patterning of neural ectoderm but possibly also for patterning of the whole A-P body axis.

In this project, evidence has been presented confirming the hypothesis that *eve1* is crucial for posterior neural development in zebrafish embryogenesis. It was shown that *eve1* is an important posterior organising gene, which regulates at least two signalling pathways that are crucial for posterior neural induction and patterning, Bmp and RA. This is in addition to its later role in tail development that has been reported for both fish and frog (Agathon et al., 2003; Barro et al., 1995; Beck and Slack, 1999; Ruiz i Altaba and Melton, 1989), and future analyses should clarify whether Eve1 function in neural development is conserved in other vertebrates too. *Eve1* itself is regulated by Bmp, Fgf and Wnt signalling and thus *eve1* may have further important functions in posterior development and that at least some of these, as well as functions that were characterised in this report, may be downstream of these signalling pathways. This study should facilitate the elucidation of these hypotheses.

5.2 Differential (maternal vs. zygotic) Fgf and BMP signalling in ectodermal patterning.

An outstanding question in vertebrate neural induction is the extent and timing of the requirement for Bmp inhibition and Fgf signalling in this process. We sought to examine these questions in zebrafish by making use of small-molecule inhibitors of the Fgf (PD173074) and Bmp (DM) pathways, which allowed for the analyses of the differential contributions of maternal (pre MBT) and zygotic (post MBT) Fgf and Bmp signalling to neural induction. The main findings of this research were that maternal Fgf signalling is critical for the induction of both anterior and posterior neural ectoderm and, using a novel explant assay, we showed that the requirement for Fgf signalling in anterior neural induction is likely to be independent of Bmp antagonism as had previously been shown for posterior neural ectoderm (Dee et al., 2004; Kudoh et al., 2004; Rentzsch et al., 2004). Further evidence was also provided that continuous Bmp signalling, including maternal, is required for ventral specification and that a requirement for tight regulation of Bmp signalling for proper neural induction is already evident pre MBT. The differential effects we observed, in addition to previously published data, led to the proposition that maternal Bmp and Fgf signalling may act as epidermal and neural capacitators, respectively.

Previous analyses of the molecular methods of neural induction in zebrafish had suggested a differential model, whereby Fgf signalling induces posterior

neural tissue independently of Bmp antagonism while anterior neural ectoderm was induced via the activity of Bmp antagonists (Dee et al., 2004; Kudoh et al., 2004; Rentzsch et al., 2004). Although the stage-specific analysis in this report suggests that Bmp antagonism is indeed necessary for anterior neural induction in zebrafish as has previously been reported, it also suggests that induction of anterior neural tissue requires intact maternal Fgf receptor signalling in a manner that is independent of Bmp antagonism and of the organiser. This novel finding is in agreement with experimental evidence in both frog and chick (Delaune et al., 2004; Streit et al., 2000; Wilson et al., 2000), although no requirement for maternal Fgf signalling has been demonstrated in the frog. Indeed, blocking maternal Fgf signalling in *Xenopus* with another Fgf receptor inhibitor, SU5402, had no affect on embryonic development (Delaune et al., 2004). While the reasons for this difference in frog and fish are unclear, these results could nevertheless be due to the use of a different Fgf inhibitor, PD173074, which may have differing affinities for Fgf receptors than SU5402. In addition, the zebrafish embryo is smaller and therefore tends to show higher sensitivity to chemical treatment. However, species-specific differences cannot be ruled out either. In the chick, meanwhile, epiblast cells isolated from embryos before the onset of zygotic gene expression differentiate into neural tissue, but this ability is lost in the presence of SU5402 (Wilson et al., 2000), suggestive of a requirement for maternal Fgf signalling similar to that observed in zebrafish. In the mouse, meanwhile, zygotic gene expression commences at the two cell stage (Schultz, 1993) making a maternal contribution to neural induction unlikely. But whether maternal or not, results from this report, as well as data from *Xenopus* (Delaune et al., 2004) and chick (Stern, 2000; Wilson et al., 2000), points to an early requirement for Fgf receptor signalling for all neural induction, and this mechanism may be conserved in vertebrates. The data from this report further suggest that this early role of Fgf may be to sensitise ectodermal cells to future neural specification signals, since both maternal Fgfs and Bmps are ubiquitously co-expressed in the pre-MBT blastula, before neural or epidermal specification.

