Analysis of Neural Development using Ligand-trap Transgenic Lines

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Abstract

Bone Morphogenetic Proteins (BMPs) are members of the Transforming Growth Factor-β (TGF-ß) signalling protein superfamily. BMPs play important and diverse roles in cell-cell signalling, including establishing cell fate during the development of vertebrate embryos. Their activity is antagonised *in vivo* by a number of proteins such as noggin, which sequester BMP ligands, preventing them from binding to BMP receptors. This thesis describes studies to establish a binary genetic approach combined with a ligand trap system to manipulate BMP signalling in the frog embryo. This system has been used to investigate the roles of BMP signalling in dorso-ventral patterning of the forebrain in *Xenopus tropicalis*.

The binary system described utilises a variety of tissue- or region-specific gene promoters to drive expression of the GAL4 transcriptional activator. Such transgenic "driver" lines can be crossed with a "responder" line in which expression of a membrane-tethered fusion protein comprising human Noggin fused to GFP is regulated by a synthetic promoter responsive to GAL4 (*UAS-flognog*).

Transient expression assays confirmed the effectiveness of the "responder" line, GAL4 transactivation of *UAS-flognog* resulted in the expression of *Flognog* and an expansion of neural progenitor tissue, indicated by the *X-Sox3* marker. In a binary cross with the *Otx2-gal4* driver line, targeted GAL4 transactivation lead to a decrease in phospho-Smad-1 staining in the anterior CNS and eye in a proportion of cross embryos. Such a cross resulted in embryos showing an open neural tube and alterations in both *Pax6* (dorsal) and *X-dll3* (ventral) forebrain markers, further indicating the efficacy of the binary, ligand-trap strategy.

In order to achieve temporal control on the activity of the *UAS-flognog* responder line in the telencephalon, an inducible driver line comprising the *Pax6* promoter driving hormone-inducible *GalPR* (an inducible chimeric GAL4) was created. In binary crosses with a *UAS-gfp* reporter line, GFP expression was detected in the forebrain, hindbrain and spinal cord only in the presence of the steroid hormone, RU486. Similarly, a second driver line, *N-tubulin-GalPR* yielded inducible GFP expression in the developing brain, spinal cord and lens tissue in the presence of RU486.

In conclusion, these findings are evidence that the binary ligand trap approach is functional and can cause targeted knockdown of BMP signalling, resulting in alterations in neural development and patterning. Furthermore, using an inducible version of this approach, *Flognog* (or any other target gene) can be expressed in the telencephalon in a RU486-inducible manner.

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Table A Abbreviations	
ANB	anterior neural boundary
A-P	Anterior-posterior
BCNE center	a signalling centre
bHLH	basic Helix-Loop-Helix
BMP	Bone Morphogenetic protein
CAR	cardiac actin gene reporter
Co-Smad	Smad 4
DP	dorsal pallium
DTA	Diptheria Toxin A
DTT	dithiothreitol
D-V	Dorso-ventral
ECFP	Enhanced Cyan Fluorescent Protein
EGFP	Enhanced Green Fluorescent Protein
F0	primary transgenic animals generated via the nuclear transplantation
F1	the offspring population generated by a cross between F0 and wild type animals
F2	homozygous populations made by a cross between two sibling F1's
FGF(8)	Fibroblast Growth Factor (8)
GAL4	a yeast regulatory protein
GalPR	an inducible chimeric GAL4

GDF	Growth and differentiation Factor
GFP	Green Fluorescent Protein
GS domain	glycine and serine-rich domain
HH	Hamburger and Hamilton chick staging
HMG-box	High Mobility Group-box
	(a conserved DNA binding sequence)
HPE	holoproscencephaly
IGF	Insulin-like Growth Factor
I-Smad	Inhibitory Smad
LP	lateral pallium
L-R	Left-right
MAPK	mitogen-activated protein kinase
MBT	Mid Blastula Transition
M-L	Medio-lateral
MHB	mid-brain/hindbrain boundary
MOH	MOH injection buffer (Offield et al., 2000)
MP	medial pallium
MZ	mantle zone
P-Smad-1	phospho-Smad-1
RP	Roof plate
REMI	Restriction Enzyme-Mediated Integration
RFP	Red Fluorescent Protein
RNAi	RNA interference

Ser	Serine
Shh	Sonic Hedgehog
SLT	secreted ligand trap
SVZ	sub-ventricular zone
Thr	Threonine
UAS	Upstream Activation Sequence
UAS-HIP	UAS-Hedgehog-Interacting Protein
V-D	Ventro-dorsal
VP	ventral pallium
VP16	potent viral transcription factor with a transcriptional activation domain
Wnt	a secreted protein
WT	Wild-type
X. tropicalis	*Xenopus tropicalis*

Crosses:

Otx2-gal4 X UAS-gfp	cross of heterozygous *Otx2-gal4* transactivator to heterozygous or homozygous *UAS-gfp* effector (as indicated)
N-tubulin-gal4 X UAS-gfp	cross of heterozygous *N-tubulin-gal4* transactivator to heterozygous or homozygous *UAS-gfp* effector (as indicated)

Rx-gal4 X UAS-gfp	cross of heterozygous *Rx-gal4* transactivator to heterozygous or homozygous *UAS-gfp* effector (as indicated)
UAS-flognog X WT	cross of heterozygous *UAS-flognog* effector to WT
Otx2-gal4 X UAS-flognog	cross of heterozygous *Otx2-gal4* transactivator to heterozygous *UAS-flognog* effector

CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

The aim of this study was to gain insight into the basic dorsal patterning mechanisms that act to define neuronal subdivisions in the vertebrate forebrain. If disrupted, these mechanisms are ultimately involved in many disorders, one of which is holoproscencephaly (HPE). This disorder is characterised in its most severe form by the cerebral hemispheres failing to separate completely into two distinct halves, as well as a lack of olfactory bulbs and a common ventricular system. It is the most common developmental anomaly of the human forebrain, occurring in 1 in 10,000 live births, 1 in 250 conceptions. Although this is primarily a deficit in development of the ventral midline of the forebrain, dorsal signalling alterations have been implicated in this disorder. Understanding the mechanism(s) of dorsal patterning of the forebrain, and its possible consequences on other tissues, is therefore essential for the development of therapies.

The strategy employed here to investigate these mechanisms was to use stable transgenic lines in *Xenopus* together with a spatio-temporally controlled Ligand trap system, containing extracellular negative regulators of signalling molecules. This system enabled the roles of signalling pathways involved in neural patterning to be studied.

1.2 Transgenesis in *Xenopus*

An advance in research has been the development of transgenesis in *Xenopus* (Kroll and Amaya, 1996). This transgenic technique, which is called Restriction Enzyme Mediated Integration (REMI) transgenesis, was originally used in *X.laevis*, and involved the incubation of sperm nuclei with linearised plasmid DNA, the subsequent incubation in restriction enzyme (the same enzyme used to linearise the plasmid DNA) and egg extract (to decondense the sperm chromatin), and this resulted in the enzyme-mediated integration of DNA into decondensed sperm nuclei *in vitro*. The nuclei are then transplanted into the unfertilised egg. This approach enabled stable, spatio-temporally controlled expression of cloned gene products (dictated by the promoter construct) in targeted cells of *Xenopus* embryos. Although this original technique was successful, it was quite demanding and resulted in transgene integration at four to eight sites with 5 to 15 plasmid DNA copies. Further modifications of the transgenic procedere were then implemented in an attempt to make the technique easier and enhance survival of the transgenic embryos (Sparrow et al., 2000; Offield et al., 2000; Browder, 2002; Hirsch et al., 2002). To simplify the method of generating transgenic *Xenopus*, restriction enzyme was excluded from the original transgenic procedure to minimise damage to the chromosomes (Sparrow et al., 2000). Also, partial sperm decondensation by egg extract was omitted from the procedure. These alterations resulted in the same or better numbers of viable embryos with the correct transgene expression.

The REMI transgenesis method (Kroll and Amaya, 1996) was further modified both in *X.laevis* and in *X.tropicalis* (Offield et al., 2000). It was identified that

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the major factors involved in survival of early cleavage stage embryos were egg quality and the de-jellying procedure and for later stage embryos (after gastrulation) it was the amount of egg extract used (and not the use of restriction enzyme). Thus, the modifications included the reduction in the amount of egg extract used to decondense the sperm nuclei to one sixth of the amount in the original REMI strategy. Also, MOH (see Table A) was used as injection buffer in place of sperm dilution buffer (SDB). DTT was used to de-jelly rather than cysteine. Eggs were partially de-jellied and a lower injection volume was used. These modifications resulted in a higher amount of transgenic embryos that survived to tadpole stages, and better overall long-term survival. Also, these modifications resulted in transgene integration at a single locus most of the time. Subsequently, egg extract was also heated to denature any harmful proteins that may lead to defects in development (Browder, 2002). Heated egg extract resulted in even better long-term survival.

Hirsch et al. (2002) made a number of modifications to the original transgenic procedure by Kroll and Amaya (1996) and used X.tropicalis. These modifications enhanced the efficiency of the technique even further. These included the use of oocyte extract instead of egg extract, the omittion of the plasmid backbone from the transgene DNA, and also a combination of previous modifications, such as a better method for preparation of sperm nuclei (Huang et al., 1999), the elimination of restriction enzyme from the transgenesis reaction (Sparrow et al., 2000) and the partial de-jellying of eggs. It was found that the use of oocyte extract to decondense nuclei was better than heated egg extract. When heated egg extract was compared against oocyte extract for survival of transgenic embryos, oocyte extract was found to be much more effective at promoting

viability of transgenic embryos as assayed by a higher percentage of perfect neurulae. The alterations that were made to the sperm preparation protocol were to separate diploid cell nuclei from haploid sperm nuclei and to use digitonin for membrane solubilisation to limit chromosomal damage. Also, when promoter reporter constructs with and without vector sequence were assayed for reporter expression, the use of transgene DNA without vector sequence was found to produce expression mostly in promoter driven areas, whereas embryos transgenic for the promoter reporter contruct with vector resulted in ectopic expression outside promoter driven areas in the majority of embryos. This result indicated that the elimation of vector sequence from the transgene DNA was needed to avoid ectopic transgene expression. Additionally, it was found that partial de-jellying affords some protection to the embryos by limiting the influx of extracellular Ca^{2+} ions through the hole made by transplantation. All these alterations resulted in a higher proportion of normal transgenic embryos that reached adulthood compared to the unmodified transgenesis technique (Kroll and Amaya, 1996).

Many different transgenesis techniques have been developed in *Xenopus* since then, including ϕC31 integrase, *I-SceI* meganuclease-mediated integration and *Tol2* transposon-mediated transgenesis (Allen and Weeks, 2005; Ogino et al., 2006; Hamlet et al., 2006). These techniques were developed to increase the amount of normal transgenic F0 embryos (Allen and Weeks, 2005; Ogino et al., 2006), and to use for insertional mutagenesis techniques and germline transgenesis (Hamlet et al., 2006).

1.3 Stable Transgenic Lines in *Xenopus*

Stable transgenic lines can be made and used as *in vivo* reporters or to manipulate gene expression at later stages of development (Offield et al., 2000). *X.tropicalis* has been demonstrated to share similar developmental characteristics to *X. laevis*, allowing staging morphological criteria, probes and techniques to be adapted to *X.tropicalis* (Khokha et al., 2002). Furthermore, many transgenic lines have been made in *X. tropicalis* due to its short generation time (Offield et al., 2000; Hirsch et al., 2002). In the transgenics, the reporter transgenes behave as dominant alleles and segregate in simple Mendelian ratios. Analysis of these ratios in transgenic progeny can occasionally be complicated by the presence of two or more integration sites, however one integration site is usual and makes analysis simpler (Hirsch et al., 2002). Every transgenic founder (F0) has unique site(s) of integration and different copy numbers (and occasionally different numbers of transgene integration sites), as well as defects from the transgenic procedure (Hartley et al., 2002). This results in variable transgene expression levels and patterns and, sometimes, suppressed transgene expression, due to the transgene being hidden in heterochromatin or other position effects. Therefore, it is essential to screen through founders to make stable populations containing the same transgene integration and expression levels.

1.4 Transcriptional Binary Systems and Other Techniques for Controlled Gene Expression in *Xenopus*

As transgenesis has been adapted to *Xenopus*, this has permitted the binary GAL4/UAS system, which is widely used in *Drosophila*, to be used in *Xenopus* (Fischer et al., 1988; Brand and Perrimon, 1993; Phelps and Brand, 1998; Hartley et al., 2002; Chae et al., 2002). The advantage of this system is that it can be used to control gene transcription in living animals and it allows spatio-temporal manipulation of gene expression. This makes possible conditional manipulation of gene function at later stages of development, and avoids the lethality and pleiotropy associated with manipulation of gene function, including transgene expression on its own, or expression of lethal genes at early stages of development by other techniques, such as microinjection. The system is based on the generation and use of two distinct transgenic lines. One line is a transcriptional activator, which contains the coding sequence for the yeast transcriptional activator, GAL4, driven by a temporally and/or spatially regulated promoter element, and the other line is the effector, which carries the gene of interest fused to the target sequence of GAL4, upstream activating sequence (UAS). Upon a cross of the two lines, in the resulting progeny, there is transcription of target gene in cells expressing GAL4 and any phenotypic consequences can be studied (Brand and Perrimon, 1993).

The ability to express a gene in a directed fashion is a useful means of analysing its role in development. By targeted mis-expression of genes, the GAL4/UAS system can be used to investigate processes, such as cell fate alterations, cell-cell signalling and *in vivo* analysis of embryos or phenotypes. The system also offers

flexibility to transgenesis, as already characterised GAL4 or UAS lines can be used in numerous combinations to assess different biological processes. Furthermore, the GalPR/UAS inducible mis-expression technique has been used in *Xenopus* (Wang et al., 1994; Chae et al, 2002), and this allows temporal (including inducible), as well as spatial control over mis-expression. GalPR is a chimeric protein containing a ligand-binding domain for a steroid analogue, RU486, the DNA binding domain of GAL4, and the transactivation domain from VP16. In a cross of a promoter-GalPR transactivator to a UAS-target gene effector, upon binding of RU486 to GalPR, GalPR escapes cytoplasmic sequestration, allowing translocation of the complex to bind UAS motif, and to activate transcription of target genes (Chae et al, 2002). This approach permits RU486-inducible, tissue-specific transgene expression. By manipulating the RU486 concentration, the transgene expression level may be manipulated, thus allowing correlation of transgene expression levels with the induced biological response.

In addition to the above techniques for controlled gene expression, use of other methods to knock down expression or activity of specific genes have been investigated. Recent studies using gene silencing in *Xenopus laevis* by DNA vector-based RNA interference and transgenesis has demonstrated that transgene-driven RNAi could specifically and stably inhibit protein expression from a GFP transgenic line at later stages of development (Li and Rohrer, 2006). However, this technique is complicated by the problem that RNAi did not completely inhibit GFP expression, suggesting that it is not a good technique for strong knock-down of gene function. This emphasises the importance of finding the right knockdown technique for loss-of-function experiments.

1.5 Neural Morphology in *Xenopus*

1.5.1 Early Neural Morphogenesis

The beginning of the neural plate can be distinguished morphologically by the differentiation of the sensorial layer of the dorsal ectoderm (neuroectoderm), as it begins to elongate and form columnar epithelium (in cells lateral to the dorsal midline) (at stage 11.5, mid-gastrula) (Hausen and Riebesell, 1991). Cells are transformed into a latero-medial organisation in the neural plate and the midline of the neural plate is occupied by the floorplate. There is no sharp boundary between neuroectoderm and lateral epidermal ectoderm. The forebrain arises from the anterior neuroectoderm.

By stage 12.5 to 13 the neural plate has segregated and becomes morphologically distinct as a thickened layer of neuroepithelial cells (several cell layers thick) (Fig. 1.1) (Hausen and Riebesell, 1991). These cells are the embryonic precursor of the brain. The neuroepithelium has been specified as prosencephalic tissue. Subsequently, this specified tissue undergoes a process known as transformation, whereby the tissue is specified along the A-P axis into mesencephalic, rhombencephalic and spinal cord tissues (Nieuwkoop, 1952). There are more cell proliferation and morphogenetic movements (Hartenstein, 1993) and then, assisted by the formation of medial and lateral hinge points during neurulation, the neural plate rolls up and by stage 20 forms a dorsal hollow neural tube (Nieuwkoop and Faber, 1994). As neural tube formation progresses, a group of superficial cells lateral to the bottle cells (bottle-shaped cells derived from epithelial cells that form the neural groove) form the roof plate, a medial group of dorsal midline glial cells, which is later covered by lateral ectoderm

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L (D) ⟷ M (V)

Figure 1.1 *Xenopus* Neural Developent and BMP expression
A. Pictures depict whole *Xenopus* embryos and the development time to the respective stages at 25 degrees Celsius for *X.tropicalis* (Khokha et al., 2002), from left to right, dorsal view of early neurula (Stage 13), anterior and then dorsal view of late neurula (Stage 19), lateral and then dorsal view of tailbud embryos (at Stages 24 and 25 respectively). Black arrows point to the proscencephalon (Adapted from Nieuwkoop and Faber, 1994). B. Picture on left of prosencephalic (pr) neural plate depicting the location of expression of BMPs in the non-neural ectoderm. Picture on right depicts section through the telencephalon of adult *Xenopus*, indicating the location of expression of BMPs (red) in the roof plate (rp). The dorsal telencephalon consists of the roof plate, the medial pallium (MP), the dorsal pallium (DP), lateral pallium (LP), ventral pallium (VP). The ventral telencephalon, the subpallium, consists of the lateral ganglionic eminences (LGE) and the medial ganglionic eminences (MGE) (Adapted from Shimamura and Rubenstein, 1997; Campbell, 2003). A, anterior; D, dorsal; L, lateral; M, medial; P, posterior; V, ventral; bp, basal plate; ap, alar plate; pcp, prechordal plate; me and rh, mes-or rhombencephalic; nc, notochord; ec, non-neural ectoderm; anr, anterior neural ridge

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migrating medially (Hausen and Riebesell, 1991). The movements of the different regions of the neural plate during neural tube closure also reveal a pattern in the medio-lateral direction at this stage. Neural tube closure is terminated when the edges of the lateral ectoderm fuse to form a continuous cover of ectoderm over the neural tube, which completely detaches from the neural tube at stage 21.

1.5.2 Brain Morphogenesis

Xenopus brain contains the same major compartments as other vertebrates. The brain has divided into the archencephalic and deuterencephalic region by stage 21 and the cephalic flexure in the form of the retro-infundibular fold formation occurs (Niewkoop and Faber, 1994). Ventricular formation makes progress and the archencephalic ventricle elongates dorso-ventrally, as well as widens. At stage 22, in the anterior, there is a series of ring-like constrictions, which mark the boundaries between the forebrain, midbrain and hindbrain (pros-, mes-, and rhomb-encephalon). The proscencephalon, which is the most anterior region of the CNS, segregates into the dorsally positioned telencephalon, telencephalic vesicle and eyes, and the more caudally located diencephalon (Rubenstein and Shimamura, 1998). At this stage, the brain ventricle is enlarged and the prosencephalon ventricular cavity extends through the prospective optic stalk into the eye vesicles, as two symmetrical ventricles at the end of the neural tube (Niewkoop and Faber, 1994). The roof plate extends along the A-P axis with its rostral extent covering the most posterior dorsal area of the telencephalon. It is medially invaginated in between the bulk of the telencephalic tissue, two cerebral hemispheres. The roof itself may only be one cell diameter thick and forms part of the

telencephalic epithelium. There is a rapid further progress of internal organisation of the brain that occurs from stage 23 to 28 (Fig. 1.1).

Fate mapping studies have revealed that most of the areas of the brain derive from the neural plate, and much of the telencephalon, ventral forebrain, and dorsal brain stem derives from the anterior neural ridge in the stage 15 *Xenopus* embryo (Eagleson and Harris, 1990; Shimamura et al., 1995; Eagleson et al., 1995). The lateral and dorsal telencephalic primordia are located in the antero-lateral neural plate. The roof regions of the brain extend into the corresponding part of the lateral neural ridge. The most dorsal neural structure, the pineal gland, is derived from the lateral neural plate and partly from the ridge, whilst the ventral brain structures derive from the medial plate. The ventral telencephalon is derived from medial anterior neural plate. Also, more rostral brain regions derive from more anterior parts of the neural plate than more caudal regions. Overlapping regions of the neural plate give rise to overlapping regions of the brain, consistent with cell division, but not migration playing a major role in brain morphogenesis. The cell movements that do accompany neurulation are patterned deformations that do not introduce topological alterations.

1.6 The Role of Inhibition of BMP Signalling in Induction of Neural Tissue in *Xenopus*

The CNS initially develops from the dorso-medial region of the embryonic ectoderm, in a process known as neural induction, and requires the active repression of BMP (Bone Morphogenetic Protein) signals, which is carried out by the BMP antagonists, chordin, noggin and follistatin produced by the embryonic organizer, as

well as FGF and IGF activity (Pera et al., 2003; Delaune et al., 2005), and other signals (Glinka et al., 1997; Piccolo et al., 1999). During gastrulation, these signals from the involuting dorsal mesoderm induce the adjacent and overlying ectoderm to become neural plate (CNS primordium).

The transforming growth factor-β (TGF-β) superfamily of secreted ligands contains a number of structurally-related proteins. BMPs belong to the TGF-β superfamily, as do other ligands, such as TGFβ's (TGFβ is the original protein from the superfamily that was found to be capable of inducing a transformed phenotype in non-neoplastic cells in culture), GDFs, Nodals and Activins (Miyazawa et al., 2002). BMPs were originally identified for their ability to induce ectopic bone formation when introduced subcutaneously in mammals (Urist et al., 1979). Since then, BMP signalling has been found to be important in regulating many processes such as early embryonic patterning, organogenesis, tissue homeostasis, cell fate decisions, cell proliferation, differentiation and cell death (Reviewed by Chen et al., 2004; Mehler et al., 1997). In some tissues BMP-related ligands are known to be involved in the generation of specific cell types (Shah et al., 1996; Lee et al., 1998), but many act in a redundant manner, possibly due to similarities in structure and function (Dudley et al., 1997; Solloway et al., 1998). BMPs (or inhibition of BMPs) are essential for the development of various tissues, including the heart, lung, kidneys, limb cells, the gametes and the nervous system (Reviewed by Chen et al., 2004).

The need for BMP inhibition as a major part of the mechanism in neural tissue formation was elucidated in a series of studies. In transplantation experiments, tissue from the dorsal side of a *Xenopus* embryo was transplanted to the ventral side, and this

induced the formation of neural tissue (Spemann and Mangold, 1924). This indicated that signals from the tissue on the dorsal side of the embryo are needed to induce neural tissue. This dorsal tissue is the Organiser. The Organiser is dorsal blastopore lip tissue, which gives rise to notochord and prechordal mesoderm tissue. It is called the Organiser because it can induce host's ventral tissues to change their fate, not only to form neural tube, but also somites (dorsal mesodermal tissue). Subsequently, Noggin was cloned from a LiCl-dorsalised gastrula cDNA library, and shown to produce ectopic neural tissue when expressed on the ventral side of the embryo (Smith and Harland, 1992; Smith et al., 1993). Also Noggin was found to induce neural fate in dorsal ectoderm (Lamb et al., 1993). Further experiments demonstrated that Noggin is a BMP antagonist (Zimmerman et al., 1996). Other experiments revealed that embryonic ectodermal cells that are exposed to high BMP levels develop as epidermis, whereas cells exposed to low BMP levels develop as neural ectoderm (Wilson and Hemmati-Brivalou, 1995; Chitnis et al., 1999). Furthermore, the organizer molecules, such as Chordin and Noggin, which are expressed in the axial mesoderm, act as competing neuralising factors with the epidermalising activity of BMP to pattern the ectoderm along the dorso-ventral axis (Sasai et al., 1994; Piccolo et al., 1996). Thus, there is a morphogen gradient of BMP signalling that specifies epidermal versus neural fate.

Is there a similar mechanism for neural induction in other vertebrates? In zebrafish mutants for the *bmp2* and *bmp7* or *Smad* genes, embryos are dorsalised with an expansion of the neural plate and have ventral defects, whereas mutants for *chrd*, which encodes a BMP antagonist, result in ventralised embryos with a reduced size of the neural plate, but with normal CNS patterning (Schulte-Merker et al., 1997; Hild et

al., 1999; Kishimoto et al., 1997; Dick et al, 2000). Thus, Chordin is needed for proper neural development. It has also been reported in mouse that inhibition of BMP signalling has a role in neural induction (Di-Gregorio et al., 2007). Thus inhibition of BMP signalling plays a role in neural tissue formation across vertebrates.

1.7 The Bone Morphogenetic Protein (BMP) Signalling Pathway

How do BMPs signal? BMPs act by a cell signalling pathway mechanism. The BMP signalling pathway initiates when homo- or heterodimers of BMP ligands bind to type II and type I Ser/ Thr kinase receptors and then heterodimerize to form a tetramer complex of these receptors (Fig. 1.2) (Shi and Massague, 2003; Heldin et al., 1997). The type II receptor phosphorylates a serine or threonine on the type I receptor (GS domain) and type I receptor phosphorylates receptor-Smad proteins, Smad1, Smad5 or Smad8, at carboxyl-terminal SS(V/M)S consensus motifs and thus activates them (Heldin et al., 1997). Activated Smads 1, 5 or 8 bind to the co-factor Smad4 (common Smad4) and translocate as a complex to the nucleus where they regulate target gene transcription. The oligomeric Smad complexes regulate target gene transcription through interaction with various transcription factors and transcriptional coactivators and corepressors. The target genes then execute further sequential molecular events (Reviewed by Miyazono, 2002; Miyazono et al., 2005).

There are different BMP sub-families based on similarities in structure and function (Kawabata et al., 1998), and these include the BMP2/4 sub-group, which contains BMP2, BMP4 and the *Drosophilia decapentaplegic (dpp)* gene product. The

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Figure 1.2 The Bone Morphogenetic Protein Signalling (BMP) Pathway Diagram illustrates the mechanism of action of BMPs/GDFs and their regulation. BMP signalling initiates when homo- or heterodimers of BMP ligands bind to type II and type I Ser/ Thr kinase receptors and then heterodimerize to form a complex. The type II receptor phosphorylates a Ser or Thr on the type I receptor. The type I receptor phosphorylates receptor-Smads, such as Smad-1, at a SS(V/M)S motif. Activated, Smads, such as Smad-1 bind to the co-Smad, Smad4, and translocate to the nucleus where they regulate transcription of target genes, such as *Msx-1, BmprII, BMP4* and *Ids* (Figure adapted from Altmann and Brivanlou (2001)).