For similar reasons, it is also proposed in this report that maternal Bmp signalling acts as a capacitator of epidermal induction, while the data also show that this capacitation function also serves to inhibit ectopic neural induction on the ventral side of the embryo later during gastrulation. In addition, this report has also further highlighted the differential requirement for Bmp inhibition in the

specification step of anterior and posterior neural induction after the MBT. Because of these and our Fgf data, a model was suggested whereby, after the maternal capacitation step, epidermis would be specified by the continuing activity of zygotic Bmp signalling on the ventral side of the embryo; anterior and posterior neural ectoderm, meanwhile, would be specified by the zygotic activity of organiser-derived Bmp antagonists and marginal, non-organiser-derived Fgf signalling, respectively. However, differences in the results obtained between the *ichabod* explants and wildtype embryos suggests that not all anterior neural induction may be dependent on maternal Fgf. Whilst in the *ichabod* explants the neural marker *sox3* was never expressed under conditions where maternal Fgf and continuous Bmp signalling were suppressed, in wildtype embryos *sox3* expression was never completely lost in anterior neural ectoderm, even at 24 hpf, and epidermal markers never expanded to cover the presumed organiser domain. However, epidermal induction in the whole ectoderm was achieved in some of the embryos when Fgf signalling was continuously inhibited until fixation (see chapter 2). Since zygotic Fgf expression is not activated in the explants due to the absence of mesoderm, a requirement for zygotic Fgf signalling in anterior neural induction could explain these different results. Although anterior neural ectoderm remained largely unaffected when zygotic Fgf signalling alone was suppressed, this could be due to 'leakage' of maternal Fgf activity after the MBT.

What the mechanisms of maternal capacitation by Fgf and Bmp may be was not tested in this report, but there are some clues, at least for anterior neural ectoderm. In this report, evidence was presented that Bmp inhibition could rescue anterior neural induction in organiser-deficient *ichabod* embryos, suggesting that the only role of the organiser in neural induction is to suppress Bmp signalling. However, data from zebrafish and other organisms has shown that neural tissue can still be specified with surgical removal or molecular suppression of the organiser (Ang and Rossant, 1994; Dufort et al., 1998; Kudoh et al., 2004; Stern, 2005; Weinstein et al., 1994). There is some evidence that this may be due to the expression of factors that directly or indirectly antagonise Bmp signalling and/or expression in both prospective organiser and prospective anterior neural cells on the dorsal side of the blastula, such as *boz/dharma* and *squint/ndr1* in fish and the BCNE centre and its precursors in the frog (Kuroda et al., 2004; Leung et al., 2003; Rebagliati et al., 1998; Wessely et al., 2001). Although in *Xenopus* it has not been tested whether Fgf signalling plays any role in the regulation of BCNE

centre genes, in zebrafish Fgf signalling has been shown to function in the maintenance of *boz/dharma* expression and upstream of *squint/ndr1* (Maegawa et al., 2006), two factors that are expressed maternally (Leung et al., 2003; Rebagliati et al., 1998). So, at least in part, maternal Fgf-mediated neural capacitation may function by regulating factors involved in the clearance of Bmp signals from the dorsal part of the embryo.

Similar to what is observed with the role of Fgf signalling in neural induction, there appear to be differences in the timing and extent of the role of Bmp antagonism in neural induction in other vertebrate model systems. Evidence from the chick suggests that the role of Bmp antagonism in neural induction is only as a late, maintenance step (Linker and Stern, 2004), while the importance of suppressing Bmp signalling in neural induction has also been shown in both the frog and mouse (see introduction). In the mouse, like in fish, this has only been demonstrated for anterior neural ectoderm, while in the frog Bmp antagonism may be necessary for induction of the whole neural plate, although this may still require intact Fgf signalling. However, no direct requirement for maternal Bmp signalling in D/V patterning has been shown for the frog. Whether these differences mean that, although the molecular pathways in this process may be conserved, their temporal and spatial functions may have diverged, or that differences may be partly down to experimental procedures used with different organisms, remains unresolved and the subject of much research.

In order to analyse the stage-specific activity of Bmp signalling, a recently characterised Bmp type I receptor inhibitor, Dorsomorphin, was used in this report. Bmp type I receptors function by forming complexes with type II receptors, leading to phosphorylation and activation of effectors of the Bmp pathway, the Smads (1, 5 and 8) (Shi and Massagué, 2003). In zebrafish, it has been shown that maternal activity of the Gdf6a/Radar, which is closely related to the Bmps, is essential for ventral specification (Sidi et al., 2003) and is required for zygotic Bmp expression upstream of the Bmp receptor, Acvr1l. However, which effector mediates Bmp signals downstream of Acvr1l is not clear. Although *smad5sbn* is the only *smad* shown to be expressed maternally, data from the same authors suggests that Smad5 does not mediate signals downstream of Acvr1l (Sidi et al., 2003). As such, Acvr1l activity may be mediated by as yet unidentified *smad* genes or via a different pathway that could be indirect. Maternal Smad5 activity is itself indispensable for ventral specification possibly

downstream of different Bmp receptors (Hild et al., 1999; Kramer et al., 2002; Sidi et al., 2003). Interestingly, *acvr1l* is required for activating the zygotic expression of *bmp2b* and *bmp4*, but not *bmp7* (Sidi et al., 2003), while only *bmp7* expression is initially downregulated in *smad5/sbn* mutants (Kramer et al., 2002). This supports the existence of distinct and parallel pathways functioning in ventral specification, including induction of epidermis, although it is not yet known which ligand or receptor, if any, is responsible for activation/phosphorylation of the Smad5 protein. But it also raises the possibility that either some zygotic Bmp activity remains in *acvr1l* morphants and/or in *smad5/sbn* mutants, or that at least it may be active for longer than if both pathways were simultaneously inhibited. By using DM, which is a specific inhibitor of all three Bmp type I receptors known to be expressed maternally (Acvr1l, Bmpr1aa and Bmpr1ba), not only were we able to perform a stage-specific analyses of Bmp signalling but it is likely that DM impacts on the function of all maternal, and possibly zygotic, Bmp-related receptors.

Finally, although we have shown that suppressing Bmp signalling cannot induce neural markers if maternal Fgf signalling is compromised, this does not, however, shed any further light on the long standing dispute on whether suppressing Bmp signalling is sufficient, as well as necessary, for anterior neural induction. We have only looked at one neural marker, *sox3*, in the explant assay. Two very recent papers (Marchal et al., 2009; Wills et al., 2010) have suggested that, in *Xenopus*, Fgf signalling is required for induction of some early neural markers, while Bmp antagonism induces other neural markers, independently of Fgf signalling. Since both epidermal (*p63*) and neural (*sox3*) marker expression is absent in the caps exposed to both DM and PD (maternally), the possibility remains that other neural markers could be expressed. This report should facilitate further research into this field as well as other issues that have been raised here.

5.3 A zebrafish ectodermal explant assay to study neural development.

The purpose of this project was to generate an ectodermal explant assay in zebrafish that could provide a basis for future analyses of early zebrafish neural development, without interference from other germ layers and from organiser signalling. Initial molecular characterisation showed that wildtype embryos were not suitable in general for this purpose, as expression of both neural and epidermal marker genes were patchy and D/V patterning was evident in these embryos. This