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OP-1 sub-group (known as OP-1 sub-group due to its members being closely structurally-related to osteogenic protein-1 (OP-1), a protein known to induce bone formation (Sampath et al., 1990)), which contains BMP5, BMP6, BMP7 (also known as OP-1), BMP8 and *Drosophila gbb60A* product, and the GDF-5 sub-group which contains GDF-5, 6 and 7. BMPs bind to three distinct type I receptors, which include ALK2, ALK3/BmprIA, and ALK6/BmprIB and they also bind to three distinct type II receptors, which include BmprII, ActRIIA and ActRIIB (Shi and Massague, 2003; Heldin et al., 1997).

Different TGFβ superfamily ligands bind with differing affinities to different type I and type II receptors and activate signal transduction through the specific activation of different members of Smad family proteins (Miyazono, 2002). Most BMPs bind to three distinct type II receptors, BmprII, ActRIIA and ActRIIB. BmprII is specific for BMPs, whereas ActRIIA and ActRIIB are shared by activins and BMPs. Of the type I receptors, there is more specificity, BMP2 and BMP4 preferentially bind to BmprIA and BmprIB, whereas the OP-1 sub-group bind to ALK-2 and BmprIB. The GDF-5 sub-group bind to BmprIB, but not the other type I receptors (Miyazono, 2002). The activin/TGFβ pathway induces Smad2 and Smad3 signals (Faure et al, 2000). Activin cannot induce phosphorylation of Smad-1, but BMP4 and BMP7 can induce phosphorylation of Smad2, indicating activation of the activin/TGFβ pathway by BMPs. Mostly, however, the BMP/Smad-1 and the activin/TGFβ pathways only converge by the common use of co-Smad and I-Smads. Ligands in the TGFβ superfamily are active as dimers, and the subunit composition of these molecules can dramatically affect signalling activity (Hazama et al., 1995; Suzuki et al., 1997; Nishimatsu and Thomsen,

1998). The activity of BMP heterodimers is more potent than homodimers (Suzuki et al., 1997; Nishimatsu and Thomsen, 1998).

There are different mechanisms of BMP signalling regulation. BMP signalling can be positively regulated by Smads regulating their own activation. Smads can either directly or through interactions with other transcription factors bind to regulatory DNA sequences and positively regulate *BMP* genes. For example, these interactions can regulate a subset of BMP target genes, the BMP4 synexpression group (Karaulanov et al., 2004). The synexpression group is set of genes that share similar expression profiles in some biological processes. The promoter elements in this group include, the BMP-responsive element, bre7 (which is important for the synexpression), SBEs (Smad binding elements) and Smad-cofactor. Consequently, for example, BMP4 can regulate expression of the *Bmp4* gene, as well as its receptor *BmprII* (among other target genes too, such as *Ids* and *Msx-1*) (Fig. 1.1). BMP signalling can also be negatively regulated by various mechanisms. BMP signalling can be inhibited by extracellular antagonists, such as Noggin, Chordin, Follistatin and Cerberus, which act by preventing BMPs from binding to their receptors (Zimmerman et al., 1996; Miyazono, 2000; Massague and Chen, 2000). Moreover, BMP signalling can regulate these antagonists (Gazzerro et al., 1998; Piccolo et al., 1997), thereby modulating the strength and duration of its own signal. Noggin is induced by BMPs in rat osteoblastic cell cultures (Gazzerro et al., 1998). Also, Xolloid (a BMP1 homolog), a secreted metalloprotease, can activate BMP signalling by cleaving Chordin and releasing active BMPs (Piccolo et al., 1997). Therefore different parts of the BMP signalling pathway are subjected to either positive or negative regulation.

At different stages of neural development and in different parts of the CNS, BMPs act to regulate cell fate, proliferation and differentiation.

1.8 Forebrain Patterning

BMPs (among other molecules) are involved in forebrain patterning during both its early and late stages of formation (Shimamura and Rubenstein, 1997; Furuta et al., 1997). The morphological processes of development after (and during) the initial induction of neural tissue involve molecular and cellular activities occurring either in the neuroectoderm itself or exerting forces and spatial constraints from the outside. The molecular and cellular activites include transcription factors, morphogens (graded signals capable of inducing at least two distinct cell types at different concentrations) and lateral inhibition mechanism, which are involved in defining the neural precursor cell fate and/or neuronal differentiation, as well as pattern. Patterning is the process by which cells in separate regions of an embryo become different and is essential for establishing the spatial organisation of the developing embryo. Through this and other processes cells acquire their fate, and depending on the stage of development and the competence of the responding tissue these cell fates can be transformed. Formation of forebrain pattern in vertebrate embryos entails the specification of regional cell fates along the anterior-posterior (A-P), dorso-ventral (D-V) and left-right (L-R) axes, initially during gastrulation (Reviewed by Rubenstein et al., 1998; Altmann and Brivanlou, 2001). Two co-ordinated but partly independent patterning mechanisms assign positional information along the A-P and D-V axes, with the A-P axis preceding D-V axis formation. Patterning occurs during neural induction stages in gastrulation

through to the formation of the mature forebrain. The location where a particular cell is generated sets down its developmental potential. This has been indicated by transplantation and grafting experiments, and fate mapping studies, altering the orientation of neural tube tissue (Simon et al., 1995; Graff et al., 1989; Eagleson and Harris, 1990). For example, in neural tube grafts whereby the dorso-ventral axis was inverted and transplanted to a different A-P position along the neuraxis, cell types differentiated in a D-V position according to their host rather than the original graft D-V axis (Simon et al., 1995). However, the change in cell fate was restricted to the repertoire characteristic of their original A-P origin. Thus, at this stage, precursor cells had been assigned a fate, but remained multipotent in their choice of specific cell type. This experiment indicated that D-V values at this stage were still labile, whereas the A-P values were fixed.

During initial development of the forebrain, the anterior neural tissue (forebrain and midbrain fate) acquires its unrefined initial regional identity by avoiding exposure to caudalising factors, Wnt, FGF and retinoids (Reviewed by Altmann and Brivanlou, 2001; Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; Blumberg et al., 1997). A-P pattern indicated by position-specific gene expression is subsequently conferred to the neural plate soon after neural plate formation by the exposure to these caudalising factors. In *Xenopus* the early patterning of the nervous system can be visualised by position-specific expression of developmental regulatory genes, such as *OtxA* (forebrain), and *Krox20* (hindbrain) (Lamb et al., 1993; Nieto et al., 1992). The initial A-P regionalisation and growth also establishes local organising centres, the anterior neural ridge, or anterior neural boundary and the midbrain-hindbrain boundary

(ANR or ANB and MHB) in the neural plate (Shimamura and Rubenstein, 1997; Houart et al., 1998; Reviewed by Rhinn and Brand, 2001). The ANR, for example, induces the telencephalon and patterns the tissue along the anterior-posterior axis (Shimamura and Rubenstein, 1997). Using mouse neural plate explants it was found that Fgf8 signals from the ANR to induce *Bf-1* expression. Also, signals, such as inhibitors of BMPs and Wnts or Shh and TGFβ's from the prechordal mesendoderm have been shown to induce anterior neural fate and also anterior ventral neural fates in the neural plate (Kiecker and Niehrs, 2001). Thus, as the forebrain initially forms, signalling molecules and transcription factors combine to specify the identity and fate of cells in each of its regions. The mechanisms that drive regional patterning (early regionalisation of the prosencephalic neural plate) occur by interactions between the embryonic germ layers and embryo tissue before neural tube closure. The centres further define initial regional pattern and lead to the sub-division of neuronal regions in the mature CNS (Shimogori et al., 2004). A-P patterning generates transverse zones with differing competence to longitudinal and local inductive signals, this divides the anterior brain into subdivisions named prosomeres (adapted from neuromeres) (Puelles, 1995; Puelles and Rubenstein, 1993). These subdivisions can be recognised according to both histological and molecular criteria and correspond to the functional forebrain structures in the adult. Distinct programs of differentiation are implemented in different prosomeres as a result of differential fate specification between and within the prosomeres. The diencephalon (the more posterior) includes prosomeres P1-P4, and the telencephalon (the more anterior) includes prosomeres P5 and P6.

In addition to these transverse domains, patterning along the medio-lateral (M-L) axis generates longitudinally aligned domains (alar, basal, and floor plate). M-L (and then D-V) patterning occurs later than A-P patterning, and is set up by its response to inductive signals provided by specialised cellular groups that act as organising centres. In *Xenopus*, these include, vertical signals from different regions of the involuting mesendoderm of the organiser, the ADE (anterior dorsal endoderm) and possibly the adjacent ADME (anterior dorsal mesendoderm) during gastrulation neural inductive processes (Lupo et al., 2002). Signals from these tissues have been reported to be responsible for proper dorso-ventral patterning of the telencephalon. When explanted neuralised dorsal ectoderm from stage 10.5 embryos was recombined with ADE fragments, the stage 30/31 recombined tissue expressed *emx-1* (a dorsal telencephalic marker), whereas expression of *nkx2.1* (a ventral telencephalic marker) was suppressed, also when animal caps from chordin mRNA-injected embryos were conjugated with ADME explant, at stage 30/31 the recombined tissue was positive for both *emx-1* and *eomes* expression (*eomes* is another dorsal telencephalic marker). These results indicated that the ADME was able to co-operate with the action of BMP antagonists and lead to the development of the dorsal telencephalon, and also that the ADE may be important for dorsal telencephalon specification, but have an inhibitory effect on ventral forebrain specification. Additionally, in post-gastrula zebrafish *swirl* mutants, that carry a mutation in the *bmp2b* gene, there are alterations in the extent of medial and lateral expression domains (alterations in cell fate along the medio-lateral axis) in the neural plate in response to differing thresholds of BMP signalling (Barth et al., 1999). This indicates that BMP signalling regulates the boundaries of the medio-lateral domains in

the neural plate. Also, signals underlying the neural plate can regulate the molecular properties of the neural plate (Ang et al, 1994). In HNF3β -/- embryos there is a loss of organised node and notochord, and this leads to secondary D-V patterning defects of the neural tube.

Other experiments have shown that common M-L (V-D) patterning mechanisms are found in all CNS regions (Shimamura and Rubenstein, 1997). Shh can regulate specification of ventral cell types in forebrain neuroectoderm (Ericson et al., 1995) and the sub-division of the forebrain into bi-lateral compartments (Macdonald et al., 1995; Chiang et al., 1996). During early regionalisation of the forebrain, along the medio-lateral axis of the neural plate, Shh from the prechordal plate regulates the medial plate fate, and BMPs from the non-neural ectoderm regulate the patterning (dorsal fates) of the lateral neural plate (Fig. 1.2) (Liem et al., 1995; Shimamura and Rubenstein, 1997). Anterior neural plate explants were shown to be responsive to BMPs in culture, suggesting that BMPs may begin to pattern the forebrain even before neural tube closure, and that the neural plate is competent to receive dorsalising fates from BMPs (Shimamura and Rubenstein, 1997).

Dorsalising signals from the non-neural ectoderm (epidermis) have been suggested to pattern the dorsal side of the forebrain (Shimamura and Rubenstein, 1997). This was shown in experiments eliminating the anterior non-neural ectoderm resulting in a loss of *BF-1* dorsal forebrain marker. This indicated that the anterior ectoderm was necessary for *BF-1* expression. Furthermore, the specification of dorsal cell fates in the neural plate is initiated by cells of the epidermal ectoderm, which flank the neural plate, and are subsequently propagated by roof plate cells within the neural tube (Shimamura

and Rubenstein, 1997; Liem et al., 1995, 1997; Basler et al., 1993; Dickenson et al., 1995). The roof plate is a dorsal signalling centre that occupies the dorsal midline of the developing CNS along its entire A-P axis. Along the D-V axis, cell fate determination occurs during and following neural tube closure, and involves the action of two opposing signalling pathways: SHH ventrally from the notochord and prechordal plate and BMP/GDF dorsally from the boundary of the neural and non-neural ectoderm and later from the roof plate (Reviewed by Liu and Niswander, 2005). Thus, BMP signalling is involved in medio-lateral, and then D-V patterning.

Patterning along the D-V axis of the neuraxis is generated by two processes: the assignment of regional identity and sub-division of these regions into discrete domains of gene expression (and subsequently assigning cell fate) (Briscoe et al., 2000; Ericson et al., 1996; Pierani et al., 1999). In the neural tube, there is a broad expression of regional identity developmental regulators, which suggests that these genes act to restrict potential fates adopted by cells within that region (Briscoe et al., 2000). These regional identity genes include for example *Pax6*, *Nkx2.2*, *Nkx6.1* or *Dbx2*. At later stages these regions give rise to many different classes of differentiated neurons, suggesting that these regions are subdivided into discrete cell populations. The differentiated neuron subtypes include, for example, motor neurons (MN). The overlapping and individual expression of specific combinations of developmental regulators within a given region has been shown to generate the discrete populations. Even though the regionalisation and sub-division can be viewed as two distinct processes, there is overlap between the two processes, as the *Pax* gene family has been shown to contribute to dorsal identity and sub-divide a population of intermediate cells

into two distinct populations in the neural tube (Mansouri and Gruss, 1998; Pierani et al., 1999). Thus, the D-V patterning mechanisms in the neural tube generate discrete subdivisions set up by the differential expression of regionally expressed genes.

The M-L and D-V organisation of the neural plate and neural tube is characterised molecularly with several genes located at different positions along the D-V or M-L axis, such as, *Dbx, Msx-1, Nkx2.2, Pax3, Pax6, Pax7, Shh, Wnt-1, Wnt3* and *Wnt3a*, some of which extend all along the neuraxis into the prosencephalon, for example, *Pax6, Msx-1, Shh* and *Nkx2.1* (Shimamura et al., 1995). This continuation of expression of molecular markers from the spinal cord through to the forebrain provides evidence for the continuation of longitudinal properties from the spinal cord into the forebrain. These gene expression patterns can provide a read out and a link to the basic mechanisms of the inductive processes that generate regional specification within the developing CNS.

Furthermore, the conservation of the markers in zebrafish (Barth and Wilson, 1995) and across other non-mammalian vertebrates including *Xenopus* (Bachy et al., 2001, 2002) supports the notion that there is a conservation of some patterning mechanisms, and allows these other vertebrates to be used for analysis of such processes. Thus, the regionalisation of the anterior neural tube and forebrain in *Xenopus* has begun to be dissected due to the discovery of these conserved transcription factors which are involved in patterning (Papalopulu and Kintner, 1993; Bachy et al., 2002; Bachy et al., 2001; Brox et al., 2003; Brox et al., 2004; Medina et al., 2005). The telencephalon of *Xenopus* shows the same major organisation of prosomeres, and other divisions (pallium and subpallium), characterised by differential expression of the same

regulatory genes (*x-Dll3*, *x-Nkx2.1*, *x-Emx1*, *x-Pax6*, *x-Eomes*, *x-Tbr-1*, *x-dll4*, *x-Nkx2.1*, *x-Lhx5* and *xLhx7*), as in mouse (Bachy et al., 2001; Brox et al., 2003; Brox et al., 2004; Papalopulu and Kintner, 1993; Medina et al., 2005; Bulfone et al., 1993, 1999). Based on these observations, this has led to the proposal that the proscencephalon is functionally and structurally conserved in all vertebrates. However, there are some differences in forebrain expression in *Xenopus* compared to mouse. There are divergences in the LIM-homeodomain expression code (for *Lhx1/5* and *Lhx2/9*) in *Xenopus* telencephalon compared to mouse (Bachy et al., 2001). LIM-homeodomain expression marks regional specification, as well as axonal projection patterns and neurotransmitter phenotypes (Jessell, 2000). These differences could reflect divergences in cell types, connectivity and size of telencephalon (being small in *Xenopus* in comparison to mouse and other vertebrates). Hence, *Xenopus* may not contain all the same functional telencephalic domains as higher vertebrates. However, despite these major differences in size and morphology, *Xenopus* telencephalon is sub-divided into distinct progenitor domains along the dorso-ventral axis, and these are marked by the restricted expression of transcriptional regulators.

By analysis of the regionally restricted markers along the D-V axis, the telencephalon in *Xenopus* and other vertebrates was found to be divided along the D-V axis into two major subdivisions, the pallium which gives rise to the cortex (the dorsal telencephalon) and the sub-pallium which gives rise to the basal ganglia (part of the amygdala, part of the septum, the bed of the stria terminalis, the extended amygdala and the corticopetal cholinergic neurons) (Bachy et al., 2002; Medina et al., 2005). The prospective basal ganglia is in the most anterior position and the pallium in a more

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caudal and dorsal position. The basic organization of telencephalic subdivisions is conserved during embryogenesis in all vertebrates. The pallium in *Xenopus* is divided into four regions, the medial pallium (future hippocampus), the dorsal pallium (or isocortex), the lateral pallium (future olfactory cortex and part of the amygdala) and the ventral pallium (the claustrum and another part of the amygdala) (Fig. 1.1) (Bachy et al., 2002). The subpallium contains three regions, the lateral and medial ganglionic eminences (lge and mge) (that gives rise to the striatum and the pallidum) and the telencephalic stalk (the future septum and cholinergic nuclei). The lateral regions of the developing pallium become the hippocampal field and neocortex in higher vertebrates, but only the hippocampal field in *Xenopus*. Although *Xenopus* does not contain all the functional forebrain domains such as neocortex, the presence of the major D-V subdivisions may allow analysis of major D-V patterning events. It should be noted that there is some disagreement on where the D-V and A-P axes are positioned within the developing prosencephalon. Studies have suggested that both axes become colinear at the anterior margin of the neural plate; thus the A-P and D-V axes become indistinguishable most anteriorly (Barth et al., 1999; Rubenstein et al., 1998). Later, once the primordia are specified, proliferation, differentiation and migration occur in cells of the forebrain. The telencephalon develops in a modular manner whereby progenitor cells from different modules, such as the dorsal midline and cortical hem, develop independently prior to the mixing of their progeny in the mature cerebral cortex. In the telencephalon cells migrate ventro-dorsally across the pallio-subpallial boundary, from the ganglionic eminences into the cortex and other parts of the pallium (Chapouton

et al., 1999). The outcome of these processes is functionally distinct structures in the telencephalon.

Thus, there is a precise organisation and formation of the appropriate numbers and types of differentiated neurons along the A-P and D-V axes in the forebrain. This organisation requires several signalling molecules and transcription factors. BMP ligands are the major signals involved in dorsal pattern formation.

1.9 *BMP* Expression

BMP genes are maternally encoded and are differentially regulated after fertilisation (Nishimatsu et al., 1992). The expression of *BMP2* and *BMP4* (and *BMP7*) in ectodermal and neural tissue was analysed in *Xenopus laevis* embryos by whole mount *in situ* hybridisation (Hemmati-Brivanlou and Thomson, 1995; Hawley et al., 1995). During blastula stages all *BMP* expression is localised to the ventral side of the embryo. Subsequently, in the late blastula and mid-gastrula (stage 11), *BMP2* is expressed in the embryonic ectoderm and throughout the marginal zone. In the stage 18 neurula, *BMP2* is expressed in anterior ventral patch of mesoderm and ectoderm, *BMP2* expression then diminishes in the ectoderm, and *BMP2* continues to be expressed in the anterior neural folds as they close at the top of the embryo. At stages 18 to 22, *BMP2* is expressed in the neural folds. By tailbud through to hatching tadpole stages, *BMP2* is expressed in a variety of structures in the brain, pineal gland, inner portions of the forebrain ventricle and head. Forebrain sites of expression are also bilaterally symmetrical. These dynamic patterns of expression suggest that *BMP2* performs multiple functions in early development.

BMP4 is expressed in animal cap ectoderm in the late blastula (Hemmati-Brivanlou and Thomson, 1995). During gastrulation *BMP4* expression is gradually excluded from the dorsal ectoderm, in a region that is the prospective neural plate. At the end of gastrulation and through neurula stages, *BMP4* is expressed in ventral-posterior regions of the embryo and in the epidermis. At the junction between the neural plate and epidermis, the staining in the epidermis is amplified and there is a sharp delimitation of expression where the neural crest forms. *BMP4* is expressed in the non-neural ectoderm up to the boundary with the anterior neural plate (Fig. 1.2). Furthermore, at neural plate stages, *BMP4* and *BMP7* are also expressed in the prechordal mesoderm underlying the rostral-most neural plate (Hawley et al., 1995; Hartley et al., 2001). Expression of *BMP4* and *BMP7* is particularly strong underlying the medial part of the anterior neural plate, fading off to the lateral sides. When the neural folds are closing there is a lack of expression in the neural plate and neural folds. *BMP4* is expressed at the edge of the neural plate and this corresponds to cells that will form the roof plate of the closed neural tube. The anterior limit of *BMP4* expression in the roof plate of the just closed neural tube was not indicated. However, by stage 24 there are small patches of *BMP4* expression in the anterior spinal cord and across the top of the head. Thus, in *Xenopus* either *BMP2* or *BMP4* are expressed in the neural folds during neurula stages, and then in the dorsal neural tube, as well as dorsal surface ectoderm and the forebrain. Furthermore, the expression of *BMP2* and *BMP4* in *X.tropicalis* in these regions is very similar to *X.laevis* expression (Knochel et al. 2001).

Another BMP of the GDF5 subgroup, *GDF6* (Growth and differentiation factor 6) is also expressed during *Xenopus* neurulation at the edges of the neural plate, within

the neural plate and eye fields (Chang and Hemmati-Brivanlou, 1999). After neural tube closure, *GDF6* is expressed in the neural tube, and in the retina expression becomes restricted to the dorsal side. Therefore, GDF6 ligand may be involved in neural development in *Xenopus*.

An important issue regarding the function of BMPs in early development is whether the protein is present at the relevant tissue and place. There is no evidence for direct detection of BMP proteins, due to a lack of BMP antibodies. However, BMP activity was measured in the neurula and tailbud stage *Xenopus* embryo by immunostaining for phospho-Smad1 (Kurata et al., 2001; Wawersik et al., 2005). It was found that, at early neurula stage, no phospho-Smad-1 was detected in the entire neural plate. At stage 14, as the neural fold developed, the signal began to be detectable in the sensorial layer beneath the neural fold. At stage 17, the phospho-Smad-1 staining was observed in the eye primordial, including optic vesicle and overlying ectoderm. Upon neural tube closure, the phospho-Smad-1-positive cells appeared to move to the dorsal part of the forming neural tube and neural crest. The anterior limit of phospho-Smad-1 staining in the dorsal neural tube is not indicated. However, at stage 20, it is known that phospho-Smad-1 staining localises to the dorsal neural tube in regions above the notochord. At tailbud stage, it was observed that phospho-Smad-1 staining was found in the dorsal part of the neural tube. Thus BMP signalling activity is located in a similar location as the expression of its ligands.

The expression of BMPs (2, 4, 7) and BMP signalling components in the developing neural folds, epidermal ectoderm, dorsal surface ectoderm, dorsal neural tube and forebrain in *Xenopus* and other vertebrates (Fig 1.2) (Furura et al., 1997; Liem

et al., 1995; Faure et al., 2002) during neurulation, neural tube and tailbud stages (in *Xenopus*) support the hypothesis that BMPs have a role in dorsal patterning. It is likely that there is a co-operative BMP signalling mechanism.

1.10 Dorsal Patterning and BMPs

1.10.1 Dorsal Patterning in the Spinal Cord

In the spinal cord, distinct populations of neural progenitor cells exist in defined locations along the dorso-ventral (D-V) axis (Helms and Johnson, 2003; Briscoe and Ericson, 2001). The relative position along the D-V axis of the progenitor cells in the neural tube dictate the neural subtype that will be generated. Neural crest, commissural neurons and some groups of sensory neurons are formed dorsally (namely dorsal interneurons, dI1-6), whereas motor neurons (MN) and some interneurons are formed ventrally (ventral interneurons V0-3).

In loss-of-function studies, genetic ablation or loss of the roof plate, in mice, reveals that non-autonomous signals from the roof plate are essential for dorsal interneuron (IN) differentiation (Lee et al. 2000; Millonig et al., 2000). In *Gdf7-DTA* ablated mice the dorsal *Pax7* domain is reduced, while the ventral *Pax6* domain expands, and *Math1* and *Ngn* expressing progenitor cells are absent, as are their respective neuronal populations, dorsal interneurons dI1 and dI2 in the dorsal third of the neural tube (Lee et al., 2000). Concomitantly, dorsal neural progenitors expressed *Mash1*, a gene that is normally expressed in the progenitors in the intermediate region of the neural tube, and there was an expansion of more ventral dI3 cell types throughout the dorsal third of the neural tube including the dorsal midline. Furthermore, in the

dreher mouse mutant, the roof plate defects as a result of a loss of *Lmx1a* transcription factor results in a reduction of dI1 interneurons (Millonig et al., 2000). These experiments support the idea that the roof plate is required in a non-autonomous manner for inducing dorsal neuronal cell types (and pattern) in the spinal cord. The differences in the severity of their effects on dorsal neural gene expression are possibly due to residual roof plate signal, differences in the timing of roof plate ablation or due to alteration of surrounding tissue, which express TGFβ ligands and other factors. A role for BMPs as the signal from the roof plate is speculated due to the high abundance of *BMP* expression in the roof plate.

Gain-of-function and loss-of-function analysis, and gene expression profiles support the idea that early lateral and later dorsal signalling in the caudal neural tube is regulated by BMPs (Liem et al., 1995, 1997; Lee et al., 1998; Basler et al., 1993; Lee and Jessell, 1999; Jessell, 2000; Briscoe and Ericson, 2001). The differentiation of dorsal cells types is initiated at the neural plate stage by BMPs in the epidermal ectoderm (epidermis) (Liem et al., 1995). Subsequently, the epidermis (then surface ectoderm), containing *BMP4, 5, 7* and *dorsalin-1*, establishes neural crest and a secondary signalling centre by inducing *BMP4* expression in the roof plate cells of the neural tube (Chizhikov and Millen, 2004; Liu et al., 2004; Lee et al., 2000; Liem et al., 1995, 1997; Lee et al., 1998; Basler et al., 1993). The roof plate subsequently expresses multiple *BMPs* (*BMP 4, 5, 7*) and other TGFβ signals, *Gdf7, activin* and *dorsalin*. The specification of distinct neuronal progenitor cell domains at defined positions (pattern) and the dorsal specification of dorsal and intermediate interneurons in the dorsal neural tube (and then the spinal cord) depends on BMP signalling from the

dorsal midline organising centre (Lee and Jessell, 1999; Liem et al., 1995, 1997; Jessell, 2000; Briscoe and Ericson, 2001). BMP4 protein from the roof plate acts as a morphogen, and induces a cascade (or gradient) of TGF-β proteins (BMP4, BMP7, dorsalin and activin) in adjacent cells (Liem et al., 1995, 1997, 2000; Basler et al., 1993). Different sets of cells are exposed to different concentrations of TGF-β proteins at different times, the most dorsal being exposed to more factors at higher concentrations and at earlier times. This sets up regional-specific expression of homeobox genes. Then, the temporal and concentration gradients of the TGF-β proteins induce different types of transcription factors, basic helix-loop-helix (bHLH) and homeodomain genes, in cells at different distances from the roof plate, thereby giving them different identities.

The the gain-of-function evidence for this chain of events include, firstly that, BMP4 and BMP7 (and other members of the TGFβ family) can induce dorsal markers such as *Pax3* and *Msx*, and dorsal neuronal subtypes when cultured with chick intermediate neural plate explants or ventral neural explants (Liem et al., 1995, 1997). Also, exposure of intermediate explants to low concentrations of BMP4 induced dI2 neurons and no dI1, whereas high concentrations of BMP4 induced many dI1, but only some dI2 neurons. The changes in expression of developmental regulators expressed in the neural plate and dorsal neural tube in response to BMPs suggests that dorsal patterning of the neural plate then the neural tube is mediated by BMPs; BMPs are sufficient to induce early dorsal identity and dorsal neuronal subtypes in the neural plate then neural tube. Other *in vivo* evidence to support a role for thresholds of BMP signalling setting the expression boundaries of dorsal regulators is shown by *in ovo*

electroporation studies. BMP signalling activation is sufficient to alter dorsal and intermediate pattern in the neural tube. When constitutively activated BMP receptor (Bmpr1a or Bmpr1b) was electroporated or virally transfected into the chick neural tube after neural tube closure from different expression constructs, this produced different BMP expression levels, and lead to the transformation of ventrally located cell types to dorsal cell types (Timmer et al., 2002). *Pax7*, which is expressed dorsally in the neural tube, was ectopically expressed ventrally. *Pax6*, which is expressed in the intermediate region of the neural tube, was reduced at low levels of BMP signalling, exhibited a ventral shift of *Pax6* at moderate levels of BMP signalling, and at high levels of BMP signal *Pax6* was repressed. Also, intermediate neural tube progenitor genes (*Dbx1, 2*) were repressed by BMP signalling. These alterations in progenitor specification genes in turn altered neuronal specification genes and finally neuronal subtypes. For example, the decrease in *Dbx1, 2* correlated with a reduction of interneurons.

Thus, BMP signalling regulates the expression boundaries of homeobox proteins Pax6, Dbx2 and Msx1 to generate precursor populations with distinct developmental potential. Within the resulting populations, thresholds of BMP act to set expression domain boundaries of developmental regulators of the homeobox (in the intermediate cells) and basic helix-loop-helix (bHLH) families (in the dorsal cells), ultimately leading to the generation of a diversity of differentiated neural cell types.

Direct targeted disruption in mouse of *BMPs* or their receptors in the dorsal midline has either been hampered by early lethality, as is the case for *BMP2, BMP4, BmprIA* and *BmprII* null mutants (Winnier et al., 1995; Zhang and Bradley, 1996; Mishina et al., 1995; Beppu et al., 2000) or functional overlap of mutations in *BMP5, 6,*

7 or *BmprIB* mutants leading to no neural phenotype (Kingsley et al., 1992; Dudley et al., 1995; Luo et al., 1995; Solloway et al., 1998; Yi et al., 2000). *Smad 4* null mice also die before E7.5 with gastrulation defects and an abnormal visceral mesoderm, and separate *Smad1* and *Smad5* null mutants also die with defects in various tissues (Sirard et al., 1998; Lechleider et al., 2001; Chang et al., 1999). However, other loss-of-function studies do indicate a requirement of BMPs for dorsal cell fate in the neural tube (spinal cord) (Wine-Lee et al., 2004; Liem et al., 1997; Chesnutt et al., 2004; Lee et al., 1998). Deletion of both *BmprIA* and *BmprIB* using a conditional *Brn3a-Cre* allele, results in the loss of dI1, *Math-1* sensory interneurons (dorsal-most neurons), and a decrease and dorsal shift in dI2 neurons (dorsal neurons just ventral to dI1 neurons) (Wine-Lee et al., 2004). This indicates that a loss of BMP signalling results in spinal cord patterning defects. The defects may involve later stages of neurogenic precursor maintenance because there is a loss of pre-existing roof plate and dI1/dI2 dorsal precursor identity. As there was no spinal cord patterning defects in knockouts of either Bmpr1a or Bmpr1b alone, the phenotype in these double mutants indicate that there is receptor redundancy in the maintanence of dorsal cell identity.

Other studies have found that the secretion of BMPs and other TGFβ's by the epidermal ectoderm, and the roof plate are required for the induction of dorsal interneurons (Liem et al., 1997). BMP4, 7 and activin induction of dorsal neuronal subtypes in neural plate explants is blocked in the presence of BMP inhibitors, Noggin and Follistatin, demonstrating a requirement for BMPs in the induction of dorsal neurons. Recently, it has been found, using mis-expression of Noggin in the spinal cord, that loss of BMP signalling causes loss of *Math-1+* dI1 interneuron, and

concurrent expansion of dI2-4 population towards the roof plate (Chesnutt et al., 2004). Furthermore, the roof plate was lost via Noggin overexpression in some embryos, indicating the requirement of BMP signalling in roof plate maintanence. Also, in Gdf7 mutant mice, only a specific subset of dI1 interneurons are affected (Lee et al., 1998). As there was only an alteration in dI1 interneurons this suggested that functions of BMPs/GDFs in patterning the neural tube is divided between different *BMP/GDF* genes and is not part of a general combinatorial cascade. Other studies have found that Id proteins that are induced by BMP can repress transcription of Mash-1 and neurogenin, which is induced by basic HLH heterodimers, resulting in the inhibition of neurogenesis (Nakashima et al., 2001). Based on all these findings it is established that BMPs act as morphogens differentially regulating cell fate in the neural tube (and spinal cord). The different effects on dorsal interneuron populations, dI2 to dI4, such as a decrease and shift, an expansion or no effect, from a loss of BMP signalling, may be due to the different techniques and areas of knockdown or knock out, or due to qualitative differences in the effects of different TGFβ signals. However, the fact that not all dorsal interneurons are affected by roof plate ablation or loss of BMP signalling indicates that there must be additional inputs into dorsal patterning.

Originating from mesodermal derivatives, studies in mouse and chick have shown Shh induces ventral interneuron and motor neuron progenitor cells by a concentration-dependent secretion from the notochord and floor plate (Roelink et al., 1995; Briscoe et al., 1999; Ericson et al., 1996, 1997; Marti et al., 1995). The case being such, it is the interaction of inductive factors derived from both mesodermal (Shh) and ectodermal (BMP) tissues that flank the neural plate and neural tube that initiate the

specification of neural cell types. This is supported by results in chick showing that neural plate explants can lose their dorsal identity throughout neural plate stages by the exposure to notochord or SHH (Liem et al., 1995), meaning cells in the lateral plate are not committed to dorsal fates prior to neural tube closure. Then, in the spinal neural tube, activation of the SHH pathway can transform dorsal tissue to a ventral fate (Epstein et al., 1996; Hynes et al., 2000; Roelink et al., 1995). Conversely, the activation of BMP pathway in chick intermediate neural plate explants can dorsalize SHH-induced explants (Liem et al., 2000). Also, if notochord is removed or if signalling from the notochord is blocked, there is an expansion of two dorsal markers, Msx1 and Pax3, indicating that the cells of the neural plate can adopt a dorsal fate (Goulding et al., 1993; Liem et al., 1995).

Moreover, BMP/GDF signalling dorsally, combined with SHH signalling ventrally, acts to define the differential expression domain boundaries of additional genes Pax7, Msx2, Pax6 and Gli1, 2, 3. This interaction in the neural tube is further controlled by the action of secreted Noggin. BMP signalling is regulated by Noggin, which is expressed in the mesodermal cell types that flank the ventral neural tube, such as the notochord, as well as in the roof plate along the entire A-P axis (Liem et al., 2000; McMahon et al., 1998; Shimamura et al., 1995). The inhibition of the BMP/GDF ligands by Noggin is required for the proper formation of ventral cell types in the presence of normal Shh signalling (McMahon et al., 1998). In Noggin mutants, dorsal neural tube patterning is unaltered, however, ventral neural tube patterning was altered, indicated by a failure of motor neurons and ventral interneurons to develop.

Other factors also play a role in the establisment of neurons in defined positions in the neural tube (spinal cord). In chick, the expression of *Bmp2, 7* and *Bmpr1b* in the ventral neural tube, suggests a role for BMP signalling in ventral patterning. Also, the expression of several BMPs in nested domains in the epidermal ectoderm and the dorsal neural tube may suggest that different combinations of BMP hetero-dimers act through different sub-classes of BMP receptors to confer qualitatively or quantitatively distinct inductive activities. In addition to BMPs, the roof plate produces other secreted proteins, such as Wnts and Ephrins, and other TGFβ proteins, which act to specify several classes of adjacent dorsal interneurons (Reviewed by Lee and Jessell, 1999 and Nakamoto, 2000). The roles of the Wnts and Ephrins have not been assessed here. BMP signalling acts together with Wnt and Shh pathways to co-ordinate patterning and proliferation of cells.

1.10.2 Dorsal Patterning in the Forebrain

In contrast to more caudal CNS regions, the forebrain undergoes dramatic morphological changes during its development. In *Xenopus*, the posterior telencephalic and diencephalic roof curves around with the cephalic flexure at around stage 24. Only at later stages does the dorsal midline become hidden between two cerebral hemispheres as they expand. It may be that patterning is not the same in the forebrain as in the neural tube because there are these substantial cell movements (Eagleson and Harris, 1990), as well as differences in the competence of forebrain tissue to respond to inducing signals (Shimamura and Rubenstein, 1997). However, the identification of regionally expressed genes in defined patterns in the forebrain along the D-V axis suggest that the

mechanisms of D-V patterning in the forebrain may be similar to more posterior regions of the CNS (Shimamura and Rubenstein, 1997; Shimamura et al., 1995; Bachy et al., 2001; Brox et al., 2003; Brox et al., 2004; Papalopulu and Kintner, 1993; Medina et al., 2005).

In addition to their earlier roles during neural plate formation, BMP signalling has later roles in dorsal patterning of the forebrain (Furuta et al., 1997; Golden et al., 1999; Monuki et al., 2001; Cheng et al., 2006). There are several *BMPs* expressed in and surrounding the dorsal telencephalon in mouse and chick (Furuta et al., 1997; Golden et al., 1999). The roof plate at the level of the forebrain (up to and including the dorso-medial telencephalon) co-expresses multiple *Bmp* genes of the Bmp2/4 and 60A subgroups, *Bmp2* and *Bmp4*, and *Bmp5, 6* and *Bmp7*. Studies using explant cultures indicate that BMPs are sufficient to alter forebrain patterning (Furuta et al., 1997; Golden et al., 1999; Monuki et al., 2001). Exogenous recombinant BMP4 or BMP5 soaked beads were implanted into the rostral neural tube of the chicken forebrain and assayed by *in situ* hybridisation for forebrain patterning markers (Golden et al., 1999). The resulting embryos either maintained dorsal forebrain markers *Pax6, Otx1, Wnt-1, Wnt-3a, Wnt5b, Wnt-7a* or up-regulated one dorsal forebrain marker *Wnt4*, whereas there was a loss or marked reduction of ventral forebrain markers (*Pax2, Nkx2.1, Dlx-2, Shh*). The embyos showed a loss of basal telencephalon that resulted in holoprosencephaly (a single cerebral hemisphere), cyclopia (a single midline eye), a loss of ventral midline structures and cranio-facial defects. This experiment suggests that BMPs are sufficient to induce some aspects of dorsal pattern in chick forebrain explants,

and supports a local and global participation of BMPs in dorso-ventral patterning of the telencephalon, similar to the mechanisms of BMPs in the caudal neural tube.

In mouse it has been demonstrated that BMPs can regulate lateral telencephalic (cortical progenitor) gene expression. Application of exogenous BMP2 and 4 peptides to telencephalon explants induced *Lhx2* expression at low concentrations and repressed *Lhx2* expression at high BMP concentrations (Monuki et al., 2001). Thus cortical progenitor tissue expression can be differentially regulated by the concentration of BMP it receives. In other studies, application of exogenous BMP4 and BMP2 to explants from lateral telencephalic neuroectoderm induced *Msx-1* expression, inhibed *Bf-1* (also known as *FoxG1*) expression, inhibited cell proliferation and increased apoptosis (Furuta et al., 1997). Therefore, BMPs can induce roof plate genes, whereas repress genes expressed in the dorsal lateral forebrain. Both these studies support a role of BMPs in dorsal telencephalon development. Thus, BMPs can regulate midline fate, as well as dorsal forebrain molecular pattern. Conversely, a later study in chick telencephalic explants has indicated that BMP4 is not able to induce dorsal specific markers, although other BMPs were not assessed (Gunhaga et al., 2003). Furthermore, in another study, by increasing BMP signalling via *in ovo* electroporation of *BMPRIa* and *BMPRIb* into the embryonic chick forebrain, this resulted in alterations in the expression of patterning genes in the diencephalon (Lim et al., 2005). This study indicated that BMP signalling is sufficient to alter diencephalic patterning.

Clarifying the precise role of BMPs *in vivo* has proven difficult due to the large number of TGFβ ligands expressed in neural tissue, suggesting functional redundancy. Attempts to directly assess the role of BMPs in patterning of the dorsal telencephalon

with mouse knockouts have failed for various reasons including expression outside neural tissue (Winnier et al., 1995; Zhang and Bradley, 1996; Mishina et al., 1995; Kingsley et al., 1992; Dudley et al., 1995; Luo et al., 1995; Solloway et al., 1998; Yi et al., 2000; Bachiller et al., 2000; Anderson et al., 2002).

In other loss of function studies it has been found that BMP signalling has a role in dorsal midline development (Hebert et al., 2002) and in eye, craniofacial and neural tube development (Lim et al., 2005). It has been found that BMP signalling is required for induction and patterning of the dorsal telencephalic midline (Hebert et al., 2002), and this role for BMP signalling has also been supported by other studies (Panchision et al., 2001). Both of these studies indicated that BMP signalling has a role in development (including specification) of the choroid plexus (epithelium), which is the most dorsal structure of the telencephalon. In addition, it has been found that BMP signalling is necessary for eye and craniofacial development, as well as neural tube closure. However, both of these studies lack any D-V patterning alteration in the forebrain in response to a decrease in BMP signalling. Thus they do not support the role of BMPs in patterning of the forebrain in a concentration-dependent manner (Monuki et al., 2001; Golden et al., 1999). The reasons for a lack of D-V patterning alteration in the telencephalon may be due to functional redundancy between BMP receptors, leaving residual BMP signalling. In the studies by Hebert et al. (2002) explanted telencephalic tissue from the BMPRIa-deficient mouse was still responsive to BMP treatment, and this may have been because BMPRIb was still present throughout the telencephalon possibly compensating for the loss of BMPRIa. Futhermore, even though both BMPRIa and BMPRIb were knocked down in the studies by Lim et al. (2005), other studies have

shown that BMP signalling can still occur in the absence of these two receptors, by signalling through other receptors, such as Alk1 and Alk2 (Chen and Massague, 1999; Ebendal et al., 1999). Other reasons for a lack of telencephalic patterning phenotype may be because BMP signalling could have patterned the cortex prior to *FoxG1-Cre* mediated deletion of *BMPRIa* (Hebert et al., 2002) or prior to the dominant negative knockdown (Lim et al., 2005). Furthermore, the dorsal telencephalon could be specified by other factors either independently or in co-ordination with residual BMP signaling.

In roof plate ablation studies, it has been found that the roof plate is required in a non cell-autonomous manner for both induction of the telencephalic dorsal midline and for patterning of the dorsal telencephalon (Monuki et al., 2001; Cheng et al., 2006). A role for BMP signalling in patterning of the dorsal telencephalon was implied because there was a reduction and alteration in the gradient of Phospho-Smad-1 staining in the roof plate ablated mutant telencephalon, and also because the roof plate expresses many *BMPs* (Cheng et al., 2006; Monuki et al., 2001). These studies have indicted that BMP signalling plays a role in dorsal telencephalic patterning, however they are not evidence for a direct role for BMP signalling in dorsal telencephalic patterning. Other studies have found a direct role for both BMP and Wnt signalling in the regulation of *Emx2* (a dorsal telencephalic gene) (Theil et al., 2002). An enhancer for the *Emx2* gene was identified, and it contained binding sites for Smad and Tcf proteins, which are mediators of BMP and Wnt signalling respectively. They found that activation of both BMP and Wnt signalling pathways lead to ectopic activation of the *Emx2* enhancer. This study indicated that BMP signalling could be redundant with Wnt signalling in the specification of dorsal telencephalic cell fates.

Ultimately, the role of BMP signalling in modifying dorsal telencephalon patterning cannot be assessed alone due to the possibility of cross-talk with other signalling pathways. Recent data indicates that there is a co-ordination between patterning centres in the forebrain whereby ventrally derived Shh promotes and maintains Fgf8, Fgf8 then regulates a balance of dorsally-derived Bmp and Wnt signalling, which then regulates telencephalic growth and patterning (Storm et al., 2006). The paleocortex, which is the olfactory cortex located at the interface of the dorsal and ventral telencephalon, is specified in mice in which dorsal telencephalic patterning is severely disrupted (Vyas et al., 2003). Therefore, this suggests that signals, other than BMPs, may specify this region. These may include, retinoids, which have been found to regulate dorso-ventral patterning of the forebrain (Halilagic et al., 2007). In vitamin-A-deficient quail embryos there is a reduced and caudally shifted *Pax6* expression domain in the telencephalon. Also, Gli, a transcriptional mediator of Shh signalling, is involved in dorsal-ventral telencephalic patterning, possibly indicating another co-ordinating signal. Eventually, the downstream effects of BMP and/ or Wnt signalling in the dorsal telencephalon involves the combinatorial actions of transcription factors such as *Emx1, 2* and *Lhx2*, which function to specify and expand the medial and dorsal pallium. Therefore, the correct identity of D-V pattern relies on the superimposition of several genetic pathways.

1.11 Aims

The objectives of the studies described in this thesis were to establish whether a binary transgenic approach combining GAL4/UAS with a novel "Ligand Trap" knockdown strategy could be used to knockdown signalling pathways involved in development. In particular, the binary ligand trap approach was used to assess whether knockdown of BMP signalling in a targeted manner could be achieved, and then, secondly, to address the role of BMP signalling in forebrain development.

An effector line containing Flognog, a membrane tagged noggin GFP fusion protein, downstream of UAS, was used to antagonise BMPs and GDFs (BMP2, BMP4, BMP5, BMP6, BMP7 (slightly) and GDF6) in a cell-autonomous manner and hence knockdown BMP signalling by preventing BMPs/GDFs from binding to their receptors (Zimmerman et al., 1996; Chang and Hemmati-Brivanlou, 1999). In crosses of this effector line to the neural-tissue transactivator, *Otx2-gal4*, *Flognog* could be expressed in the anterior CNS and hence used to knockdown BMP signalling in the anterior CNS.

In a cross of *Otx2-gal4* to *UAS-flognog*, the roles of BMP signalling in neural patterning and differentiation could be investigated.

Finally, a *Pax6-GalPR* transactivator tool to drive *Flognog* target gene expression in the telencephalon in a hormone-inducible manner was used. This could be used to study the roles of BMP signalling in forebrain development at any stage throughout early embryogenesis after gastrulation.

CHAPTER 2: EXPERIMENTAL PROCEDURES

Table B Abbreviations for Reagents and Materials Used

MMR	0.1M NaCl, 2.0mM KCl, 1mM $MgSO_4$, 2mM $CaCl_2$, 5mM HEPES (pH7.8) (pH7.4)
SDB	Sperm Dilution Buffer (Sive et al., 2000)
MEMFA	0.1M MOPS, 2mM EGTA, 1mM $MgSO_4$, 10% formaldehyde
RU486	(Mifepristone, $C_{29}H_{35}NO_2$) Progesterone receptor antagonist
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
1X PBS	137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4.7H_2O$, 1.4mM KH_2PO_4
NBT/ BCIP	Nitro blue tetrazolium chloride/ 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt
BM purple	NBT/BCIP ready-to-use solution
GS	Goat Serum
DMSO	Dimethyl sulfoxide
2X Sample buffer	125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 1.8% β-mercaptoethanol
SDS	Sodium dodecyl sulphate
PVDF	polyvinylidene difluoride (membrane)
TBS	10mM Tris pH8, 150mM NaCl

TBST	10mM Tris pH8, 150mM NaCl, 0.2% Tween 20
BSA	Bovine Serum Albumin
1X SDS Running buffer	25mM Tris, 250mM Glycine, 0.1% (w/v) SDS
Transfer buffer (1X)	10mM CAPS pH11.0, 10% Methanol

2.1 Embryo Manipulations

2.1.1 Ovulation, *in vitro* Fertilisation, Natural Matings, Rearing of Embryos and Embryo Collection and Staging

Ovulation of adult *X. tropicalis* (Cambridge or NASCO), *in vitro* fertilisation of eggs, natural matings, rearing of embryos and embryo collection were conducted as described by http://tropicalis.berkeley.edu/home and

http://www.nimr.mrc.ac.uk/devbiol/zimmerman/protocols.

Fertilised eggs were de-jellied in 2.5% cysteine in 0.05X MMR, pH8.0 for 3-6 minutes. Embryos were incubated at 22°C or 25°C in 0.05X MMR with 100μg/ml gentamicin sulfate and staged according to morphological criteria described in Niewkoop and Faber, 1967, 1994 (Nieuwkoop and Faber staging is applicable to *X.tropicalis* (Khokha et al., 2002)). For raising embryos, after 3-4 days embryos were gradually transferred from 0.05X MMR with 100μg/ml gentamicin sulfate to ELGA water and raised according to standard husbandry procedures for *X.tropicalis* described in the above websites.

2.1.2 Transgenesis by Oocyte Extract-Mediated Integration

Oocyte extract-mediated integration transgenesis by nuclear transplantation into unfertilised eggs was carried out as described by Hirsch et al. (2002). Briefly, sperm

nuclei are incubated with linearised transgene DNA construct in sperm dilution buffer (SDB) for five minutes. Then, *Xenopus* oocyte extract and SDB are added to the reaction and incubated for a further fifteen minutes. The reaction is then diluted in SDB and subsequently transplanted by microinjection into unfertilised eggs. This results in fertilisation. *Pax6-GalPR* and *N-tubulin-GalPR* hormone-inducible transgenics were made by this method. The *Pax6-GalPR* and *N-tubulin-GalPR* constructs were linearised with SfiI and used directly in the transgenesis reaction as described above (see section 2.2.1 for construct generation).

2.1.3 Generation of Transgenic Lines and Use in Transgenic Crosses

All transgenic frog stocks were made by crossing *Otx2-gal4*, *N-tubulin-gal4*, *Rx-gal4*, *Pax6-GalPR* or *UAS-flognog* transgenic founders with wild-type frogs (either NASCO or Cambridge) (see section 2.2.1 for construct generation). The *Pax6-GalPR* line was raised by Zimmerman, L. All different types of transactivator transgene had a secondary reporter cassette containing γ-crystallin promoter (Offield et al., 2000) upstream of ECFP. The different UAS effector transgenes both had a secondary reporter cassette containing CAR promoter upstream of RFP (Mohun et al., 1986).

A detailed comparison of the promoter-GAL4 driven expression patterns to both the respective promoters and endogenous gene expression patterns are described in the results. *Otx2-gal4* was used for expression of *UAS*-transgenes in the anterior neuroectoderm; *N-tubulin-gal4* was used for expression of *UAS*-transgenes in the primary neurons; *Rx-gal4* was used for expression of *UAS*-transgenes in the retina and early anterior forebrain; *Pax6-galPR* was used for RU486-inducible expression of *UAS-*

transgenes in telencephalon (amoung other CNS regions). *UAS-flognog*, which encodes

Flognog, a GFP tagged version of human Noggin was used to block BMP signalling in

tissues directed by GAL4; *UAS-ECFP* (enhanced cyan fluorescent protein) was used as

a *UAS*-reporter in tissues directed by GAL4.

Otx2-gal4, *N-tubulin-gal4* or *Rx-gal4* transgenic lines or founders, or *Pax6-*

GalPR F0 were subsequently crossed with *UAS-gfp* or *UAS-flognog* transgenic frogs to

yield double-transgenic embryos. All crosses were between heterozygous parents unless

otherwise stated. Homozygous *UAS-gfp* reporters were used in some cases. Reporter

cassettes, *γ-crystallin* driving ECFP and *CAR* driving RFP, were used to determine the

transgenic content of the embryo. chi-square test was used to analyse the probability of

a significant deviation from Mendelian segregation ratios in embryos displaying reporter

cassette transgene expression.

When determination of the transgenic content of the individual embryo was

needed, embryos were monitored in individual well dishes over time until reporter

cassette transgene could be easily visualised.

2.1.4 RU486-inducible *Pax6-GalPR* Transactivator Cross and Drug Treatment

RU486 (mifepristone) (BIOMOL) was dissolved in DMSO at 25mg/ml concentration.

Final concentration was 0.5μM RU486. To test the ability of *Pax6-GalPR* transgenic to

transactivate UAS transgenes in a spatially-restricted and conditional manner, embryos

from a cross of *Pax6-GalPR* to either *UAS-gfp* or *UAS-flognog* were sorted at the 2 to 4-

cell stage, and subsequently incubated in either 0.5μM RU486 or DMSO alone control.