suggested that some neural inductive activity had been initiated in the explants, possibly due to early activity of prospective organiser cells, and would therefore bias any results that would be obtained in a manner similar to what has been described for *Xenopus* explants (Linker et al., 2009). To address this, an explant assay was developed that made use of the organiser-deficient zebrafish *ichabod* mutant. Molecular analyses showed that expression of Bmp epidermal-specific downstream targets were ubiquitous in these explants, providing a cell mass of homogenous epidermal characteristics that could be induced to a neural fate by the application of exogenous factors with known neural inducing capability. Importantly, the utility of the *ichabod* mutant for this purpose highlights one of the great strengths of zebrafish as a model organism, the wide availability of mutants of many of the important signalling pathways involved in embryonic development. Mutants offer a more homogenous genetic environment and more likely reflect true loss-of-function phenotypes than exogenous applied gene-inhibiting substances. Injected morpholinos, for example, can often be unevenly distributed in the embryo and may also have non-specific effects such as toxicity. Application of chemicals can often have similar effects, and furthermore may affect pathways other than the one intended. In addition to the *ichabod* mutant used in this assay, other mutant lines could be used from which to excise animal caps. These include the *tokkaebi* mutant, which, like the *ichabod*, is also deficient in dorsal nuclear accumulation of *β-catenin2* (Nojima et al., 2004). However, the *β-catenin2* locus is unaffected in *tokkaebi* mutants, meaning that this line could be used as an alternative to *ichabod* in neural induction assays where there may be concerns about loss of maternal Wnt signalling.

Apart from the usefulness of using a maternal-effect mutant for the assay, the *ichabod* animal cap explants also offer the possibility of finding as yet uncharacterised factors that may be important for D-V patterning. For example, since *ichabod* caps should consist only of cells of epidermal (hence ectodermal) character, transcriptome analyses following ectopic alterations to key signalling pathways involved in neural induction, such as *noggin* or DM, should reveal changes in gene expression that occur only in the ectoderm in response to neural inducing signals.

The usefulness of using the *ichabod* explants for analyses of neural induction has already been demonstrated in a previous report, where exposure of caps to concentrations of chemicals that were found to be lethal to embryos helped

uncover a maternal requirement for maternal Fgf and Bmp signalling in neural and epidermal induction, respectively (see Research Chapter 2). However, analyses of molecular mechanisms of neural induction could be further extended. Both anterior and posterior neural cell types can be generated with this assay, with anterior being generated as a default in response to noggin and DM, for example, while posterior fates were shown to be induced in response to RA exposure. Although this wasn't specifically investigated, it suggests that the mechanisms underlying the induction and development of anterior neural structures, such as the forebrain, can be investigated. Induction and proper development of the forebrain in the embryo has been shown to be dependent on Wnt inhibiting molecules such as Dickkopf1 (Dkk1) (Shinya et al., 2000) and Tlc (Houart et al., 2002). Analyses of neural marker gene expression following ectopic alterations to these and other genes should help to unravel the extent to which anterior neural induction is dependent on these other germ layers.

Transplantation is a powerful tool for use in embryology, and evidence has been provided that this method can be applied to the explants. Transplantation has been shown in relation to neural patterning of the ectoderm into neural/non-neural domains, but more local patterning events may also be analysed. For example, the neural crest, which gives rise to, among others, the peripheral nervous system (PNS) in vertebrates, originates from a group of cells located at the border of the neural plate/non-neural ectoderm and is thought to be specified by intermediate levels of BMP signalling (LaBonne and Bronner-Fraser, 1999), and modifying signals specific to neural crest specification, such as levels of Bmp signalling, should help to unravel the molecular mechanisms underlying this process. Although this was not analysed, transplantation of *noggin*-expressing cells may have induced neural crest markers at the border between *foxi.1* and *otx2* positive and negative domains. Furthermore, this also suggests that our assay may be useful for analysing other morphogenic effects of BMP signalling, which is known to specify different cell fates at different concentrations.

In addition to the above, in this report it was demonstrated that in response to neural inducing signals, both neurulation and neurogenesis can be recapitulated to a certain extent in the animal caps. However, the extent to which this explant assay would be useful to analyse these two processes, specially neurulation, is uncertain. Although both *sox3* and *otx2* were expressed as expected and at the right time when compared to wildtype embryos, nevertheless two observations