Embryos were then raised at 25°C and monitored for the presence of GFP by

fluorescence microscopy until tadpole stages. Embryos from the crosses were also fixed

at neural plate and neural tube stages and analysed by whole-mount *in situ* hybridisation.

2.1.5 Photomicrography

All GFP and RFP were observed by epifluorescence using a standard fluorescence

stereo-microscope with GFP2 (and GFP1) and RFP filter sets, respectively. Photos

were taken with a camera assembled on the microscope. In time-course experiments,

fluorescence was observed in individual live embryos that were separated in well dishes.

Embryos were monitored by fluorescence until the transgenic identity of the transgenic

embryos could be observed by the expression of their reporter cassette transgene, either

CAR-RFP (Mohun et al., 1986) or *γ-crystallin-ECFP* (Offield et al., 2000).

Whole-mount *in situ* hybridisation images were obtained using a stereo-

microscope fitted with a camera. Images were altered for brightness and contrast in

Adobe Photoshop.

2.1.6 Microinjection of Synthetic mRNA

Template for *GAL4* was linearised with NotI (SP6), *Flognog* (1950bp) with NotI (SP6),

Noggin (800bp) with NotI (SP6), *BMP4* (1104bp) with Asp718 (SP6), *FLAG-Smad-1*

(SP6) and *LacZ* with XbaI (SP6). Then capped mRNA for these genes were synthesised

in vitro using the mMESSAGE mMACHINE SP6 or T7 kit (Ambion). Synthesised

mRNAs were injected in a 2nl volume into specific blastomeres of early dividing

embryos, as stated in the results. Embryos were injected in 3% Ficoll in 0.1XMMR and

100μg/ml gentamicin sulfate, and subsequently incubated in 0.1XMMR and 100μg/ml

gentamicin sulfate until collection and use for analysis. In the *Sox3* assay, the width of

the injected side and uninjected side of the embryo was measured for each embryo. A

paired student's t-test was used to test whether there was any significant variation in

width on the injected side compared to uninjected side of the embryo.

2.1.7 X-Gal Staining

For, tracing the injection site in microinjection experiments, embryos were injected with

LacZ mRNA (50pg) into specific blastomeres as indicated in the results. Embryos were

then incubated as above, and upon collection were fixed in MEMFA for 1 hour. X-Gal

staining for β–galactosidase activity was carried out as described by Amaya et al.

(1993).

2.1.8 Whole-mount *in situ* Hybridisation

IMAGE and *X. tropicalis* EST library clones were obtained from HGMP MRC

Geneservice. Antisense digoxygenin (DIG)-labelled RNA probes were synthesised (see

section 2.2.2) and whole-mount *in situ* hybridisation was performed as described by

Harland (1991) and Sive et al. (2000), using either BM purple or NBT/BCIP as the

chromogenic substrate (Roche).

2.1.9 Immunohistochemistry

Anti-Phospho-Smad1/5/8 (Cell Signalling Technology) and anti-GFP (Molecular

probes, A11122) antibodies were used for immunohistochemical analysis. The vitelline

membrane was removed from embryos. Embryos were fixed in MEMFA for 1 hr, then

washed four times in PBS containing 0.1% Triton (PBT) for 10 minutes each. For

Phospho-Smad-1 detection, embryos were digested with DNase1 for 75 minutes at 37°C.

DNase buffer contained 66mM Tris pH 7.5, 5mM $MgCl_2$, 1mM β-Mercaptoethanol,

50U/ml DNase1 (Promega). Embryos were then washed in PBT four times for five

minutes each, and subsequently blocked for 1 hr in 10% goat serum (GS) (in PBS)

containing 1% Triton. Embryos were then incubated in Phospho-Smad-1/5/8 antibody

at 1:50 dilution in 10% GS (in PBS) containing 1% DMSO and 1% Triton, at 4°C

overnight. The embryos were washed in PBT five times for 1hr. Then embryos were

then incubated in AlexaFluor-488-coupled goat anti-rabbit fluorescent secondary

antibody at a 1:500 dilution in 10% goat serum (in PBS) containing 1% Triton

overnight. Embryos were washed in PBT for 2 to 3 hrs, then phototed. The same

strategy as above was used for Flognog (GFP) detection, except PBS containing 0.1%

Triton was used for washing and antibody incubation (also no DMSO was used in

antibody incubation), the DNAse digestion step was omitted, and the primary antibody

used was anti-GFP (Molecular probes, A11122).

2.1.10 Histology

Embryos were processed by whole-mount *in situ* hybridisation, then vibratome

sectioned. NBT/BCIP (Roche) was used as the chromogenic substrate when embryos

were sectioned. Embryos were embedded in 2% agarose, and 25μm sections were

taken. Sections were collected onto slides, dried, mounted in 70% glycerol and then

photos were taken on a Zeiss microscope fitted with an Axiocam camera.

2.1.11 Western Blot

Anti-Phospho-Smad-1 (Ser463/465) (Upstate), anti-GFP (Molecular probes, A11122), anti-α-tubulin (DM1A) (Sigma) and Anti-FLAG-M2 peroxidase conjugate (Sigma, A8592) antibodies were used for western analysis. Embryos were individually snap-frozen at the stages indicated in the results and transferred to -80°C. Embryos were homogenized in Phospho-extraction buffer (Novagen) containing protease inhibitors, EDTA-free mini-complete tablets (Roche); yolk was then extracted with an equal volume of 1,1,2-trichloro-1, 2, 2-trifluoroethane (Fluka). Lysates were centrifuged at 14,000g for 3 minutes at 4°C, suspended in twice their volume 2X Sample buffer and boiled for 3 minutes. Samples (one whole embryo per sample) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane. Blots were blocked in 5% BSA in TBS for 1 hr. Blots were transferred to primary antibody (dilutions were: anti-phospho-Smad-1 at 1:250, anti-GFP at 1:1000, anti-α-tubulin at 1:5000) in 5% BSA (0.1% Tween) and incubated overnight at 4°C. Following primary antibody incubation blots were washed in TBST (0.1% Tween), and subsequently incubated in their respective secondary antibody (either ECL Rabbit IgG, HRP-linked F(ab')$_2$ Fragment (Amersham Biosciences) for the Phospho-Smad-1 and the GFP antibodies or ECL Mouse IgG, HRP-linked Whole Ab (Amersham Biosciences) for the α-tubulin antibody) for 1hr at RT. Blots were washed in TBST (0.1% Tween) and developed with chemiluminescent reagents (PIERCE). Blots were scanned and images were adjusted for brightness and contrast in Adobe Photoshop.

2.2 Molecular Biology Techniques

2.2.1 Constructs

pCS2-gal4 was generated by inserting the 3694bp *GAL4* coding sequence from *pGaTB* (Brand and Perrimon, 1993) digested with BamH1 and SpeI into *pCS2* digested with BamHI and XbaI. *Pax6-GalPR* was generated by inserting a fragment containing the 3.6kb *Pax6* promoter (Hartley et al., 2001), from *Pax6-gal4* (from Hartley, K) digested with SalI, then blunted with Klenow (Promega), then digested with SfiI into *GalPRBicB* (see below) digested with Eco47III then SfiI. *GalPRBicB* was generated by inserting the 1.7kb *GalPR* coding sequence from *pGL-VP* (Wang et al., 1994) digested with BamHI, then blunted as above, then digested with Asp718 into *pBicB* vector (*BicB* containing the *γ-crystallin* promoter upstream from *ECFP* coding sequence, Zimmerman L, Price B) that was digested with NotI, then blunted as above, then digested with Asp718. *Pax6-GalPR* was linearised with SfiI for transgenesis. *N-tubulin-GalPR* was generated by inserting the 1.7kb *GalPR* coding sequence from *pGL-VP* digested with BamHI, then blunted as above, then digested with Asp718, into a construct containing *N-tubulin* and *BicB* digested with SalI, then blunted as above, then digested with Asp718 (*N-tubulin-gal4*, Zimmerman L, Price B). *N-tubulin-GalPR* was linearised with SfiI for transgenesis. *pKS.GAL4* was generated by inserting the 3694bp *GAL4* coding sequence from *pGaTB* (Brand and Perrimon, 1993) digested with SalI and HindIII into *pKS+* digested with SalI and HindIII. Molecular biology techniques were carried out as described in Sambrook et al. (1989).

Constructs used in transgenic founders (lines) contained either *GAL4* or *GalPR* coding sequences downstream from the specified promoter for transactivators, or

Flognog downstream from GAL4 responsive elements, *UAS* and the *hsp70* minimal promoter for effectors. There was a secondary reporter cassette in these constructs, transactivator transgenes contained *γ-crystallin-ECFP* and effector transgenes contained *CAR-RFP*. These constructs are *Otx-2-gal4BicB* (Zimmerman L, Price B), *N-tubulin-gal4BicB* (Zimmerman L, Price B), *Rx-gal4BicB* (Zimmerman L, Price B), *Pax-6-galPRBicB* (constructed by myself) and *UAST-flognogBic3* (constructed by Zimmerman L, *Flognog* Dionne, M and Harland, R) or *UAS-gfpBic1*, *Bic1* has a cassette containing the cardiac actin promoter upstream of RFP (Zimmerman L, Price B). The promoters in these constructs are 2000bp fragment of the *Xenopus laevis Otx2* promoter (Blitz and Cho, 1995), the *Xenopus laevis* 1.8kb *N-tubulin* promoter (neural-specific β-tubulin promoter) (Richter et al., 1988), a 2200bp proximal fragment from the *Xenopus tropicalis Rx* gene (Chae et al., 2002; Hirsch et al., 2002), proximal 3500bp from the *Xenopus laevis Pax6* promoter (Hartley et al., 2001), 2.2kb *Xenopus laevis γ-crystallin* promoter (Smolich et al., 1993), *Xenopus laevis CAR* (cardiac actin) promoter (Mohun et al., 1986).

2.2.2 Table of Templates for Antisense RNA Probes

Gene	Linearisation site	RNA polymerase	IMAGE or *Xenopus tropicalis* EST ID or Reference
GFP	BamH1	T7	pCS2.GFP (Mohun, T)
GAL4	Eco47III	T7	pKS.GAL4 (constructed myself)
x-Sox3	EcoR1	T7	Genbank ID #BG512766
Pax6	NsiI	T7	IMAGE 6992220

X-dll3	EcoR1	T7	Burd, G (University of Arizona)

2.2.3 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from 20 pooled *Otx-2-gal4 X WT* cross embryos, each at the stages specified in the results, by lysis in Trizol (Invitrogen) and subsequent isolation as described by Invitrogen. RNA was quantified on an agarose gel and 0.5-1.0μg of each was used for subsequent cDNA synthesis reaction. cDNA were made using SuperScript™ First-Strand cDNA synthesis reverse transcriptase kit according to the protocol described by Invitrogen. cDNA was used directly in PCR reaction for *GAL4* and *EF1a*. The oligonucleotide primers used were, for *GAL4* (F:5'-CATGCGATATTTGCCGACTT-3'; R:5'-GCTGTCTCAATGTTAGAGGC-3') and for *EF1a* (F:5'-CAG ATT GGT GCT GGA TAT GC-3'; R: 5'-ACT GCC TTG ATG ACT CCT AG-3'). The PCR reaction mix included, 1X PCR buffer (Abgene), 1.5mM MgCl$_2$, 0.2mM dNTPs, 0.05 Units/μl Taq, 0.5μM Forward primer, 0.5μM Reverse primer, 1.0ng/μl DNA up to 10ml with dH$_2$O. The PCR amplification protocol was carried out as described by http://tropmap.biology.uh.edu/PCRprotocol.html, and was as follows, initial denaturing at 94°C for 4 min, denaturing 94°C for 1 min, annealing 58°C for 1 min, elongation at 72°C for 1 min, for 30 cycles, then final elongation 72°C for 5 min. PCR products were analysed on a 1.5% agarose gel.

CHAPTER 3: TRANSACTIVATOR CHARACTERISATION

3.1 Aim and Introduction

The transactivator transgenes contain the yeast transcriptional activator, *GAL4*, under the control of neural promoters *Otx-2* (Blitz and Cho, 1995; Pannese et al., 1995), *N-tubulin* (Richter et al., 1988) or the retinal tissue-specific promoter, *Rx* (Hirsch et al., 2002) (Fig 3.1B). Using GFP reporter transgenic lines, these promoters have been previously characterised in *Xenopus tropicalis*, and spatial expression patterns similar, but not identical, to that of the endogenous gene were observed (Hirsch et al., 2002). The exact gene expression profile of these promoters remains unclear. Hence, due to a lack of precise gene expression profile data for these promoters and due to variable GAL4 driven expression, the aims were to define whether these transactivators were able to transactivate a *UAS-gfp* reporter, and to define the exact timing and location of promoter driven transactivation of target gene expression at stages during development of the forebrain. In a screen, founder transactivator transgenics were crossed to *UAS-gfp* reporter transgenic lines. The strength and location of transactivation was monitored and compared for each founder. Once a founder was identified that could transactivate in the correct tissue-specific manner for its respective promoter, stable lines were generated.

3.1.1 *Xenopus* Tissue-Specific Promoters

The use of tissue-specific promoters allows transcription to be limited to a subset of cells, but is restricted by the limited numbers of cloned and characterised promoters.

Fortunately, most identified promoters display no difference in the pattern of GFP expression between *X. laevis* and *X.tropicalis* in GFP reporter lines (Offield et al., 2000). The *Xenopus Otx2* promoter is among these characterised promoters (Hirsch et al., 2002). It has been shown to drive transgene expression in a similar pattern to the expression of the X*otx2* gene. The *Xotx2* (*Xenopus Otx2*) gene is homologous to the *Drosophila* gene *Orthodenticle* and is expressed in anterior neuroectoderm, including the eye and forebrain (Panesse et al., 1995). *Xotx2* is expressed at blastula stages. Subsequently, *Xotx2* is strongly expressed in the anterior mesendoderm (and in cells of the Spemann's organizer) at the onset of gastrulation (stage 10+), and in the prospective anterior neuroectoderm as gastrulation proceeds. In the late gastrula embryo (stage 12), *Xotx2* expression is in a region of columnar cells, which constitute the future brain, and also in a region anterior to the columnar cells. At open neural plate stages, *Xotx2* expression is confined to cells of anterior dorsal regions and ectodermal expression overlaps the anterior border of the neural plate. At stage 18, *Xotx2* is expressed in the anterior-most part of the ectodermal derivatives, which includes the brain anlage (Kablar et al., 1996). *Xotx2* expression persists in the anterior regions of the newly closed neural tube. Also, expression persists weakly in the cement gland anlage until early tailbud stages. At stage 23, when the encephalon is sub-divided into three vesicles, there is expression in the whole forebrain and midbrain regions, as well as prospective ventral diencephalon. Xotx2 is also expressed in the lamina terminalis at the base of the forebrain (the most anterior part of the vertebrate neuraxis) and prospective anterior commissure at stage 23.

The central nervous system expression in a GFP reporter line for the *Xenopus* Otx2 promoter (*Otx2-gfp*) seems to generally recapitulate the expression seen in prospective anterior CNS cells (Hirsch et al., 2002). GFP was detected in the anterior neuroectoderm and sensorial ectoderm by stage 19 and expression persisted in these regions throughout development. The apparent delay of transgene expression may have been caused by embryonic pigmentation or by growth at 25°C, which can reduce levels of fluorescence in transgenic embryos. GFP expression was also detected at stage 10.5-11 in the prechordal mesoderm, and persisted in this region until stage 35-36. This maintainence of expression in the notochord tissue is unlike the endogenous expression pattern. Presumably 2kb of the *Otx-2* promoter does not include the regulatory elements responsible for turning off expression in this area.

There are also other characterised promoters, some of which include, *γ-crystallin*, *Pax-6*, *Xenopus neural-specific β-tubulin* (*N-tubulin*) and *Rx*, and these promoters have been shown to drive transgene expression in *Xenopus* in the lens, CNS, primary neurons and retina respectively (Offield et al., 2000; Hartley et al., 2001; Paul Krieg, unpublished). Moreover, this expression is reproducible in *X. tropicalis* (Hirsch et al., 2002; this thesis). The *γ-crystallin* and *Pax6* promoters driving GFP have been bred to homozygosity. The second filial generations (F2) of tadpoles from the *Pax6-GFP* line have been shown to display a consistent pattern of GFP expression (Hirsch et al., 2002). The *Rx* promoter was originally cloned from *X. tropicalis* (Hirsch et al., 2002). *Rx-GFP* transgene expression is reported to be similar to that of the endogenous *Rx* gene. GFP expression was first detected in the presumptive eye fields in the anterior neural plate. Expression continued in the eye field throughout neurulation and tadpole

stages and low level *Rx* transgene expression was also detected in the forebrain. *Xenopus neural-specific β-tubulin* (*N-tubulin*) promoter has been reported to drive the expression of a reporter gene in the primary neurons of transgenic lines (Paul Krieg, unpublished).

The spatio-temporal expression pattern from the *Pax6* promoter was analysed in more detail. In the *Pax-6-GFP* transgenic line it was found, via direct observation of GFP fluorescence or by whole-mount *in situ* hybridisation to *GFP RNA*, that expression is first detected at the beginning of neurulation (stage 12.5), indicating for this promoter that the expression is temporally correct. However, further analysis of the *GFP RNA* pattern revealed that there were differences in expression driven from the *Pax-6-GFP* transgene compared to endogenous *Pax-6* expression. Differences in transgene expression compared to their endogenous counterpart expression has also been demonstrated from other *Xenopus* promoters, such as the noggin promoter, where expression was located to fusing neural tube instead of notochord cells (Geng et al., 2003). These studies indicate that these characterised promoters can recapitulate spatio-temporal expression patterns of the endogenous genes to some extent, but others can drive expression in different areas.

3.1.2 GAL4/UAS Transgene Driven Expression in *Xenopus*

Analysis of the spatio-temporal profile of target gene expression in binary crosses has been assessed (Hartley et al., 2001; Hartley et al., 2002). In a cross of a *CMV-gal4* transactivator to a *UAS-gfp* reporter, there was ubiquitous GFP expression in a proportion of the F1 embryos. GFP fluorescence was observed from mid-late neurula

stages, indicating a time delay between the accumulation of GAL4 protein and its transactivation of GFP. The *Pax-6* promoter was used to make a *Pax-6-gal4* transgenic transactivator and in a binary cross with a *UAS-gfp* reporter tissue-specific expression of GFP was observed in a proportion of the F1 embryos. GFP fluorescence was observed at stage 25, mainly in the eye primordia, whereas *GFP mRNA* was detected at stage 20 by *in situ* hybridisation in prospective eye, hindbrain and spinal cord regions. They show that the expression of *GFP* mRNA correlated with the expression of *Gal4* mRNA, and with expression of *GFP* mRNA in embryos transgenic for the *Pax-6* promoter driving GFP. They conclude that there is spatially and temporally controlled transactivation of the reporter, GFP, in a cross of founder activator and effector lines, using the GAL4 system in *Xenopus*. An obvious limitation of these binary crosses is that there are delays in target gene expression and hence alterations in the promoter driven expression pattern. These studies indicate that the analysis of the exact spatio-temporal expression profile of transgene driven expression is needed to interpret the effects of gene manipulation using this system.

3.2 Results

3.2.1 Germline Transmission of *Otx2-gal4*, *N-tubulin-gal4* and *Rx-gal4* Transgenes

Germline transmission rate of transgenes was estimated in progeny from founder transactivators to identify lines with one stably expressed transgene integration site. To facilitate identification of the transactivator transgenics, each transactivator type transgene contained a secondary reporter cassette, *γ-crystallin-ECFP* (Fig. 3.1B).

Figure 3.1 Germline Transmission of *Otx2-gal4*, *N-tubulin-gal4* and *Rx-gal4* Transgenes

A. GFP fluorescence microscopy images illustrating lateral (i, iii, v, vi, viii, x) and dorsal (ii, iv, vii, ix) views of stage 40-42 F1 tadpoles from a cross of either *Otx2-gal4* (i, ii, vi, vii), *N-tubulin-gal4* (iii, iv, viii, ix) or *Rx-gal4* founders (v, x) to heterozygous *UAS-gfp* line. i-v show ECFP in the lens of the eye driven from the γ-*crystallin* promoter. ECFP expression is in embryos only carrying the GAL4 transgene, as assessed by the absence of RFP in the somites from the CAR-RFP reporter cassette. vi-x show no ECFP in the lens. B. Diagram of transactivator construct in transactivator lines. The yeast transcriptional activator, GAL4 is under the control of either the *Otx-2* (*X. laevis*), *N-tubulin* (*X. laevis*) or *Rx* (*X. tropicalis*) promoters. A secondary reporter cassette containing γ-*crystallin* promoter linked to ECFP is placed downstream of the GAL4 transactivator transgene. C. Table I illustrates the numbers of embryos displaying GFP in the lens in the *Otx2-gal4* (T165), *N-tubulin-gal4* (T104) and *Rx-gal4* (T196 and T191) transactivator founder crosses to a homozygous *UAS-gfp* line. It illustrates the percentage of GFP+ embryos (containing GFP in the lens and RFP in the somites) and their estimated transgene integration number, which is based on statistical analysis of the segregation ratios in each cross. The expected segregation ratios and *P* values are indicated, and the *P* values, based on chi-square test, were $P > 0.05$ in all crosses. Each of the tabulated values, GFP+ and GFP-, represent numbers obtained from counting GFP expressors and no GFP-expressors from one cross. It should be noted that transactivator transgenics drive expression of the *UAS-gfp* reporter in the eye, therefore GFP in the lens may result from combined transactivator driven GFP expression with γ-*crystallin* reporter transgene ECFP expression. (T165, T104, T196, T191 refer to the nomenclature used for the lines subsequently raised from these founders after further analysis).

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A Figure 3.1

| Otx2-gal4 X UAS-gfp | N-tubulin-gal4 X UAS-gfp | Rx-gal4 X UAS-gfp |

GFP: i, ii, iii, iv, v

no GFP: vi, vii, viii, ix, x

B

Otx-2
Ntubulin
Rx   GAL4   SV40pA   γ-crystallin   ECFP   SV40pA
                            promoter

kanamycin vector
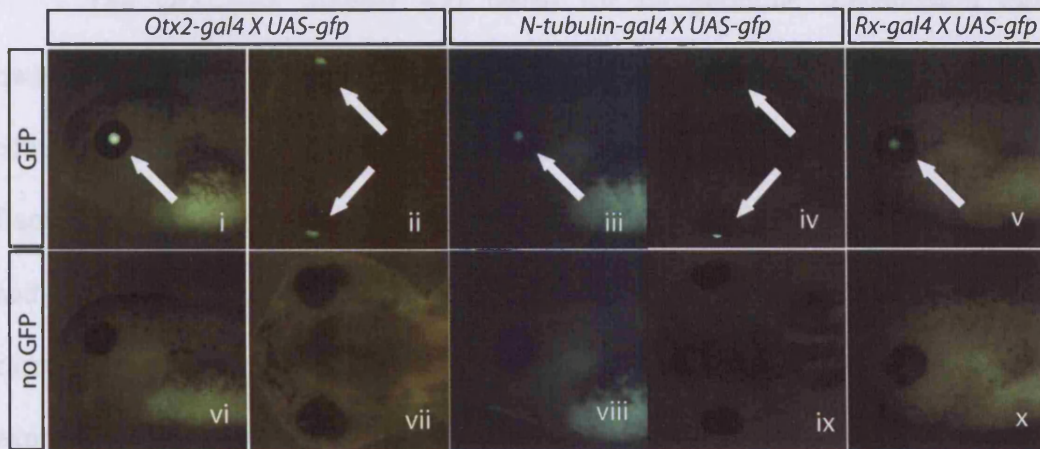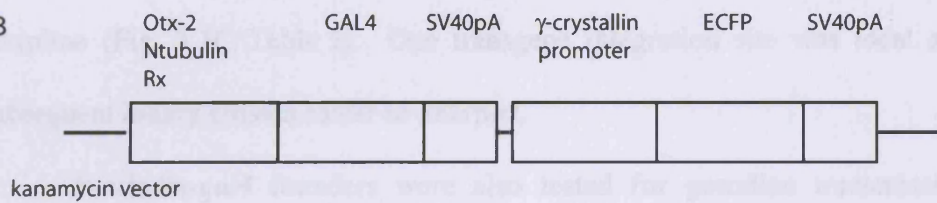
C

GFP detection in the lens in *Otx2-gal4 γ-crystallin-GFP*, *N-tubulin-gal4 γ-crystallin-GFP* and *Rx-gal4 γ-crystallin-GFP* F1 embryos

Transactivator (Line)	GFP+	GFP-	Total Sample Number	% GFP+ embryos	P	Expected Segregation Ratio	Predicted Integration number
Otx2-gal4 (T165)	31	28	59	53	0.70	1:1	1
N-tubulin-gal4 (T104)	37	45	82	45	0.38	1:1	1
Rx-gal4 (T196)	65	169	234	27			1
Rx-gal4 (T191)	157	82	239	65			2

TABLE I

Embryos could be easily scored for GFP fluorescence in their lens from stage 30 onwards (Offield et al., 2000).

The *Otx2-gal4* founder was tested for its germline transmission rate of transgenes by a cross to a *UAS-gfp* line. It was found that 31/59 (53%) F1 embryos contained GFP fluorescence in the lens of their eyes (Fig. 3.1A, C Table I). The GFP fluorescence appeared to be detected throughout the lens. If one transgene integration had integrated before the first cell division, only half of the embryos were expected to carry one copy of the transgene. Thus, as ~50% of the F1 embryos were GFP expressors, this may indicate that one integration event occurred during the transgenic procedure, most likely at a very early stage of development resulting in a non-mosaic germline (Fig. 3.1C Table I). One transgene integration site was ideal as it makes subsequent binary crosses easier to interpret.

N-tubulin-gal4 founders were also tested for germline transmission rate of transgenes by a cross to a *UAS-gfp* line. An *N-tubulin-gal4* founder was found to contain GFP in the lens in 37/82 (45%) F1 embryos (Fig. 3.1A, C Table I). This suggested that this founder contained one transgene integration site (Fig. 3.1C Table I).

Three *Rx-gal4* founders were tested for their germline transmission rate of transgenes by a cross to a *UAS-gfp* line. One founder was found to contain GFP in the lens in 65/234 (27%) F1 embryos (Fig. 3.1A, C Table I). A germline transmission rate of 27% GFP expression in offspring from founder is near the 25% expected Mendelian ratio for a transgene integrated at a single locus from a founder that was half-transgenic (such that the transgene integration had incorporated at the 2-cell stage). This may indicate that there had been a single transgene integration event in this founder. It could

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also indicate that multiple integrations had occurred. Southern blot analysis on F1 embryos will determine the number of transgene integrations. The two other *Rx-gal4* founders were found to transmit their transgene to produce 157/239 (65%) and 340/655 (52%) F1 embryos containing GFP in the lens, respectively. More than 50% of offspring exhibiting GFP expression from a founder indicates that multiple insertions into different chromosomes occurred in its germline. This may suggest that there were multiple integration sites for the *Rx-gal4* T191 founder (Fig. 3.1C Table I). The value of 52% (340/655, P>0.05, P=0.33) expression in the progeny is close to the expected ratio for one integration site, suggesting that the *Rx-gal4* T63L1 founder contained one transgene integration site.

3.2.2 Onset and Location of Expression from Transactivator Transgenics

To determine the onset of transcription from the promoter in the transactivator transgenics, the onset and pattern of *GAL4* mRNA expression was assessed. The GAL4 expression pattern was also assessed in comparison to the promoters' respective endogenous gene expression pattern to identify the location of promoter driven GAL4 expression. Embryos from a cross of either *Otx2-gal4*, *N-tubulin-gal4*, or *Rx-gal4* founders (F0) to WT were analysed either by whole-mount *in situ* hybridisation for *GAL4* and the appropriate endogenous mRNA expression or by RT-PCR for *GAL4* expression.

3.2.2.1 *Otx2-gal4* Transactivator

RT-PCR for *GAL4* expression was performed on embryos from an outcross of the *Otx2-gal4* transactivator to WT at a series of time-points from the onset of expression of the endogenous *Xotx2* gene. *GAL4* was expressed from blastula stages (stage 9.5) and expression was maintained throughout gastrula and neurula stages (stages 11, 13 and 17) (Fig 3.2A). These results suggest that the *Otx2-gal4* transactivator can drive expression from early stages of development, most likely from MBT (when zygotic transcription begins), consistent with the onset of the endogenous *Xotx2* gene (Pannese et al., 1995). Subsequently, promoter driven *GAL4* expression is maintained throughout neurula stages.

3.2.2.2 *N-tubulin-gal4* Transactivator

GAL4 is expressed at stage 13 in two stripes in the posterior-most region of the neural plate in 14/28 (50%) *N-tubulin-gal4 X WT* F1 cross embryos (from the *N-tubulin-gal4* founder suggested to contain one transgene integration site) (Fig. 3.2B i, iv). At this stage there is no *GAL4* expression in the anterior neural plate (Fig 3.2B iv). Endogenous *N-tubulin* is expressed in three stripes along the neural plate corresponding to the three types of primary neurons located along the medio-lateral axis (Oschwald et al., 1991) (Fig 3.2B iii). *GAL4* mRNA expression therefore does not completely recapitulate *N-Tubulin* endogenous gene expression, most likely being restricted to the posterior-most prospective ventral primary neurons at stage 13. The reasons for the altered *GAL4* expression may be due to a lack of all the regulatory elements in the *N-tubulin* promoter fragment, causing only part of the endogenous gene expression. Also,

Figure 3.2 Onset of Expression from *Otx2-gal4*, *N-tubulin-gal4* and *Rx-gal4* **Transactivators**

A. *Gal4* mRNA expression starts at mid-blastula stages in *Otx-2-gal4 X WT* cross embryos. RT-PCR on F1 embryos from an outcross of *Otx2-gal4* transactivator displaying that *Gal4* mRNA expression is transcribed from blastula stages and throughout gastrula and neurula stages. RNA was made from embryos at the stages 9.5, 11, 13 and 17 as indicated. *EF-1a* was used as a control. B. Images represent dorsal views of embryos from a cross of *N-tubulin-gal4* or *Xrx-gal4* to WT that were analysed by whole-mount *in situ* hybridisation for *Gal4* mRNA and their respective endogenous mRNA. Images iv and v are dorso-anterior views. *Gal4* mRNA is expressed in the posterior neural tube in two stripes closest to the prospective ventral midline in *N-tubulin-gal4* X WT cross embryos (i, iv). Endogenous *N-tubulin* mRNA expression at this stage is throughout the primary neurons of the neural plate (iii). *Gal4* mRNA is expressed in putative forebrain (prospective retinal fields) in the anterior neural plate in stage 13 *Xrx-gal4* X WT cross embryos (vi). Endogenous *Xrx* is expressed in a uniform field in the anterior neural plate of a stage 14 *Xenopus* embryo (image depicts an anterior view) (Mathers et al., 1997). ii, v, vii represent sibling embryos from *N-tubulin-gal4* and *Rx-gal4* crosses respectively (indicated by panels) displaying no *Gal4* mRNA expression.

Figure 3.2
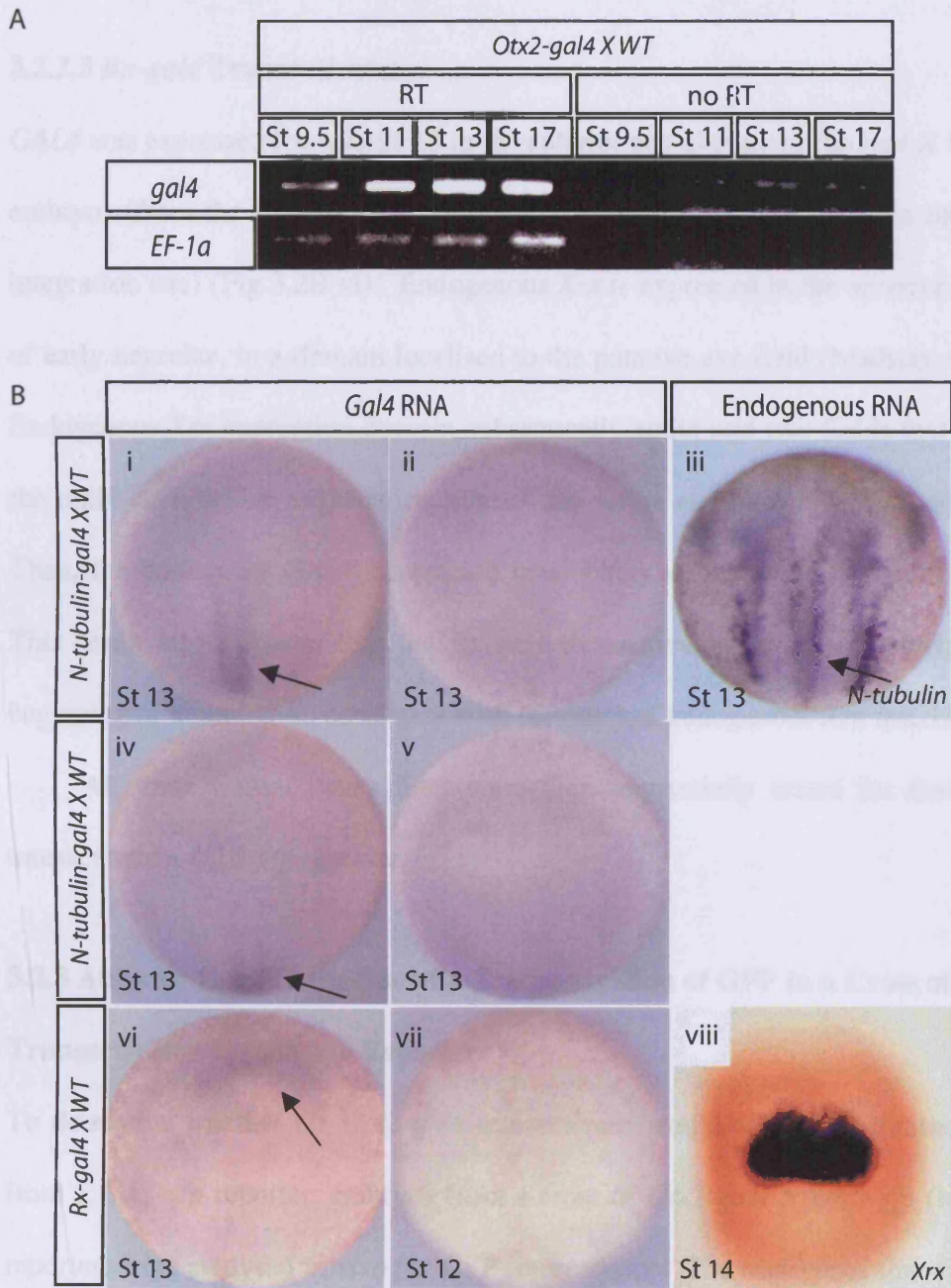
the *GAL4* mRNA expression pattern may not indicate the onset of the *N-tubulin* promoter as endogenous *N-tubulin* expression initiates at blastula stages (Oschwald et al., 1991).

3.2.2.3 *Rx-gal4* Transactivator

GAL4 was expressed from stage 13 in the anterior neural plate in *Rx-gal4 X WT* F1 cross embryos (from the *Rx-gal4* half-transgenic founder suggested to contain one transgene integration site) (Fig 3.2B vi). Endogenous *Xrx* is expressed in the anterior neural plate of early neurulae, in a domain localised to the putative eye field (Mathers et al., 1997). Endogenous *Xrx* expression domain subsequently splits into two fields that give rise to the optic cups, which is the primordial of the retina at neural tube and tailbud stages. Thus, the domain of *GAL4* expression most likely corresponds to putative forebrain. This result also suggests that the *Rx-gal4* transactivator initiates transcription at the beginning of neurulation, consistent with the onset of endogenous *Xrx* mRNA.

All these transactivator lines were then sequentially tested for their ability to transactivate a *UAS-gfp* reporter.

3.2.3 Anterior CNS Tissue-Specific Transactivation of GFP in a Cross of *Otx2-Gal4* Transactivator to *UAS-gfp* Reporter

To determine whether the *Otx2-gal4* transactivator could efficiently transactivate GFP from a *UAS-gfp* reporter, embryos from a cross of *Otx2-gal4 X UAS-gfp* (homozygous reporter) were analysed *in vivo* for GFP fluorescence. The transgenes that each embryo contained were established partly by using the detection of RFP in the somites from the *CAR-RFP* reporter (*CAR*, cardiac actin, Mohun et al., 1986) in the *UAS-gfp* reporter.

The transgenic identity could not be predicted from ECFP detection in the lens from the *γ-crystallin-ECFP* reporter, due to *Otx2* promoter driven co-expression in the lens. It was expected that two quarters of the progeny would carry one locus of each transgene.

GFP fluorescence was detected from stage 19 (neural tube stage) in anterior neural tube tissue in *Otx2-gal4 X UAS-gfp* cross embryos, whereas no GFP fluorescence was detected in *UAS-gfp* sibling control embryos (Fig 3.3A, K, N). GFP fluorescence was subsequently detected in the developing forebrain and midbrain (and the cement gland) at stages 23 to 36 in *Otx2-gal4 X UAS-gfp* cross embryos, whereas there was no GFP detected in *UAS-gfp* single transgenic embryos (Figure 3.3B, C, E, L, M, N). Due to time lags before GFP visibility, growth at 25°C reducing transgene fluorescence expression, leading to a delay in GFP detection, the stability of GFP for up to 24hrs or high pigmentation in *Xenopus,* which can cause a lack of ability to visualise GFP (Clontech; Amsterdam et al., 1996; Li et al., 1998; Hirsch et al., 2002), GFP fluorescence detection cannot be used to accurately determine the location of target gene expression at a particular stage of development because it may reflect an earlier expression profile. Nevertheless, GFP detection can be used to determine qualitatively if expression is driven in a tissue-specific manner. It should be noted that the *γ-crystallin* promoter from the transactivator transgene can drive expression in the hindbrain at stage 19, as well as ectopic expression in the forebrain (Offield et al., 2000). Thus, although it cannot be ruled out that part of the GFP fluorescence observed in the forebrain and hindbrain is due to the *γ-crystallin* promoter, the GFP observed in the midbrain must be due to *Otx2-gal4* transactivator driven expression. Furthermore, while GFP may not detect the real timing and location of expression of target gene for

Figure 3.3 GFP is Transactivated by GAL4 in the Anterior CNS and Eye in *Otx2-gal4* X *UAS-gfp* Cross Embryos

Images are fluorescence microscopy photos of embryos from a cross of *Otx-2-gal4* transactivator to *UAS-gfp* reporter. All images are dorso-anterior views of the embryos, except images E, I, J are lateral images. White arrows point to developing anterior CNS tissue. GFP fluorescence is detected at stage 17 in the anterior neural tube (A). Then GFP is detected at early tailbud stage in the anterior CNS region (B). By stage 36, GFP can be seen in the brain and eye (and cement gland) (C). GFP is in the brain and eye at stage 39 (E) (and ECFP is co-expressed in the lens from the *γ-crystallin* reporter). Images D and I are the respective images for C and E and illustrate RFP in the somites from the *CAR-RFP* reporter in *UAS-gfp*. F-H, J are the respective brightfield images for A-C, E. No GFP is detected at stage 17 (K), early tailbud (L) and stage 36 (M) in a *UAS-gfp* single transgenic embryo (N). The genotype is predicted by the presence of RFP in the somites from the *CAR-RFP* reporter (K, L, M, N). O-Q are the respective brightfield images for K-M.

GFP / Brightfield			RFP	GFP / Brightfield
A	B	C	D	E
F	G	H	I	J
K	L	M	N	
O	P	Q		
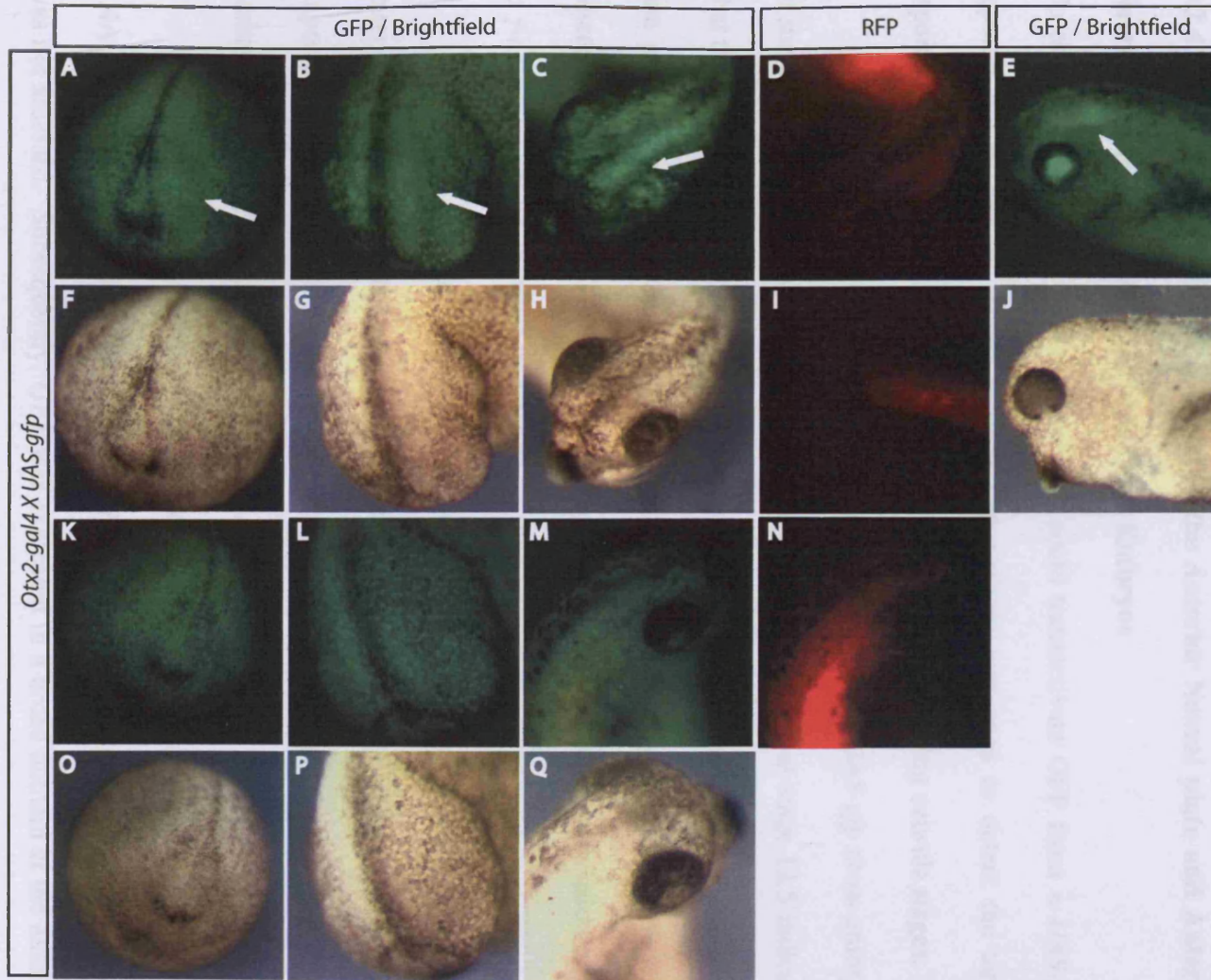
Otx2-gal4 X UAS-gfp

Figure 3.3

the above reasons, and the stages displaying strong GFP are not for subsequent analysis, the data suggests that the *Otx2-gal4* transactivator can transactivate expression from a *UAS-gfp* reporter in a tissue-specific manner.

3.2.4 Target Gene, *GFP*, is Expressed in the Anterior Neural plate and Anterior Neural Tube in *Otx2-gal4 X UAS-gfp* Cross Embryos

To establish if the *Otx2-gal4* transactivator could transactivate GFP from a *UAS-gfp* reporter during neurula stages, western blot analysis was used to detect the target reporter protein, GFP, in *Otx2-gal4 X UAS-gfp* cross embryos during neurula stages.

A GFP signal (at ~27kDa) was detected in *Otx2-gal4 X UAS-gfp* cross embryos at stage 12.5 and at stage 19 (Fig. 3.4B). The detection of GFP at stage 12.5 indicates that the *Otx2-gal4* transactivator can transactivate *UAS-gfp* GFP to protein levels during late gastrulation/ early neurula stages. At stage 19, it is not possible to distinguish whether the detection of GFP is due to *Otx2-gal4* transactivator driven expression or due to *γ-crystallin* promoter driven expression.

To further examine if the *Otx2-gal4* transactivator could drive expression in the anterior CNS anlage, the location of *Otx2-gal4* transactivator driven target gene expression was analysed by whole-mount *in situ* hybridisation for *GFP* RNA in embryos from the *Otx2-gal4 X UAS-gfp* cross.

GFP was expressed broadly throughout the anterior neural plate at stage 13 (Fig. 3.4Ai). Whether or not there was earlier *GFP* expression in prospective neuroectoderm was not assessed. Subsequently, *GFP* was expressed in a broad domain at the anterior of the neural tube at stage 19 (Fig. 3.4A iii). The *GFP* expression at stage 19 is likely to

Figure 3.4
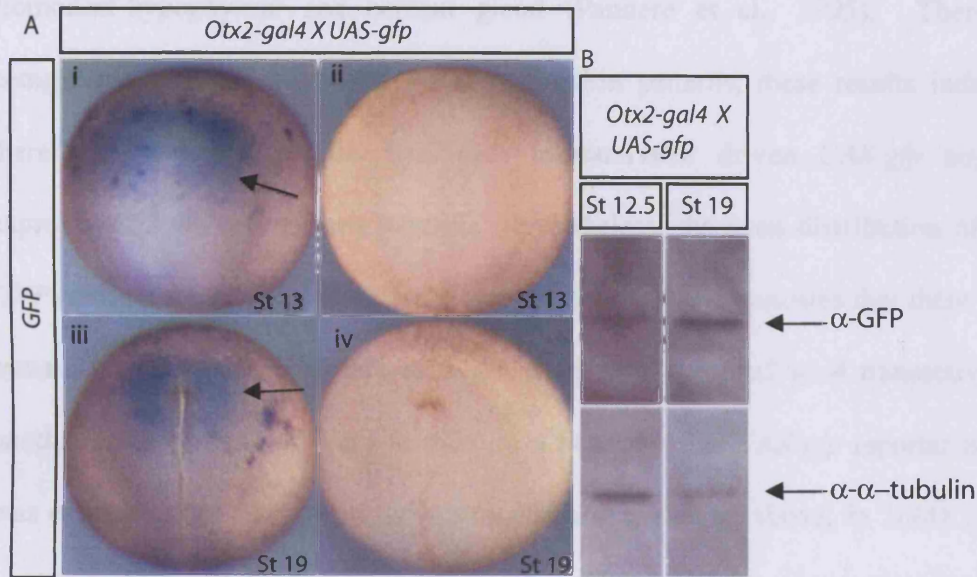
Figure 3.4 *GFP* **is expressed in the anterior neural plate and anterior neural tube (and other anterior-most regions) in** *Otx2-gal4 X UAS-gfp* **cross embryos** A. Images depict embryos from a cross of *Otx-2-gal4 X UAS-gfp* (i-iv) analysed by whole-mount *in situ* hybridisation for *GFP* (i-iv). Images are whole-mount dorsal views (i, ii) or anterior views (iii, iv). *GFP* is expressed in the anterior neural plate of stage 13 embryos, as well as other anterior regions (i), and then in the anterior neural tube and other anterior regions at stage 19 (iii). No *GFP* is expressed in sibling control embryos (ii, iv). B. Lysates of single embryos from a cross of *Otx-2-gal4 X UAS-gfp* made at the stages indicated, stage 12.5 (St 12.5) and stage 19 (St 19), and used for western blot analysis. A single embryo was loaded per lane. GFP is detected at stage 12.5 (n=12) and stage 19 (n=12) in *Otx2-gal4 X UAS-gfp* cross embryos. α-tubulin signal indicates loading of samples. Images represent a typical signal in one embryo for its stage of development.

be due to *Otx2-gal4* transactivator driven expression, because in other GAL4 transactivator crosses that do not drive expression in the forebrain at stage 19 (Fig. 3.7b C), there is no *GFP* expressed in the forebrain from *γ-crystallin* promoter driven *GFP* expression. At these stages endogenous X*otx2* is expressed in a defined domain of ectodermal cells in the anterior dorsal regions (including neural plate), as well as stomodeal-hypophyseal and cement gland (Pannese et al., 1995). Therefore, in comparison with the published *Xotx2* expression patterns, these results indicate that there is a difference in the *Otx2-gal4* transactivator driven *UAS-gfp* target gene expression to the endogenous pattern. Nevertheless, the even distribution of reporter *GFP* transgene expression within the anterior neural tissue indicates that there is a non-mosaic targeted mis-expression of target gene from the *Otx2-gal4* transactivator. In another cross of the *Otx2-gal4* founder to a heterozygous *UAS-gfp* reporter line, *GFP* was expressed consistently in the anterior neural tissue, as above, in 10/41 (24%) F1 embryos. Although it was not confirmed that *GFP* expression was due to the presence of both *Otx2-gal4* transactivator and *UAS-gfp* effector transgenes, *GFP* was expressed in approximately one quarter of the mating embryos. This indicated that expression was close to Mendelian ratios, and thus supported that *GFP* expression was due to embryos containing both *Otx2-gal4* transactivator and *UAS-gfp* effector transgenes.

Embryos from a cross of *Otx2-gal4* X *UAS-gfp* were further analysed by vibratome sectioning after whole-mount *in situ* hybridisation for *GFP* RNA. *GFP* expression was seen within the dorsal-anterior region of the embryo in ectodermal tissues (Fig. 3.5A-E). *GFP* expression was seen in the brain and eye anlage, as well as the epithelial layer of neuroectoderm. These results indicate that *GAL4* expressed from

Figure 3.5

I

II   Otx2-gal4 X UAS-gfp

GFP   A   B   C   D   E

no GFP   F   G   H   I

D
A —— P
V
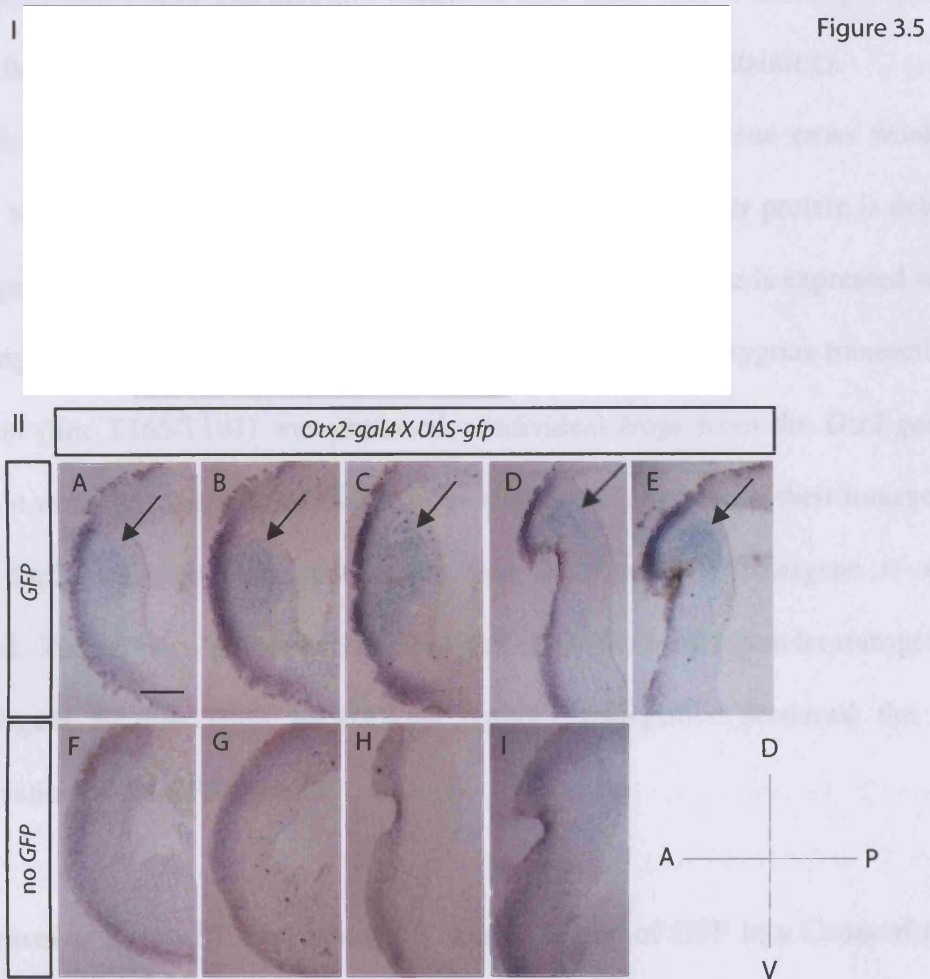
Figure 3.5 *GFP* is expressed in the anterior brain and eye anlage (and other ectodermal tissue derivatives) in *Otx2-gal4 X UAS-gfp* cross embryos I. A picture to illustrate a para-sagital section of a stage 17 *Xenopus* embryo. The brain and eye anlage, and epithelial layer of the neuroectoderm are indicated by the black lines (Hausen and Riebesell, 1991). II. Images represent stage 17 embryos from a cross of *Otx2-gal4 X UAS-gfp* analysed by whole-mount *in situ* hybridisation for *GFP* and subsequently sagitally sectioned. Dorsal is at the top and anterior is at the left. Both A to E and F-I represent a stack of sections from the para-sagittal plane (left) to sagittal plane (right) through a representative *GFP* express-ing embryo (A-E) and a sibling, non-*GFP* expressing embryo (F-I). A-E *GFP* expression is in the brain and eye anlage, as well as the epithelial layer of neuroectoderm. Black arrows point to the brain and eye anlage expression. F-I display background staining in a a sibling, non-*GFP* expressing embryo in the epithelial layers surrounding the embryos. Scale bar 50μm. Axes are indicated and are the same for the section picture in I and the images in II.

the *Otx-2* promoter is capable of activating transcription from *UAS-gfp* in anterior CNS cells. (It should be noted that BM purple was used as the substrate for alkaline phosphatase. BM purple can give two shades of blue stain with abundantly expressed RNA's. Both the dark purple and the turquoise stain represent real staining).