require some caution in the interpretation of these results. The explants are supposed to consist only of ectodermal cells, which should be induced to a neural fate on inhibition of Bmp signalling. This is indeed the case when the caps are fixed at the corresponding late gastrula stage, at least with *sox3*. However, both in the caps that express *otx2* and those that express *sox3* at 24 hpf, there are domains that are free of expression of both genes. Epidermal marker analyses with *p63* suggested that these expression-free domains are not epidermal in nature, and analyses of *ntl* expression, to check for posterior mesoderm contamination, as well as *myoD*, which would be more generally indicative of the presence of mesoderm (Dworkin et al., 2007) suggested that they are not mesoderm in nature. However, staining for *p63* or for mesoderm markers was not done with double staining for neural markers, so neither possibility, although unlikely, can be discounted. But if the unstained regions of the caps are neither epidermal nor mesodermal in nature, then what is the character of the unstained domains? This issue requires further examination, but since the caps ubiquitously express neural markers at the late gastrula stage in response to Bmp inhibition, they should in principle also express them at 24 hpf. Similarly, more examination and fine tuning is required if this assay is to be applicable to analyses of neurogenic events. There are also domains in the caps that are free of expression of the neuronal marker, *elavl3,* and the same caution as above should apply. In addition, although neurons do indeed appear to have been generated, they are few in number. This could perhaps be explained by a delay in development, as we observed that *ichabod* embryos complete gastrulation slower when compared to their wildtype counterparts. This may simply mean that staging may have to be better coordinated with wildtype embryos. However, *elavl3* expression is specific to differentiating neurons (Kim et al., 1996), but in the caps ectopic staining seems to have been activated. Background staining cannot account for this due to the presence of staining-free regions in the caps. If this is found to be due to the absence of factors that limit the expression of this neuronal marker, another marker may have to be found that will specifically be expressed in neurons. This limitation may be overcome by the use of fluorescent microscopy, however, a method of identifying neurons that was already used successfully in this project. If conditions can be optimised for both processes, then it is certainly feasible that the molecular mechanisms involved in both neurulation and neurogenesis could be studied with this assay. One goal would be to generate specific types of neurons,

such as sensory or inter-neurons, within a background of relatively patterned neural tube. For example, this could be attempted in response to activation of signalling pathways such as sonic hedgehog (*shh*), which is known to be required for ventral neuronal identity in the spinal cord (Litingtung and Chiang, 2000), in addition to exposure to RA at the appropriate time which would confer posterior identity on the explants.

5.3.1 Comparisons with a mammalian system

The strength of the zebrafish ectodermal explant system is its direct link to embryonic development. There is already a large amount of knowledge about early neural development, mutants are readily available and gene expression modification is routine. However, to better understand the molecular mechanisms of neural patterning and neurogenesis, it would be extremely useful if direct comparisons could be made to a mammalian system which would have as its main strength a link to possible applications for therapeutic purposes. Although it does not form part of this project, an attempt was made to generate a mammalian system similar to zebrafish, based on p19 mouse embryonal carcinoma cells which, although not true stem cells, nevertheless possess many of the attributes of stem cells. Formation of p19 cell aggregates is a well established technique (van der Heyden and Defize, 2002) and application of exogenous retinoic acid is known to induce neural fates in these cells. Following on from our data with zebrafish explants, we found that exposing p19 cell aggregates to DM in addition to RA induced neurogenesis more strongly than RA alone, although neurogenesis took longer. These and other preliminary observations suggest that such a mammalian system, using mouse embryonic stem cells, for example, could be adapted in a similar manner for comparison with the zebrafish explant system.

5.4 Conclusion

The major findings of this PhD have significantly contributed to our understanding of the molecular mechanisms of neural induction and patterning. The role of the *eve1* gene, which had hitherto been difficult to analyse in vertebrates, was characterised and found to be critical posterior neural development. The temporal analysis of Bmp and Fgf signalling in neural induction has contributed to the understanding of the earliest molecular

mechanisms involved in this process while the ectodermal explant assay, meanwhile, while still requiring further work, provides a tool for future analyses of many aspects neural development. Knowledge gained from this may then be compared to a mammalian system, possibly developed in parallel.

The *eve1* project was recently published in a major journal (Cruz et al., 2010) and the maternal Bmp/Fgf will have been submitted to Development by the time of the *viva*. With some extra work and time permitting, ectodermal explant assay data may be publishable at a later date.

Chapter 6. References

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Chapter 7. Appendix

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