These results suggest that an *Otx-2-gal4* X *UAS-target gene* cross would be useful to analyse neural development, as a UAS-target gene reporter protein is detected from stage 12.5 onwards during neural development and target gene is expressed within developing CNS tissue. Therefore, a viable *Otx2-gal4* F1 heterozygous transactivator population (line T165/T191) was produced. Individual frogs from the *Otx2-gal4* F1 population were then outcrossed, and all frogs tested stably expressed their transgene in the F2 *Otx2-gal4* population, confirming that the *Otx2-gal4* transgene is stably expressed. Furthermore, progeny from crosses of either *Otx2-gal4* founder transgenic or all *Otx2-gal4* F1 line frogs to *UAS-gfp* reporter transgenics produced the same transactivation of the GFP reporter.

3.2.5 Neural or Retinal Tissue-Specific Transactivation of GFP in a Cross of either *N-tubulin-gal4* or *Rx-gal4* to *UAS-gfp* Reporter

3.2.5.1 *N-tubulin-gal4* X *UAS-gfp* Cross

The three *N-tubulin-gal4* founders were compared for their ability to transactivate a *UAS-gfp* reporter. The founders were crossed to a *UAS-gfp* reporter and the F1 embryos from these crosses were monitored for GFP fluorescence. In order to predict the transgenic identity of sibling embryos, individual embryos were grown in individual

wells and monitored for their reporter cassette expression. By stage 40, secondary reporter cassettes containing CAR driving RFP in the heart and somites were visible.

In *N-tubulin-gal4* X *UAS-gfp* cross embryos from the *N-tubulin-gal4* F0 4 founder there was no GFP in the nervous system (Fig 3.6 D-F, D'-F'). This indicated no transactivation for this line. In *N-tubulin-gal4* X *UAS-gfp* cross embryos for another founder, *N-tubulin-gal4* F0 5, there was strong GFP in the pharyngeal arches together with background GFP throughout the embryo and some GFP along the spinal cord (Fig 3.6 G-I, G'-I'), implying ectopic transactivation outside the promoter-driven expression domain, possibly due to a leaky promoter construct. In *N-tubulin-gal4* X *UAS-gfp* cross embryos from one *N-tubulin-gal4* F0 3 founder there was relatively strong CNS-specific GFP fluorescence (including along the spinal cord), with minimal background fluorescence (Fig. 3.6 A-C, A'-C'), indicating for this founder that there was CNS-tissue specific transactivation of GFP from *UAS-gfp* reporter. Consequently, due to the CNS-specific transactivation, this *N-tubulin-gal4* line (F0 3) was chosen for further analysis. Spinal cord or neural tube GFP fluorescence was evident from stage 23 in *N-tubulin-gal4* X *UAS-gfp* cross embryos (Fig. 3.7B). GFP is maintained along the spinal cord throughout tailbud stages in *N-tubulin-gal4* X *UAS-gfp* cross embryos (Fig. 3.7G). Only by late tailbud stages can GFP expression be seen to be reaching more rostral areas (Fig. 3.7C). By stage 40 and stage 42 there is GFP expression in the brain (Fig. 3.7E, D). No GFP was detected in sibling *UAS-gfp* single transgenic embryos (Fig 3.7K, P, L-N).

To analyse the exact location of transactivation of *GFP* target gene, *in situ* hybridisation for *GFP* RNA was performed. *GFP* expression was evident from stage 13 in two stripes in the posterior-most neural plate domain (Fig. 3.7b A), analogous to the

Figure 3.6 Different Areas of GFP Transactivation in Crosses between Different *N-tubulin-gal4* Founders and a *UAS-gfp* Reporter

Fluorescence microscopy images of *N-tubulin-gal4* X *UAS-gfp* cross embryos from three different *N-tubulin-gal4* founders F0 3, F0 4 and F0 5. Images are taken at three sets of stages 26-28, 28-34, 38-39, see box above column. Panels on left indicate the founder that each image represents; images are of the same embryo from each different founder monitored over time. There was CNS tissue-specific GFP (A-C, see white arrows), no observable GFP in the anterior CNS (D-F) or non-specific GFP (G-I, see arrow), with expression located in areas just anterior and posterior to the eye (H) in *N-tubulin-gal4* X *UAS-gfp* cross embryos from the three different founders F0 3, F0 4 and F0 5. In G-I, in addition to the high levels of non-specific GFP speckles throughout the embryo, there is also a lack of GFP in the CNS (although some GFP is still present in the anterior spinal cord (G, see arrow)). A'-I' are the brightfield images for A-I respectively.

Figure 3.6

N-tubulin-gal4 X UAS-gfp

	F0 3		F0 4		F0 5	
	GFP	Brightfield	GFP	Brightfield	GFP	Brightfield
stage 26-28	A	A'	D	D'	G	G'
stage 28-34	B	B'	E	E'	H	H'
stage 38-39	C (GFP/RFP)	C'	F (GFP/RFP)	F'	I (GFP/RFP)	I'

Figure 3.7 GFP is Transactivated by GAL4 in the CNS in *N-tubulin-gal4* *X UAS-GFP*
Cross Embryos

Images depict fluorescence microscopy images or brightfield, as indicated. Images A, F, J, O, G, P, L, D, I, M, R are dorsal views, all the remaining images are lateral views. GFP is detected in the developing nervous system at stage 23 to stage 42 in *N-tubulin-gal4* *X UAS-gfp* cross embryos (B, G, C, D, E white arrows point to nervous system). No GFP is detected at equivalent stages in a *UAS-gfp* single transgenic embryo (K, P, L, M, N). No GFP is detected during neurula stages in *N-tubulin-gal4* *X UAS-gfp* cross embryo (A, same embryo as D) or *UAS-gfp* single transgenic embryos (J). F, H, I and O, Q, R are the brightfield images for A, C, D and J, L, M respectively. Images A, C, D represent the same embryo. Images J, L M represent the same embryo. GFP fluorescence was observed using with the GFP2 filter set, except images B, G, L, Q, which were observed with the GFP1 filter set.

Figure 3.7

N-tubulin-gal4 X UAS-gfp
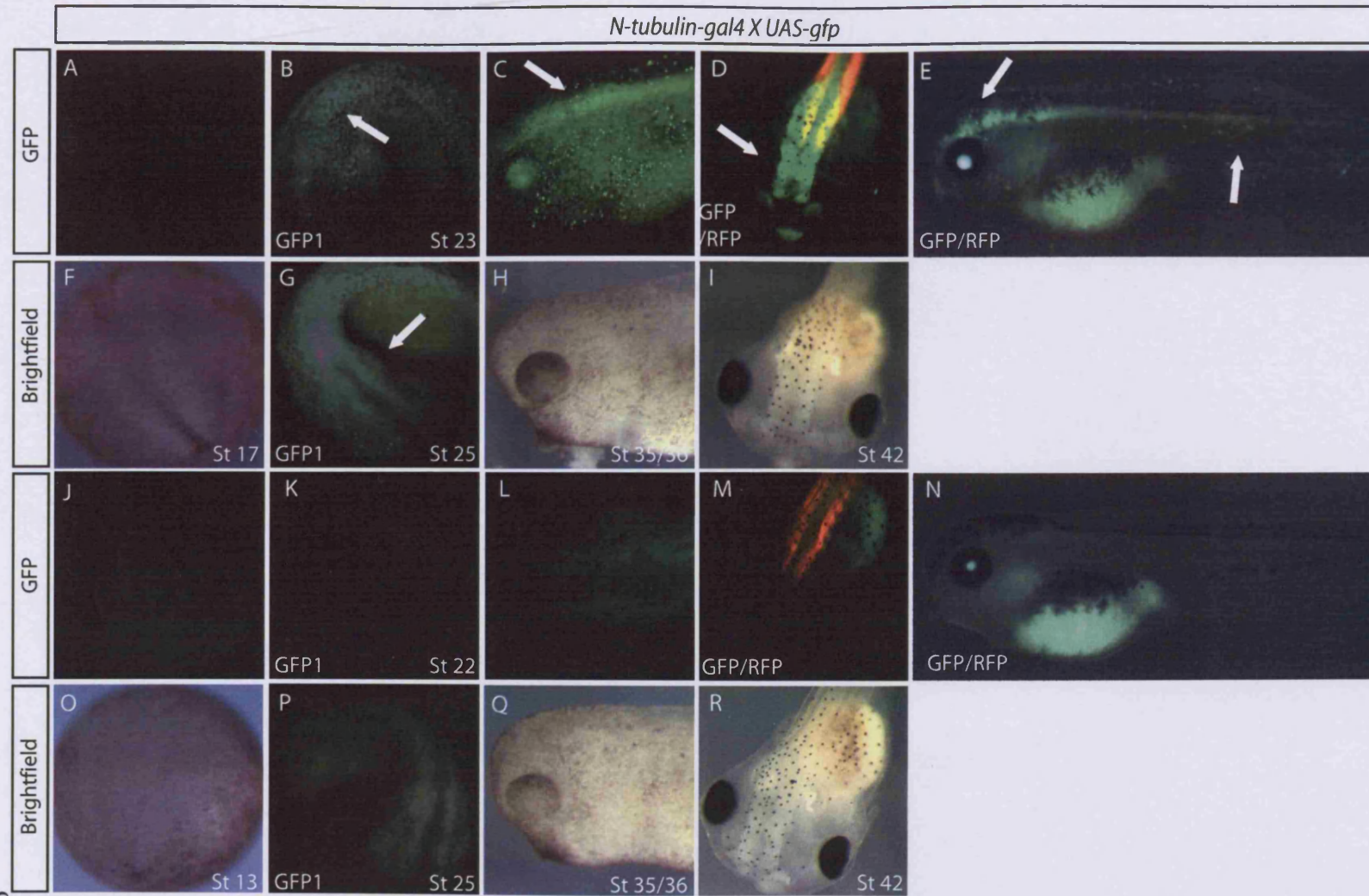
Figure 3.7b *GFP* **is Expressed in the CNS in** *N-tubulin-gal4 X UAS-gfp* **Cross Embryos**

Images depict embryos analysed by whole-mount *in situ* hybridisation for *GFP* or endogenous *N-tubulin*. *GFP* is expressed in developing primary neurons in the posterior neural plate (A, B), neural tube (C, red asterisk illustrates the level of the prospective anterior brain) and spinal cord (D) in *N-tubulin-gal4 X UAS-gfp* cross embryos (see black arrow). *GFP* is also expressed in hindbrain regions, which is an artifact from *γ-crystallin* driven *ECFP* (C, D). Sibling *N-tubulin-gal4 X UAS-gfp* cross embryos do not express *GFP* (B", D"), but illustrate background staining. Endogenous *N-tubulin* is expressed in developing primary neurons throughout the neural plate (and lateral ectoderm) (A', B'), neural tube and brain (C') and spinal cord and brain (D') in *N-tubulin-gal4 X UAS-gfp* cross embryos.

Figure 3.7b

N-tubulin-gal4 X UAS-gfp

A — GFP — St 13
A' — N-tubulin — St 13
B — GFP — St 16
B' — N-tubulin — St 17
B'' — GFP — St 16
C — GFP — St 20
C' — N-tubulin — St 20
D — GFP — St 25
D' — N-tubulin — St 25
D'' — GFP — St 25
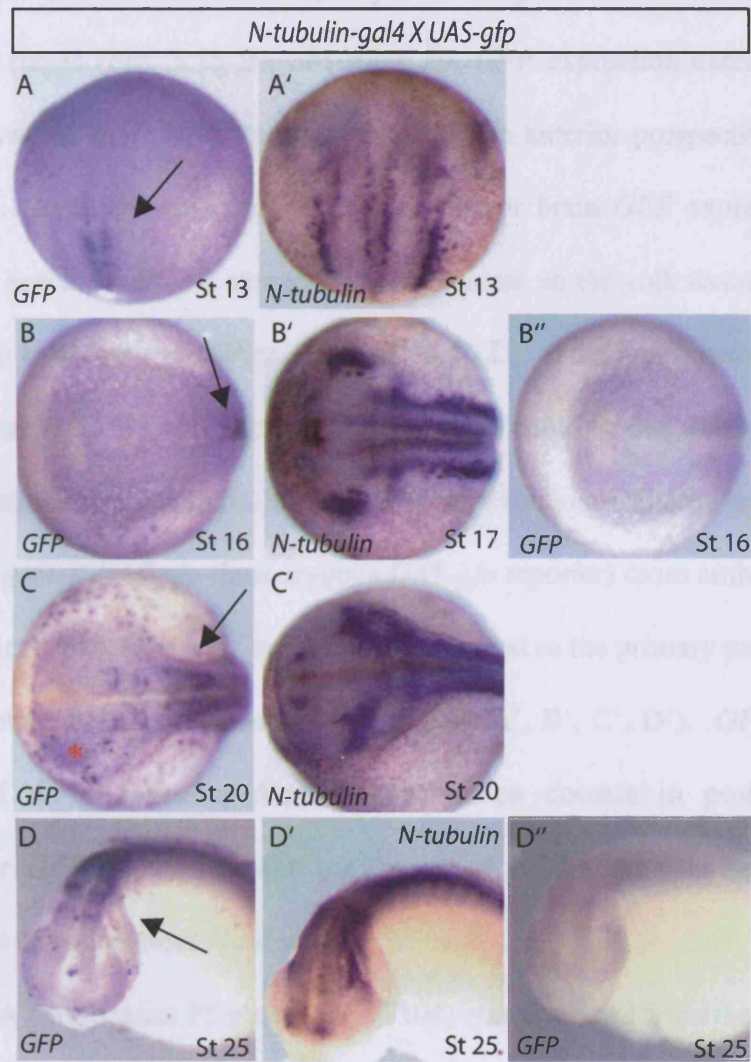
GAL4 RNA expression pattern (Fig. 3.2B i, iv). There appeared to be no time lag between GAL4 transactivation of *GFP* in this posterior domain. Again, analogous to the *GAL4* expression pattern for this promoter, the expression domain of target gene expression, *GFP*, is not located in the anterior neural plate of the stage 13 embryo (Fig. 3.7b A). *GFP* continued to be expressed in posterior neural tube areas thoughout neurula stages (Fig. 3.7b B). At stage 20, *GFP* expression extends along the neural tube, however there is still no expression in the anterior prospective brain region (Fig. 3.7b C). Even by stage 25 there is no anterior brain *GFP* expression (Fig. 3.7b D). Ectopic speckles of *GFP* expression were evident on the yolk tissue in *N-tubulin-gal4 X UAS-gfp* cross embryos (Figs. 3.7C; 3.7b C, D). This is not seen in single transgenic sibling embryos (Figs. 3.7L; 3.7b D"), therefore could be due to slight non-specificity of GAL4 transactivation from this *N-tubulin-gal4* founder. There were 11/24 (46%) of *N-tubulin-gal4 X UAS-gfp* (homozygous *UAS-gfp* reporter) cross embryos displaying *GFP* expression. Endogenous *N-tubulin* was expressed in the primary neurons throughout the neural plate, neural tube and brain (Fig. 3.7b A', B', C', D'). *GFP* expression was in parts of the endogenous *N-tubulin* expression domain in posterior CNS regions, however *GFP* expression was not expressed in the same anterior CNS expression domains of endogenous *N-tubulin*.

A heterozgous F1 population (T104) was produced from the *N-tubulin-gal4* F0 3 by outcross to WT frog. Eight frogs were raised in this F1 population. Individual frogs were tested for stable expression of their transgene, and all individual frogs tested were able to transactivate GFP when crossed to a *UAS-gfp* reporter. In three different crosses of *N-tubulin-gal4* heterozygous F1 frogs to a homozygous *UAS-gfp* reporter, there were

119/305 (56%), 112/348, (49%) and 161/441 (53%) cross embryos containing CNS-specific GFP respectively. This data suggests that the *N-tubulin-gal4* transgene has a stable transgene expression through generations and high transactivation efficiency.

3.2.5.2 *Rx-gal4 X UAS-gfp* Cross

The *Rx-gal4* founder transactivators were tested for their ability to transactivate expression of GFP from a *UAS-gfp* reporter. GFP fluorescence was detected in the eye fields in early tailbud stage *Rx-gal4 X UAS-gfp* cross embryos from *Rx-gal4* (T196) founder (Fig. 3.8A, D). GFP fluorescence can be seen in the retina surrounding the lens by stage 39 in *Rx-gal4 X UAS-gfp* cross embryos (Fig. 3.8E, E"). No GFP fluorescence was observed in a sibling *UAS-gfp* control embryo (Fig. 3.8F-H, H"). In another cross of *Rx-gal4 X UAS-gfp* from *Rx-gal4* (T196), GFP was detected throughout the retina at stage 35/36 embryos (Fig. 3.8B, C). These results indicate that *Rx-gal4* founder (T196) can transactivate GFP from a *UAS-gfp* reporter in a retinal tissue-specific manner.

In contrast, no GFP fluorescence was found in the eye fields in embryos from a cross of founder *Rx-gal4* (T191) to a heterozygous *UAS-gfp* reporter (Fig 3.9B, C). Also, no GFP was detected in *Rx-gal4* sibling single transgenic embryos (Fig 3.9J, K, L). However, using *in situ* hybridisation for *GFP* RNA, *GFP* expression could be detected in anterior neural plate in prospective eye fields at stage 13 (Figure 3.9O). Subsequently, through neurula stages, *GFP* target gene expression is maintained in eye anlage (Fig 3.9P). This supports that Rx-gal4 founder (T191) can transactivate in a retinal tissue-specific manner, and that transactivation of expression from *UAS-gfp* does occur from the beginning of neural plate stages for the *Rx-gal4* founder (T191), even

Figure 3.8 GFP is Detected in Retinal Fields in *Rx-gal4* X *UAS-gfp* Cross Embryos

Fluorescence microscopy images of F1 embryos from a *Rx-gal4* F0 (T196 line) cross to *UAS-gfp* effector. GFP is detected in the fields that give rise to the retina throughout early tailbud stages in *Rx-gal4* X *UAS-gfp* cross embryo (A, D, E (represents ECFP in lens from γ-*crystallin* reporter and *Rx* promoter driven GFP) (E'' represents detection of RFP in the somites in embryo E, at stage 39)). B, C GFP can be seen in the retina in another embryo from the *Rx-gal4* X *UAS-gfp* cross at stage 35/36 (line T196). No GFP in a *UAS-gfp* single transgenic sibling control (F, G, H, H, H'' represents RFP in the somites from embryo H, at stage 39). B', C', D', E', H' represent the brightfield images for B, C, D, E, H respectively.
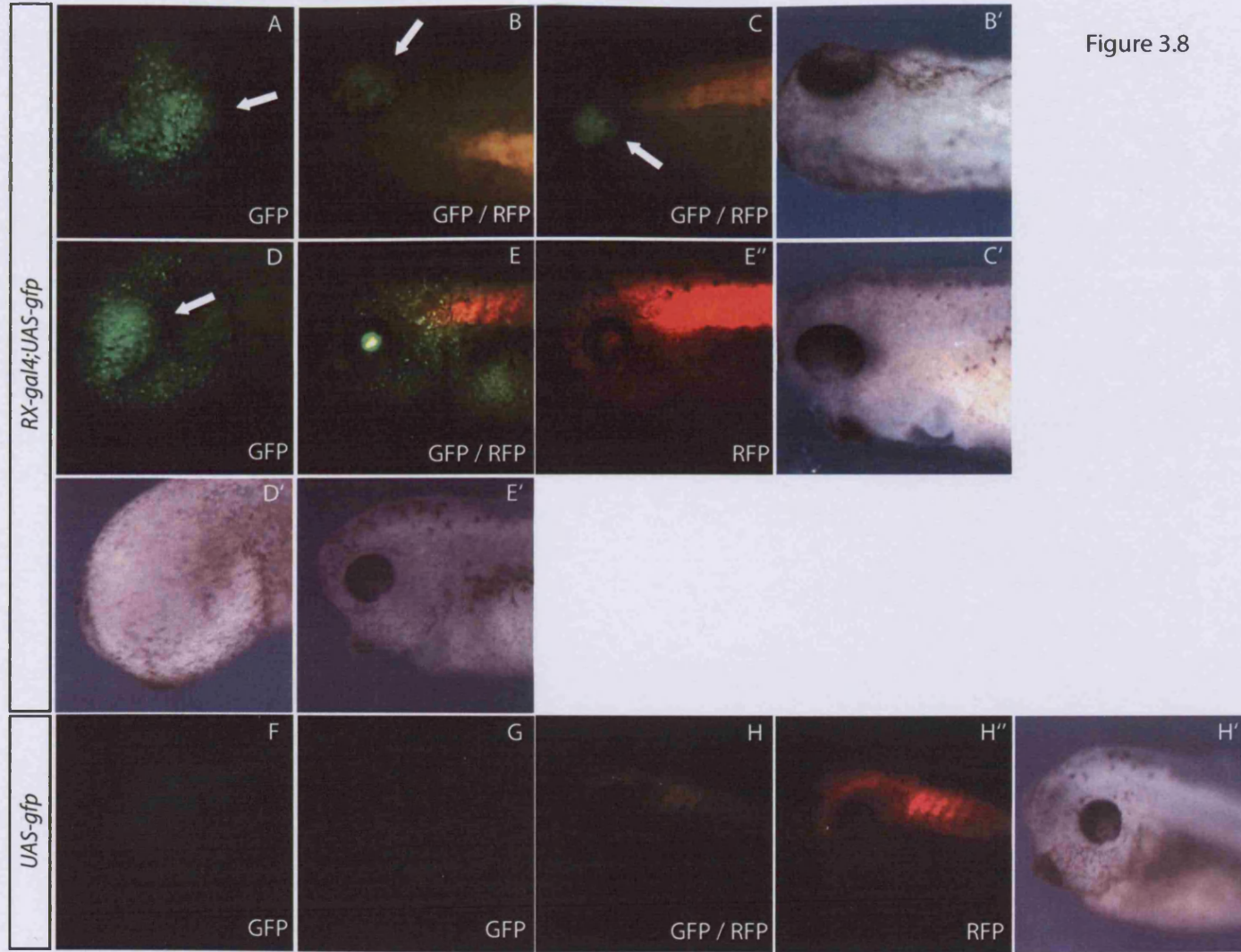
Figure 3.8

Figure 3.9 *GFP* Transcript, but not GFP Protein is Detected in Fields that give Rise to the Retina in *Rx-gal4 X UAS-gfp* Cross Embryos from another Founder

Images depict fluorescence microscopy, brightfield or whole-mount *in situ* hybridisation for *GFP* photos, as indicated, for embryos from a *Rx-gal4 X UAS-gfp* cross (T191 line). GFP is not detected in the developing retina in a *Rx-gal4 X UAS-gfp* cross embryos (A, B, C, (D, H indicating ECFP in lens from the *γ-crystallin* reporter and *Rx* promoter driven GFP and RFP in the somites respectively to suggest that the embryo is double transgenic)). No GFP is detected in a *Rx-gal4* sibling single transgenic embryo (I, J, (K, L ECFP in lens from the *γ-crystallin* reporter and no RFP in somites suggests that the embryo is a *Rx-gal4* single transgenic)). E, F, G, M, N are brightfield images to stage embryos of A, B, C, M, N respectively. O-P *GFP* is expressed in developing retina through neurula stages (13 – 16).

Figure 3.9

107
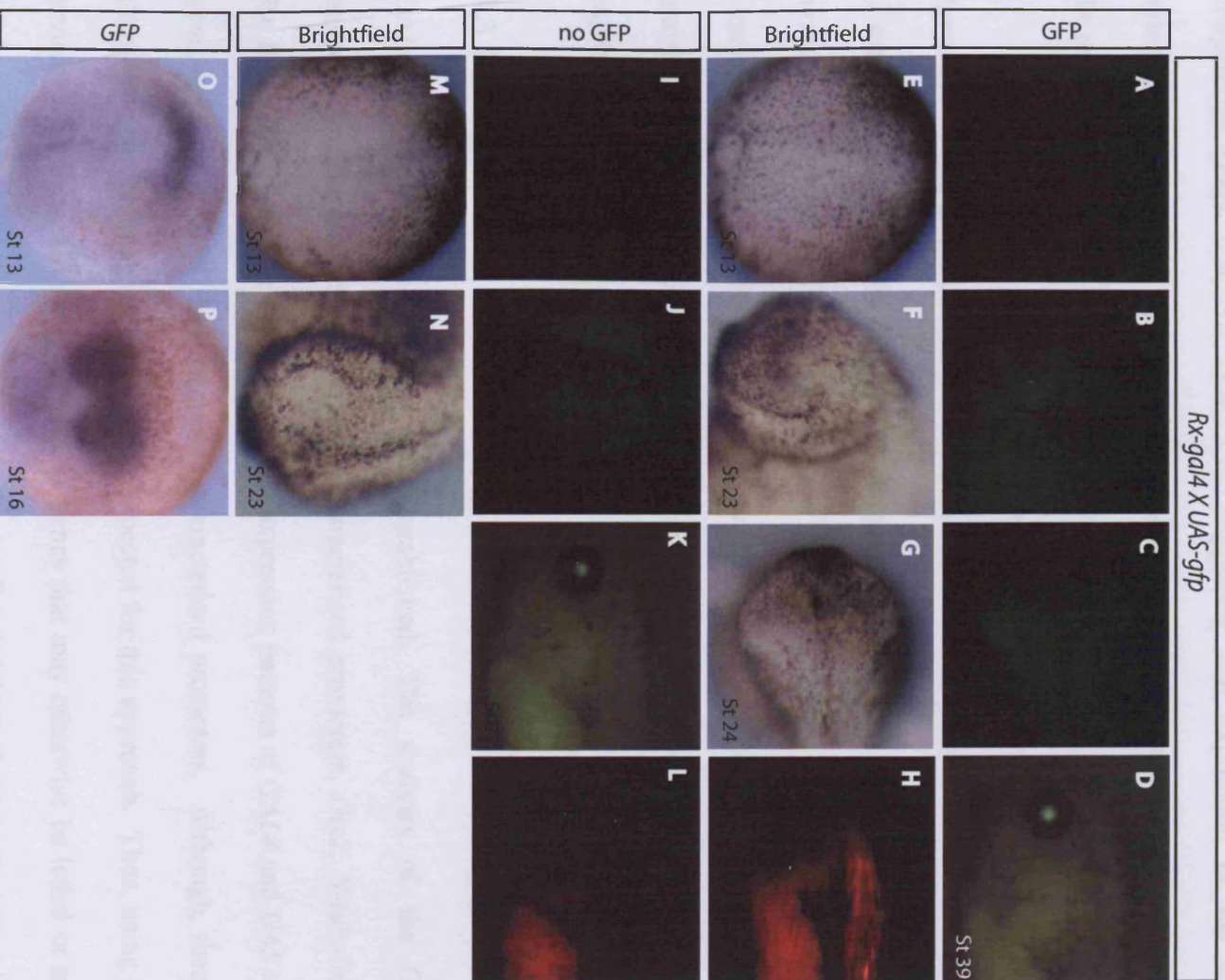
though there is no apparent transactivation of GFP. Viable heterozygous F1 stable lines were expanded from both *Rx-gal4* founders (T196 and T191).

Otx2-gal4, *N-tubulin-gal4* and *Rx-gal4* lines were viable and displayed normal morphology (Figs. 3.3; 3.7; 3.8; 3.9), suggesting that expression of the GAL4 transcription factor and *UAS* target gene expression throughout the specific tissues from the respective promoters is not deleterious in itself.

BMP signalling has a role in neural patterning at early stages of neural development (Barth et al., 1999). In order to test the binary approach it was necessary to use a transactivator that drove target gene expression during early stages of anterior neural development. The *N-tubulin-gal4* transactivator did not drive target gene expression in the anterior CNS during neurula or tailbud stages. The *Otx-2-gal4* transactivator was chosen for further characterisation due to its anterior neural plate expression.

3.3 Discussion

GAL4 transactivator lines have been established. The analysis of the GAL4 transactivator lines containing different characterised promoters, *Otx2*, *N-tubulin* and *Rx*, has indicated that their spatio-temporal expression patterns of *GAL4* and *UAS target gene*, resembles that of their respective characterised promoters. Although, there are time delays in target gene expression, as expected for this approach. Thus, using these transactivator lines, UAS target genes, of genes that may otherwise be lethal or multi-functional, may be mis-expressed in the tissues directed from these promoters, and the effects of gene function in these areas can be assessed at later stages of development.

3.3.1 Prospective Anterior CNS-targeted Reporter Gene Expression

Analysis of the transactivator line, *Otx-2-gal4*, by crosses, has established that it can be used as part of a two-part Gal4/UAS system for targeting gene expression to specific tissues in the developing anterior CNS (Fig. 3.3; 3.4; 3.5). The role of neural development and GAL4-mediated expression in this targeted mis-expression is discussed.

The expression of *GAL4* transcripts in early blastula and gastrula, as well as the detection of GFP protein in late gastrula *Otx-2-gal4 X UAS-gfp* cross embryos, suggests that there is a minimal time-lag between accumulation of GAL4, its transactivation of the *UAS-gfp* effector, and the resulting accumulation of GFP. The anterior CNS is initially specified during gastrulation, at which point target gene product is made (Fig. 3.4B). Target gene expression is detected throughout the neural plate; expression is in antero-lateral and lateral regions of the neural plate in regions which give rise to the telencephalon and dorsal neural structures, such as the pineal gland (Eagleson and Harris, 1990). Target gene expression in more medial regions of the neural plate is in regions which give rise to ventral brain structures. At later stages, when the neural tube has closed, target gene expression is still localised to the anterior of the embryo in brain anlage. Previous studies mis-expressing developmental regulatory genes within neural tissue during early stages of its development have found severe malformations in neural development (Hartley et al., 2002). Thus, mis-expression of developmental regulatory genes, in crosses of the *Otx2-gal4* transactivator to *UAS-target gene* effectors (containing target genes such as *Flognog*), has the potential to interfere with forebrain development.

At all stages of development assessed, the *GAL4* and *GFP* mRNA expression pattern is distinct from the endogenous *Otx-2* expression pattern (Fig. 3.4; 3.5). The reasons why transgene-mediated expression is not the same as endogenous expression may be due to transgene position effects, whereby the genomic site of integration affects reporter expression, with the same cis-acting sequences resulting in different subsets of the "correct" expression pattern. Alternatively, it may be due to the *Otx2* promoter fragment not containing all the necessary regulatory elements to mimic the endogenous gene; it has been reported that the *Otx2* promoter may not contain the necessary elements to down-regulate expression in the prechordal mesoderm (Hirsch et al., 2002). Also, GAL4-mediated expression may cause time lags in expression. Time lags may result from the synthesis and accumulation of GAL4. Thus, GAL4 can distort the temporal control of expression in two ways. Firstly, there may be a delay before GAL4 reaches levels sufficient to activate the UAS-gene. UAS-target gene expression will then lag behind the start of promoter *GAL4* transcription. This may result in the expression pattern in a later embryo resembling an earlier expression pattern. Secondly, GAL4 expression may carry on long after the endogenous expression has ceased, due to the stability of GAL4 protein, GAL4 protein will still be present and driving UAS-gene expression after cessation of promoter-*gal4* transcription (Phelps and Brand, 1998). Studies illustrating GAL4-mediated delays have reported that *GAL4* RNA is transcribed within 15 minutes after temperature-mediated induction from a *hsp70:Gal4* activator. Then, *GAL4* RNA decays rapidly by 90 minutes, depending on the duration of heatshock from the promoter, and subsequently GAL4 protein was detectable 1.5 hours after heat shock and persisted for at least 13 hours (Scheer et al., 2002). Other GAL4-

mediated problems include, GAL4 specificity; GAL4 transactivator lines can drive expression in other cells (Phelps and Brand, 1998). Also GAL4 can be variable, which can lead to variable phenotypes, which are difficult to interpret. These artifacts have to be taken into account when analysing the effects of a GAL4/UAS cross. Thus, in the Otx2-gal4 reporter cross, the altered target gene expression pattern from endogenous Otx2 expression in the Otx2-gal4 X UAS-gfp cross (Fig. 3.4; 3.5) (Blitz and Cho, 1995) may be due to a lack of regulatory elements in the Otx2 promoter (Hirsch et al., 2002). The target gene expression (Fig. 3.3) resembles the expression seen from the Otx-2-gfp transgene line (Hirsch et al., 2002), therefore positional effect is not affecting expression in the Otx-2-gal4 line. Due to the altered expression profile from altered regulatory elements in the Otx2 promoter, it is hard to assess whether GAL4-mediated delays have altered the expression profile in the Otx2-gal4 transactivator reporter cross, however this is likely to be a factor in the target gene expression profile. The expression patterns here do not indicate that there are problems with GAL4 expression variability because GFP was expressed consistently throughout the respective tissues for the Otx2 promoter.

The pattern of target gene expression from the N-tubulin-gal4 transactivator crosses are not recapitulating their endogenous expression patterns in primary neurons (Fig. 3.2; 3.6; 3.7; 3.7b) (Oschwald et al., 1991). Previous characterisation has shown that the N-tubulin promoter (neural specific beta-tubulin promoter) is expressed in primary neurons, as expected for this promoter (Kroll and Amaya, 1996; Richter et al., 1988). Thus altered promoter elements from the endogenous gene may not be a reason for the altered transgene expression from this promoter. Position effects could be a reason for the differences in expression (Brand and Perrimon, 1993). Also, the target

gene expression pattern in the *N-tubulin-gal4* transactivator crosses resembles an earlier expression profile for the promoter but in an older embryo (Fig. 3.7). The altered target gene expression pattern appears to gradually appear in *N-tubulin* domains over time, in a similar manner to the *Otx2-gal4* reporter cross. Thus, in addition to possible position effects modifying the *N-tubulin* promoter fragment, this delayed target gene expression may be due to a GAL4-mediated delay; GAL4 accumulation over time causing a time lag in reporter transgene driven expression.

In all transactivator type reporter crosses the levels of *GFP* target gene mis-expression were strong enough to produce detectable protein (Fig. 3.3; 3.7; 3.8). There is a high level transactivation here, which has the potential to be useful for the ligand trap technique.

Note that in figures 3.6, 3.7 and 3.7b there are ectopic speckles of GFP expression outside promoter driven areas in embryos from crosses of transactivators to reporters. As the ectopic GFP is only observed in double transgenic embryos and not in sibling control embryos, this indicates that the promoters may drive ectopic expression outside the promoter driven area. As this ectopic expression was mostly confined to yolk areas it should not be a problem.

3.3.2 Differences in Transactivation between Stable Lines

Fig. 3.8; 3.9 illustrates differences in retinal GFP expression between *Rx-gal4* transactivator lines; the *Rx-gal4* T196 line appeared to produce GFP whereas *Rx-gal4* T191 line did not display GFP. The *Rx-gal4* T196 line is the optimum line to use, as it appears to transactivate functional target gene protein (GFP). One might speculate that

the reason for such differences in transactivation could be due to transgene copy numbers, e.g. the *Rx-gal4* T196 line may contain a higher transgene copy number than the *Rx-gal4* T191 line. Thus, the overall levels of GFP via the *Rx-gal4* T191 line X *UAS-gfp* cross may not be sufficient to produce visible GFP. As the transgene copy number has not been directly established, the copy number is assumed to be anywhere between 1 to 15 tandem copies of the plasmid carrying the transgene (Kroll and Amaya, 1996; Marsh-Armstrong et al., 1999). It would be interesting to determine if this is the reason for the differences in transactivation. Other reasons for differences in transactivation (variations in expression of GAL4 occurring between activator lines containing the same construct) may be caused by the effect of the site of integration on the transgene or partial deletions of the transgene. These explanations do not seem likely as transgene-mediated expression reflects endogenous gene expression. Nevertheless, a *Rx-gal4* T196 line has been bred that has strong transactivation during retinal determination, and it will be useful to study these processes. It may also be useful to breed this *Rx-gal4* T196 line to homozygosity.

Fig. 3.6 illustrates differences in the level and location of *N-tubulin-gal4* transactivation of *UAS-gfp* reporter from different *N-tubulin-gal4* founders. One possible explanation for these transactivation differences is the transgene position. Depending on the position of the *N-tubulin-gal4* transgene integration site, it may be inhibited possibly by being buried in heterochromatin, thus resulting in a lack of transcription and a lack of transactivation from this integration site; or, transgene expression may be modified in other ways from its surroundings in the chromosome by "position effects". The action of genomic enhancers adjacent to the site(s) of transgene

insertion may perturb the transcriptional activity of the *N-tubulin* promoter, resulting in an altered expression pattern from the promoter.

3.3.3 Other Roles for Different GAL4 Transactivator Types with Useful Spatio-Temporal Expression Patterns

The characterised GAL4 transactivator lines do not have useful expression patterns to investigate dorsal telencephalon patterning. This is due to either too early, i.e. the *Otx2-gal4* transactivator (Fig. 3.3; 3.4; 3.5) or too late, i.e. the *N-tubulin-gal4* transactivator (Fig. 3.7), target gene expression during CNS development to assess initial dorsal patterning, which occurs during and after neural tube closure. Nevertheless, the GAL4 transactivator lines provide a way to drive the expression of developmentally important genes within restricted domains in the embryo. Lines have been developed that express *GAL4* in different spatio-temporal patterns that will be useful to investigate other biological questions (Fig 3.6; 3.7; 3.8; 3.9). *Xrx* gene expression is important in early eye determination (Mathers et al., 1997). Sectional analysis will verify the precise location of transactivation of the prospective retinal field of target gene expression seen in these *Rx-gal4* X *UAS-gfp* crosses (Fig. 3.7 O, P). The use of the *Xrx-gal4T196* transactivator line together with *UAS-reporters* (i.e. *UAS-gfp*) will allow expression to be visualised when retinal determination is occurring. Alternatively, *Rx-gal4* transactivator lines can be used in conjunction with UAS lines containing developmentally important genes to investigate their effects during retinal determination.

The *N-tubulin-gal4* transactivator line may be useful for studying processes of differentiation and re-organisation of identified neurones during neurogenesis (Oschwald et al., 1991).

All of these established transactivator lines can be used to induce expression of many different transgenes with a reproducible timing and pattern of expression, for example, using the *UAS-HIP* (UAS fused to Hedgehog Interacting Protein, personal communication) to trap secreted SHH, this may be useful to investigate unanswered questions in ventral CNS patterning.

CHAPTER 4: EFFECTOR CHARACTERISATION

4.1 Aim and Introduction

The effector transgene, *UAS-flognog*, contains five tandem repeats of the GAL4-binding motif UAS (Upstream Activation Sequence) along with the *hsp70* minimal promoter from pUAST (Brand and Perrimon, 1993) linked to " Flognog " (Fig. 4.1). Flognog is a membrane-tethered fusion protein containing human Noggin fused to intracellular EGFP (Clontech) via a rat CD2 transmembrane domain. Noggin is an extracellular BMP antagonist and preferentially binds to BMP2 and 4 (and also binds other GDF5 subgroup BMPs, such as GDF6) (Zimmerman et al., 1996; Chang and Hemmati-Brivanlou, 1999). Noggin cannot bind BMP7 efficiently in *in vitro* binding assays, however, it might still experience some binding *in vivo* (Zimmerman et al., 1996). This high affinity binding between noggin and BMPs prevents BMPs binding to their cognate cell surface receptors, and blocks BMP signalling. Although Flognog has been reported to dorsalize frog embryos and fluoresce (Dionne and Harland, per. comm.); it has not been established directly whether Flognog can block BMP signalling alone, as well as in the *UAS-flognog* transgenic line. Moreover, it has not been established whether the ligand trap can function in this binary system. Establishing this is therefore essential for the interpretation of any phenotype obtained in binary crosses. The *UAS-flognog* founders were screened for both their ability to block BMP signalling and for an optimum transgene integration site, in terms of copy numbers and position in the genome. Once a functional effector was identified, effector stable lines were generated, allowing a stable population containing uniform levels of transgene expression.

```
      5 X UAS    TATA   human noggin  Rat        EGFP    SV40pA   Cardiac Actin    RFP    SV40pA
                 hsp70                CD2                         promoter
```

kanamycin vector
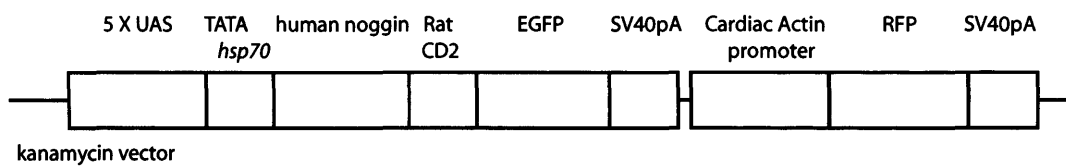
Figure 4.1 *UAS-Flognog* Effector Construct

A diagram of *UAS-flognog* effector construct. The 2-kb *Flognog* cDNA is placed under five repeats of the GAL4-responsive *UAS*. *Flognog* contains membrane-tethered human *noggin* fused to intracellular *GFP* (Dionne & Harland). A secondary reporter cassette containing Cardiac Actin promoter (Mohun et al., 1986) linked to *RFP* is placed down stream of *flognog*.

4.1.1 Noggin and BMP Signalling

BMP signalling blocks the ectoderm's ability to adopt a neural fate. An important role of neural inducers is to define an area of the ectoderm in which the anti-neural activity of the BMPs is antagonised. By blocking BMP signalling, Noggin therefore acts as a neural inducer. Neural inducers influence the fate of the ectoderm by planar or vertical signalling. Additionally, studies have demonstrated that *Noggin* mRNA injections into *Xenopus* dorsal equatorial region can cause an expansion of the neural plate, as indicated by *Sox2* expression in *Xenopus* whole embryo (Huang et al, 2007). It was unknown whether Flognog could perform this same function, therefore an aim was to determine whether Flognog misexpression in the *Xenopus* dorsal equatorial region could increase neural progenitor tissue.

Other studies have demonstrated that levels of BMP signalling can be monitored in *Xenopus* using phospho-specific antibodies directed against the carboxyl-terminal region of the transcription factor Smad1, a downstream effector of BMP signalling (Reversade et al., 2005; Faure et al., 2000; Kurata et al., 2001). For example, a block in BMP signalling using BMP2/4/7 morpholinos or a dominant negative BMPRIA receptor leads to a loss of Smad-1 phosphorylation (Kuroda et al., 2005; Reversade et al., 2005). Therefore, another aim was to determine if Flognog can act in a similar manner to block Smad-1 phosphorylation.

4.1.2 Demonstration of Efficiency of *UAS*-target Gene in Effector Transgenics

UAS-target gene effector transgenics have been characterised by determining whether the target gene transcript is transcribed (transactivated) and whether target protein

product can be detected (Brand and Perrimon, 1993). Recently, the GAL4/UAS system has been demonstrated to be effective in *Xenopus* (Hartley et al., 2002; Chae et al., 2002). Founders containing a reporter/ effector construct (*UAS-gfp*) were tested by GAL4 mRNA injections into progeny from an outcross of the *UAS-gfp* transgenic. Transactivation of the effector gene *GFP* was observed by fluorescence microscopy in embryos containing *UAS-gfp*. To test whether transactivation of the *UAS-gfp* effector could be achieved in a temporally and spatially controlled fashion, effector lines containing *UAS-gfp* were crossed to activator lines expressing GAL4 in a tissue-specific manner. Tissue-specific GFP expression was observed in a proportion of the F1 embryos from this mating (Hartley et al., 2002).

The aim of the studies described in this chapter was to determine whether Flognog is functional in the *UAS-flognog* transgenic embryos (or lines). Then, subsequently, raise the most efficient *UAS-flognog* effector line for use in experimental crosses with suitable driver lines.

4.2 Results

4.2.1 Germline Transmission of *UAS-flognog* Transgene and its Responsiveness to GAL4

In order to identify a stable effector transgenic line, four independent *UAS-flognog* effector founders were crossed to WT frogs and tested for germline transmission rate and responsiveness of the *UAS-flognog* transgene to GAL4. Initially, the F1 progeny

were analysed for the expression of RFP in the somites driven from the CAR-RFP reporter. The RFP was detected throughout the somites, demonstrating that these transgenes were fully expressed (Fig 4.2i (A, C, I, K)). The expression was consistent with that obtained from RFP pattern in the somites from the CAR-RFP reporter in another reporter line, UAS-gfp. The F1 progeny from crosses of UAS-flognog founders X WT were assessed for their number of expressors. It was observed that 28/58 (48%) embryos from T139 line, 137/286 (48%) embryos from the T201 line, 26/107 (24%) embryos from T14L2 and 117/187 (63%) embryos from T14L1 express RFP in their somites (Fig. 4.2ii Table II). Table II shows the number of embryos expressing RFP and the transgene integration number as predicted by Mendelian segregation ratios. Two UAS-flognog lines displayed RFP in approximately 50% of their embryos, suggesting both founders contain one transgene integration site (P>0.05, Table II), and that the integration event in these founder animals occurred at a very early stage of development resulting in a non-mosaic germline. One UAS-flognog line, T14L2, displayed transgene expression from CAR-RFP in 24% of its embryos. A germline transmission rate of 24% RFP expression in offspring from this founder is near the expected Mendelian ratio for a transgene integrated at a single locus from a founder that was half-transgenic (such that the transgene had incorporated at the 2-cell stage). Thus, this may indicate that there had been a single transgene integration event in this founder. The other UAS-flognog founder T14L1 had a high number of expressors, indicating multiple integrations in the founder.

To investigate whether the effector lines were functional (responsive to GAL4), GAL4 mRNA was injected unilaterally into one blastomere of two-cell stage F1 progeny

Figure 4.2 RFP is Detected in the Somites in *UAS-flognog* Lines

i. Images show stage 40 tadpole embryos from a cross between *UAS-flognog* transgenics (T139 and T201) and WT frogs. A, B, E, F, I, J, M, N are dorsal images. C, D, G, H, K, L, O, P are lateral images. A, C, I, K illustrate RFP throughout the somites driven from the *CAR-RFP* reporter. E, G, M, O illustrate sibling embryos displaying no RFP. B, D, F, H, J, N, L, P are their respective brightfield images. ii. Different founder *UAS-flognog* transgenics were crossed to WT. Table II illustrates the numbers and percentages of the F1 embryos displaying RFP in the somites; and the transgene integration number as predicted from the chi-square test. The expected segregation ratios for both the *UAS-flognog*T139 and T201 line were 1:1, and the P values were P>0.05 in all crosses. The values in the table represent data collected from one experiment (one cross for each founder). In a second cross for founder (T14L2) to WT, 9/29 (31%) F1 embryos displayed RFP in the somites.

Figure 4.2

i
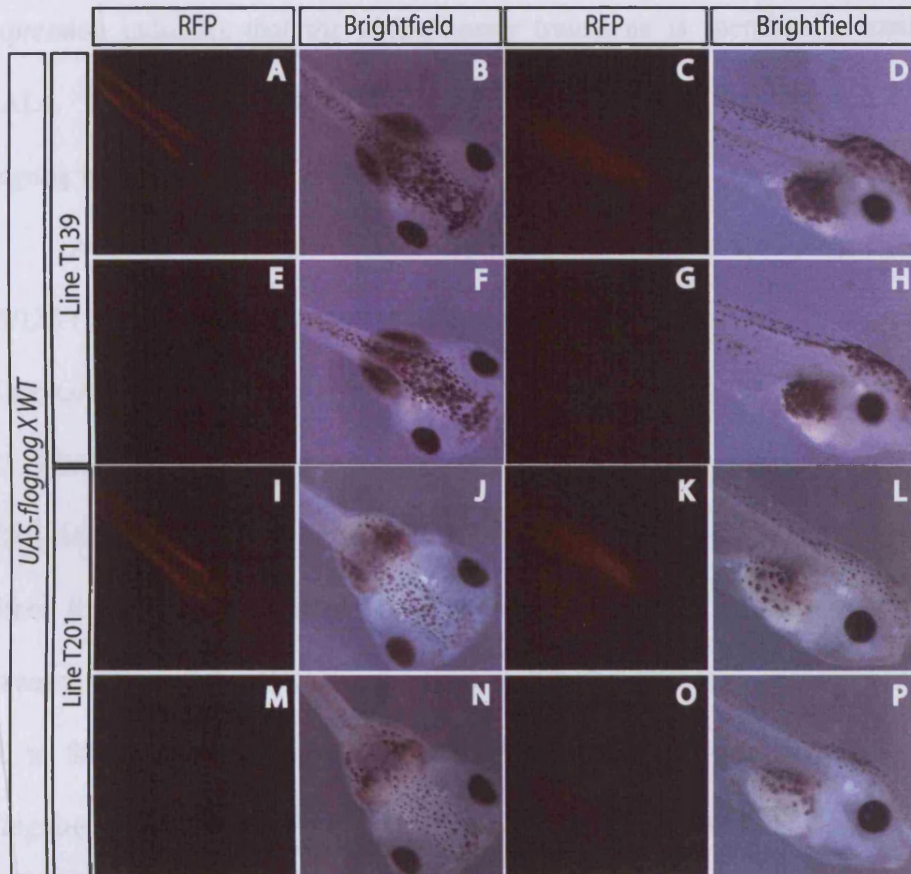
ii

RFP expression in *UAS-flognog CAR-RFP* F1 embryos

Effector Line	RFP+	RFP-	% RFP+ embryos	P	Estimated Number of Integrations
T139	28	30	48	0.79	1
T201	137	149	48	0.48	1
T14n2	26	81	24	-	-
T14n1	117	70	63	-	-

Table II

from crosses between *UAS-flognog* founders and WT. Subsequently, embryos were analysed at neurula stages for *Flognog* expression, as indicated by *in situ* hybridisation for *GFP* transcripts. After injection with *GAL4* mRNA, all lines tested showed unilateral expression of *Flognog* (*GFP*) (Fig. 4.3B i, ii, iii, iv). The unilateral *Flognog* expression indicates that the *UAS-flognog* transgene is specifically transactivated by GAL4. This suggests that GAL4 is sufficient to activate transcription from the *UAS-flognog* transgene in all *UAS-flognog* lines tested.

At 80pg *GAL4* mRNA, 8/18 (44%) embryos for the *UAS-flognog* T139 line and 25/133 (19%) embryos for the *UAS-flognog* T201 line expressed *Flognog* (Table III). At 20pg *GAL4* mRNA there is no induction of *Flognog* expression in the *UAS-flognog* T139 line, whereas *Flognog* is expressed in 28/96 (29%) embryos in the *UAS-flognog* T201 line (Table III). At 5pg *GAL4* mRNA there was no visible *Flognog* expression for either line. This data illustrates that there is transcription of *Flognog* via GAL4 transactivation of the *UAS-flognog* lines and that *UAS-flognog* requires in the region of 20 to 80pg *GAL4* mRNA to initiate transcription. The low percentages expressing *Flognog* suggest that not all of the embryos containing the *UAS-flognog* transgene are expressing *Flognog*. It should be noted that, in some cases, total amounts of embryos analysed for each condition is amalgamated data from parallel experiments that were not carried out in the same experiment (Table III).

This data confirms that *UAS-flognog* transgene is stably expressed in F1 populations from both founders. Both *UAS-flognog* T139 and T201 lines are heterozygous viable, and since both showed expression from the transgene via GAL4 transactivation, both are potentially useful. Hence, F1 animals were raised from each

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Flognog				Flognog
GAL4 mRNA				*Flognog* mRNA
i	ii	iii	iv	v
T139 line	F0 T14L1	F0 T14L2	T201 line	GFP
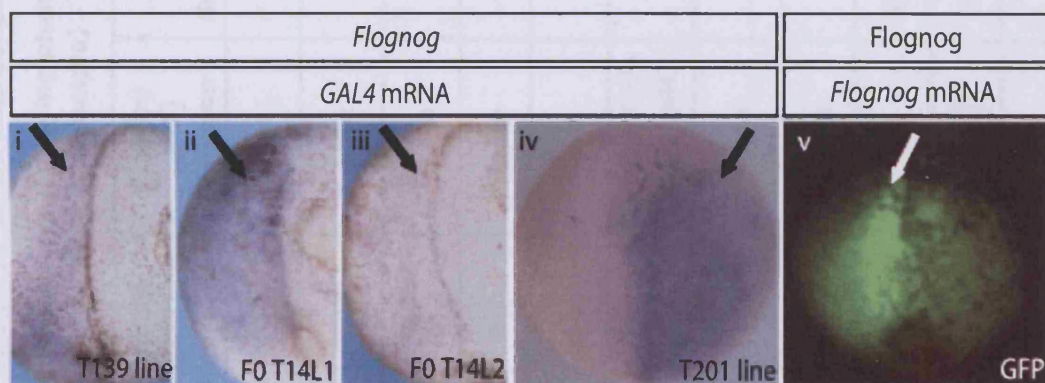
Figure 4.3 *Flognog* mRNA is expressed uni-laterally in *GAL4* mRNA-injected *UAS-flognog* embryos from different *UAS-flognog* lines

All images are dorsal views of neurula stage embryos analysed by whole-mount *in situ* hybridisation for *GFP* (i-iv) or fluorescence microscopy for GFP. i-iv represent uni-lateral *Flognog* mRNA expression in uni-laterally *GAL4* mRNA-injected *UAS-flognog X WT* cross embryos from four different *UAS-flognog* founders, T14L1, T14L2, T139 and T201. Un-injected side of the embryo represents a control displaying no *flognog* expression. v represents detection of Flognog in embryos injected uni-laterally with 50pg *Flognog* mRNA.

Table III

Cross (Line)	Type of mRNA injected (pg)	Total amount of embryos analysed	% of embryos displaying phenotype (number/sample)			Induced (I) Functional (F) Neither (N)
			Flognog (GFP) expression	Flognog (GFP) detection	Neural increase Sox3 assay	
UAS-flognog (T139) X WT	Water	47 (N [b])	N/A	N/A	0 (0/47)	N
	gal4 (80pg)	18 (N [a]) 42 (N [b])	44 (8/18)	none	12 (5/42)	I, F
	gal4 (20pg)	47 (N [b])	0	none	0 (0/47)	N
	gal4 (5pg)	30 (N [b])	0	none	0 (0/30)	N
UAS-flognog (T201) X WT	Water	27 (N [b])	N/A	N/A	0 (0/27)	N
	gal4 (80pg)	133 (N [a]) 19 (N [b])	19 (25/133)	Flognog (GFP) (100pg gal4)	5 (1/19)	I, F
	gal4 (20pg)	96 (N [a]) 20 (N [b])	29 (28/96)	none	10 (2/20)	I, F
	gal4 (5pg)	19 (N [b])	0	none	0 (0/19)	N
WT X WT	noggin (62.5pg)	27 (N [b])	N/A	N/A	81 (22/27)	F
	flognog (125pg)	34 (N [b])	Flognog expressed	Flognog (GFP)	38 (13/34)	F
	Water	29 (N [b])	N/A	N/A	0 (0/29)	N

N[a]Number of embryos analysed for GFP expression N[b]Number of embryos analysed for Sox3 expression GFP expression data for the T201 line, and Sox3 expression data for T139 and T201 lines represents combined results from two experiments. All other data represent values from one experiment.

founder. A heterozygous *UAS-flognog* F2 population was also obtained from the *UAS-flognog* T139 line.

In order to determine whether detection of fluorescence provided a viable assay for assessing the presence of Flognog protein, Flognog mRNA injections were carried out. Unilateral injection of 50pg *Flognog* mRNA resulted in unilateral neural plate Flognog (GFP) fluorescence, indicating GFP fluorescence can be detected from Flognog (Fig 4.3v). The same assay was then used to assess induction of Flognog protein via *GAL4* mRNA injection into the *UAS-flognog* line. GFP fluorescence was evident in *GAL4* mRNA-injected *UAS-flognog* embryos at 100pg *GAL4* mRNA, only in the *UAS-flognog* T201 line (Table III). This result suggests that there may be induction of protein from this *UAS-flognog* line. Further to these results, it was necessary to investigate whether or not Flognog protein was functioning.

4.2.2 Flognog Function in *UAS-flognog* Lines

Firstly, to determine the efficacy of Flognog fusion protein, Flognog was tested for its neural inducing ability in comparison to Noggin. *Noggin* and *Flognog* mRNA were injected unilaterally into the dorsal-animal equatorial region of WT embryos to induce a neural fate in neighbouring cells. Neural tissue can be identified by the *X-Sox3* gene, this gene is a member of the HMG-box containing transcription factor family and is expressed in a highly restricted pattern in dorsal ectoderm where it marks proliferating neural precursor cells (Penzel et al., 1997). Alterations in the amount of neural precursor cells in injected embryos were then assessed by *in situ* hybridisation for *x-*

Sox3 expression at neural plates stages, in an assay similar to that employed by Huang et al. (2007) to monitor Noggin activity.

In both *Noggin* and *Flognog* mRNA-injected embryos there was an expansion in domain of neural progenitor tissue in the neural plate on the injected side of the embryo, as indicated by x-*Sox3* expression, which was not seen in water-injected control embryos. Embryos were co-injected with *LacZ* mRNA to determine the injected side of the embryo. The width of the x-*Sox3* expression domain was measured, and the injected side of the embryo was compared with the contralateral uninjected side. There was background variation in the width of x-*Sox3* expression domain in water-injected control. Therefore, taking this into account, if the width of domain of x-*Sox3* expression at the mid-point of the anterior-posterior axis was at least two times the width of the un-injected side, it was classified as an expansion (an increase). Using this criterion, there were 22/27 (81%) of Noggin (62.5pg) mRNA-injected embryos displaying an increase in neural tissue on the injected side of the embryo, whereas 13/34 (38%) Flognog (125pg) mRNA-injected embryos showed an increase on the injected side (Fig 4.4 C, F, Table III). As a control for injection, the same measurements were taken for embryos injected with *LacZ* mRNA alone. There were 0/29 (0%) *LacZ* mRNA-injected embryos displaying similar alterations in x-*Sox3* expression domain. Therefore, these control embryos displayed no increase of neural progenitor tissue in the neural plate on the injected side of the embryo. In uninjected embryos, no variation in x-*Sox3* expression between embryo sides was detected using the two-fold criterion as the threshold. These results suggest that Flognog is mimicking the activity of Noggin at inducing neural tissue.

Figure 4.4
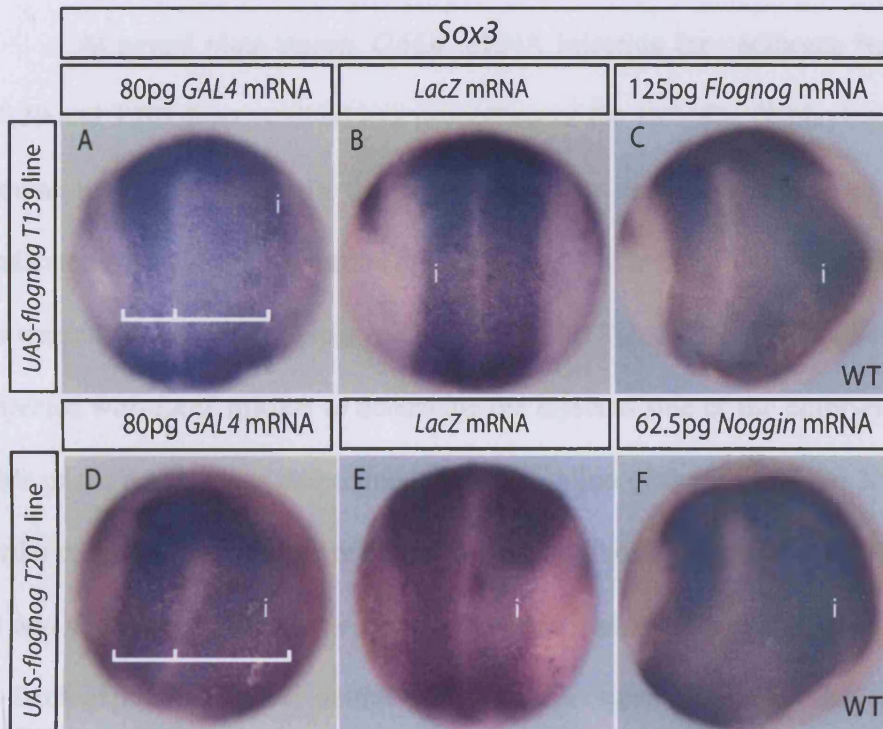
Figure 4.4 Expansion of the neural plate progenitor cells in *GAL4, Flognog* and *Noggin* mRNA-injected *UAS-flognog X WT* cross or WT embryos

Images are dorsal views of whole-mount *in situ* hybridisation neural plate stage embryos expressing *Sox3*. A, B indicate embryos from T139 *UAS-flognog line* X WT cross, D, E indicate embryos from T201 line X WT cross and C, F indicate embryos from a WT cross. Embryos from a cross of *UAS-flognog to WT* (A, B, D, E) or WT embryos (C, F) were injected with 80pg *GAL4* mRNA (A, D) or 125pg *Flognog* mRNA (C) or 62.5pg *Noggin* mRNA (F) or *LacZ* mRNA alone (B, E) into one animal dorsal blastomere of 8-cell stage embryos and assayed for the extent of domain of neural progenitor cells via *Sox3* expression. A, D show an expansion of the neural plate progenitor cells in *GAL4* mRNA-injected embryos from both T139 and T201 *UAS-flognog* lines, indicated by the expression of *Sox3* marker. There is no expansion of neural plate in *LacZ* mRNA alone-injected control embryos (B, E). C, D show an expansion of the neural plate progenitor cells in both *Flognog* (125pg) and *Noggin* (62.5pg) mRNA-injected embryos. i indicates the injected side of the embryo, as assessed by the presence of X-Gal staining (β-Gal activity)). X-Gal stained embryos were sorted for left and right and then analysed by *in situ* hybridisation for *Sox3* for *Flognog* and *Noggin* mRNA-injected embryos or embryos were analysed by *in situ* hybridisation for *Sox3* and then analysed for X-Gal stain for *UAS-flognog X WT* cross embryos. See Table III for numbers of embryos injected.

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To investigate whether the effector lines were producing functional protein, *GAL4* mRNA was injected unilaterally into the dorsal-animal equatorial region of embryos from a cross of *UAS-flognog* founders to WT frogs. Then subsequently assayed for x-*Sox3* expression at neural plate stages.

At neural plate stages, *GAL4* mRNA injection into embryos from *UAS-flognog* T139 and T201 lines resulted in a proportion of the embryos displaying an expansion of neural progenitor tissue in the neural plate on the injected side of the embryo, as indicated by x-*Sox3* expression (Fig 4.4 A, D; Table III). This expansion was not seen in water-injected control embryos (Fig 4.4 B, E; Table III). Again all embryos were co-injected with *LacZ* mRNA to determine the injected side of the embryo and the injected side of the embryo was determined by examination of the embryo for X-Gal staining. In order to assess the response of the *UAS-flognog* lines to different doses of *GAL4* mRNA, it was necessary to define the criteria for what is classified as an increase. The width of x-*Sox3* expression in the neural plate was measured for all of the embryos. It was decided that if the increase in width of domain of *Sox3* expression on the injected side, at the middle of the anterior-posterior axis, was at least 1.5 times the width of the un-injected side, it was classified as an increase (expansion). This arbitrary value of 1.5 fold increase was taken based on the fact that there was a 1.05 to 1.06 mean fold increase in water-injected (*LacZ* mRNA alone) control. At 80pg *GAL4* mRNA there were 5/42 (12%) embryos for the T139 line and 1/19 (5%) embryos for the T201 line displaying an increase in neural progenitor tissue on the injected side of the embryo (Fig 4.4 A, D, Table III), whereas 0/47 (0%, mean fold increase on injected side was 1.05, P=0.54, P>0.05) and 0/27 (0%, mean fold increase on injected side was 1.06, P=0.22,

P>0.05) water-injected (*LacZ* mRNA alone) control embryos for the T139 and T201 lines respectively, displayed an alteration in size of neural progenitor tissue (Fig 4.4 B, E, Table III). There was no severe expansion of neural progenitor tissue on one side of the embryo compared to the other in uninjected control embryos expressing *Sox3*. As the dosage of *GAL4* mRNA was decreased, at 20pg *GAL4* mRNA there were 0/47 (0%) and 2/20 (10%) embryos for the T139 line and T201 lines displaying an increase in neural progenitor tissue on the injected side of the embryo. At 5pg *GAL4* mRNA there were no drastic alterations of neural progenitor tissue on the injected side of the embryo, compared to *LacZ* mRNA-injected control embryos. These results suggest that GAL4 is sufficient to cause an increase the number of neural progenitor cells in the neural plate and indirectly suggests that BMP2, 4 and GDF6 signalling can be blocked by transactivation of the *UAS-flognog* transgene in these effector transgenic lines.

To verify that both of the *UAS-flognog* lines are functional, 80pg *GAL4* mRNA was injected into the ventral marginal zone to determine whether axis duplication could be induced. Noggin mRNA injection into the same site is known to cause axis duplication (Fang et al., 2000). It was observed that axis duplication can occur in both T139 and T201 *UAS-flognog* transgenic lines upon injection of *GAL4* mRNA, visualised by staining for N-tubulin. Axis duplication was not observed in LacZ mRNA alone-injected control embryos. Also, Flognog mRNA injections, like those of Noggin mRNA, induced secondary axis formation by injection into ventral marginal zone, providing further evidence that Flognog can mimic the function of Noggin.

For subsequent analysis, the *UAS-flognog* T139 line was used.

4.2.3 Loss of Smad-1 Phosphorylation (Activation) in *GAL4* mRNA-injected *UAS-flognog* Embryos

The phosphorylation state of Smad-1 can be used as an indicator of activation or inactivation of the BMP/ Smad-1 signalling pathway. Phospho-Smad-1 can be detected via western analysis of *Xenopus* embryo at neurula stage, and the signal is enhanced by activation of the BMP signalling pathway with BMP4 (Faure et al., 2000). The size of phospho-Smad-1 in *Xenopus* is approximately 60kDa (Kuroda et al., 2005). To determine whether BMP signalling was blocked in *UAS-flognog* embryos via GAL4 transactivation, embryos from a *UAS-flognog* X *WT* cross were co-injected at the 2-cell stage into two blastomeres in the vegetal pole with *GAL4* and *BMP4* mRNA (or *BMP4* mRNA alone), or were un-injected. Injected and un-injected sibling embryos were harvested individually at neurula stage for western blot detection with anti-phospho-Smad1 antibody.

Anti-phospho-Smad1 (Ser463/465), raised against a peptide of amino acids 455-465 from the C-terminus of human Smad1 protein was obtained. This antibody recognizes the dual serine phosphorylated Smad1 (Ser 463/465) and due to conservation of sequence it cross-reacts with *Xenopus* phospho-Smad-1 (Fig. 4.5A). When GAL4 is co-expressed with BMP4 in *UAS-flognog* X *WT* cross embryos, anti-phospho-Smad-1 antibody reveals a signal at approximately 60kDa in 7 out of 17 embryos (Fig 4.5C). Expression of BMP4 in *UAS-flognog* X *WT* cross embryos and detection with anti-phospho-Smad-1 reveals a signal in all *BMP4* mRNA injected embryos, whereas there is a faint signal in un-injected sibling embryos, supporting the specificity of the phospho-Smad1 band. I was unable to detect a Smad-1 signal with a Smad-1 antibody, so I

Fig. 4.5 Loss of Smad-1 Phosphorylation in GAL4 mRNA-injected *UAS-flognog* **Embryos**

A. Diagram of antigen sites and highly conserved carboxyl terminal sequence of Smad-1 proteins of *Xenopus* and human. The amino acid sequence of human Smad-1 shown corresponds to the sequence of the synthesised phosphopeptides against which anti-phospho-Smad-1 antibody was raised. The location of the phosphorylated serine residues are highlighted in red. B. Phospho-Smad-1 size control. Embryos from a cross of *UAS-flognog* founder to WT were either injected with Flag-Smad-1 mRNA (25pg) (FLAG-SMAD-1) or injected with GAL4 mRNA (Non). Lysates from single embryos were loaded one per lane, and detected with either α-phospho-Smad-1 or α-Flag. A phospho-Smad-1- signal is detected at approximately 60kDa, and a FLAG signal is detected between 45 and 60kDa. C. There is a loss of Smad-1 phosphorylation in *UAS-flognog* embryos co-expressing GAL4 and BMP4 from the *UAS-flognog*T139 line. Embryos from a *UAS-flognog* X WT cross were co-injected at the 2-cell stage into two blastomeres with GAL4 (80pg/embryo) and BMP4 (100pg/embryo) mRNA (GAL4/ BMP4) or BMP4 mRNA alone (100pg/embryo) (BMP4), or were un-injected (Un). Injected and un-injected siblings were harvested at neurula stage for western blot detection with anti-phospho-Smad1. Individual embryos were lysed and lysate from one embryo was loaded per lane. The embryo number is shown, the figure illustrates 17 individual GAL4/BMP4 embryos, one BMP4 alone embryo and one un-injected embryo. Phospho-smad-1 signal is detected in BMP4 alone, and reduced to a residual faint signal in un-injected embryos. Anti-α-tubulin indicates loading of samples.

Figure 4.5

A

MH2

C

GSPHNPIS**SVS** human Smad-1 protein (a.a. 455-465)
GSPHNPISSVS Xenopus Smad-1 protein (a.a. 455-465)

B

αPS1	αFLAG
Non	FLAG-SMAD1

60kDa —

— α-P-Smad-1
— α-FLAG

45kDa —

C

		UAS-flognog X WT								
Un	BMP4	GAL4 / BMP4								
1	1	1	2	3	4	5	6	7	8	9

α-P-Smad1

α-tubulin 50kDa

UAS-flognog X WT						
GAL4 / BMP4						
10	11	12	13	14	15	16	17

α-P-Smad1

α-tubulin 50kDa
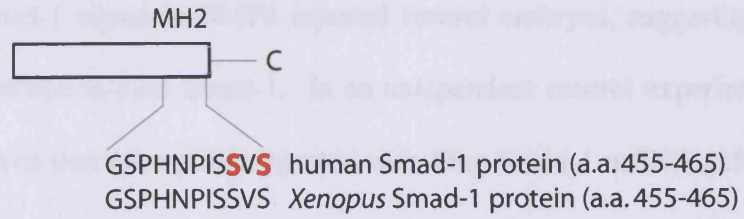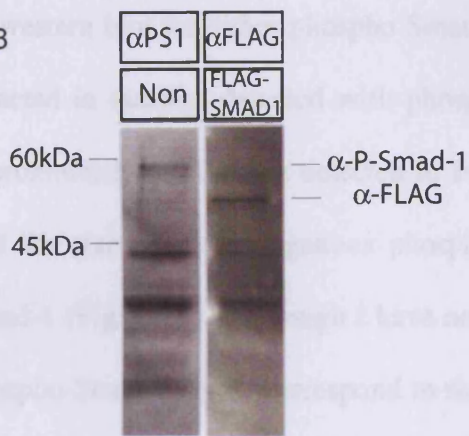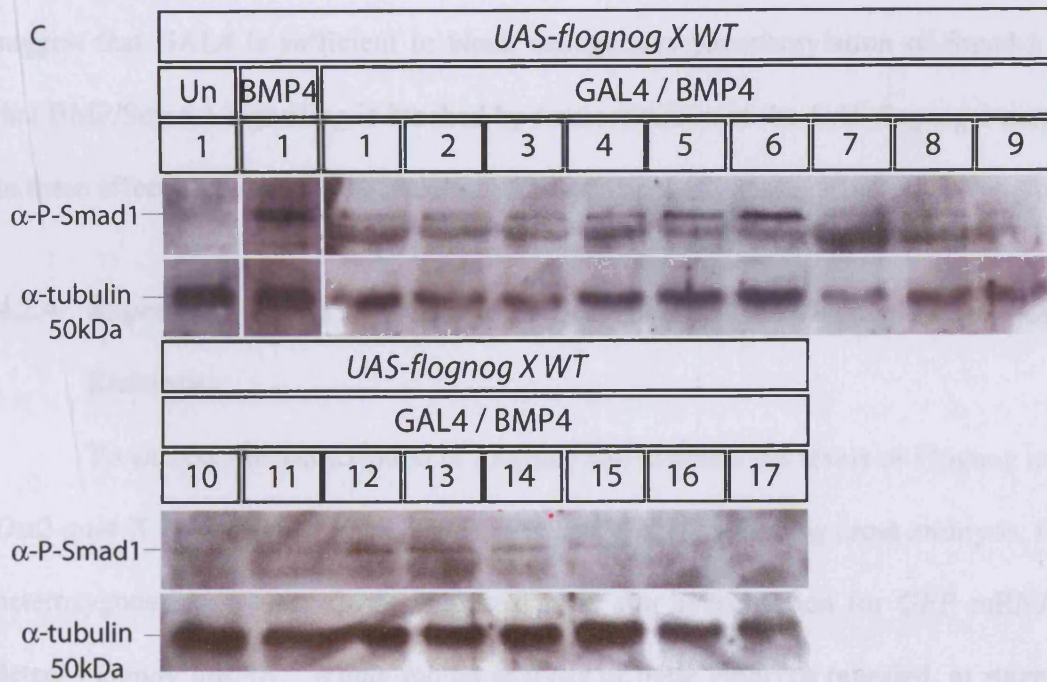
cannot rule out the possibility of alterations in total Smad-1 protein levels as a reason for alterations in phospho-Smad-1 signal. However, anti-α-tubulin reveals a signal in all embryos, controlling for loading of samples. Also, there was no variation within phospho-Smad-1 signal in BMP4 injected control embryos, suggesting that there may not be alterations in total Smad-1. In an independent control experiment, lysates from single embryos that were either injected with Flag-Smad-1 mRNA (25pg) (from cDNA encoding human Smad-1) or were not injected with Flag-Smad-1 mRNA, were analysed by western blot for either phospho-Smad-1 or FLAG antibody. A signal at 60kDa was detected in samples detected with phospho-smad-1 antibody (Fig. 4.5B). A signal at approximately 55kDa was detected in Flag-Smad-1 mRNA-injected lysates, indicating that the size of the endogenous phospho-Smad-1 was at a slightly higher size than Smad-1 (Fig. 4.5B). Although I have not demonstrated that the embryos with a loss of phospho-Smad-1 signal correspond to the transgenic embryos, the loss of the phospho-Smad-1 signal in approximately half of the *GAL4* mRNA-injected embryos could suggest that GAL4 is sufficient to block endogenous phosphorylation of Smad-1 and that BMP/Smad-1 signalling is blocked by transactivation of the *UAS-flognog* transgene in these effector embryos.

4.2.4 Expression Levels of Flognog in *Otx2-gal4* X *UAS-flognog* Binary Cross Embryos

To analyse the transcription of *Flognog* and to assess the levels of Flognog in the *Otx2-gal4* X *UAS-flognog* binary cross, *Otx2-gal4* X *UAS-flognog* cross embryos, from heterozygous frogs, were firstly analysed by *in situ* hybridisation for *GFP* mRNA to detect *Flognog* mRNA. Whole-mount analysis of these embryos revealed, at stage 11,

Flognog is expressed in the neuroectoderm (Fig. 4.6A). At stage 13, *Flognog* continues

to be expressed in ectoderm cells of anterior dorsal regions, in the presumptive fore- and

mid-brain (Fig. 4.6C), in a pattern similar to that reported for endogenous *Otx2*

expression (Pannese et al., 1995). Later, *Flognog* was strongly expressed in the anterior

neural tube, and prospective eye region at stage 19, as well as throughout the developing

forebrain and midbrain and eye tissue at stage 24 (Fig. 4.6E, G). Throughout early

development, although *Flognog* expression was maintained strongly in some embryos,

there was variation (in expression seen) in the expression domains. This suggests that

the expression seen is likely to represent the prolonged expression of *Flognog* due to

GAL4-mediated expression, and may indicate that while the mRNA is stable in some

embryos, the mRNA is not as stable in other embryos. Nevertheless, this data suggests

that *Flognog* mRNA is transcribed in the *Otx2-gal4* X *UAS-flognog* binary cross from

gastrula stages. Furthermore, *Flognog* was expressed in 15/72 (21%) embryos,

suggesting that the *Otx2-gal4;UAS-flognog* double transgenics are viable.

To test if GAL4 can activate *UAS-flognog* to levels that result in embryonic

phenotypes, GFP detection can be used as an indication of expression levels of Flognog

protein in the binary cross. Embryos from a cross of *Otx2-gal4* X *UAS-flognog*, from

heterozygous frogs, were monitored throughout development from end of gastrulation/

beginning of neurulation stage up to tailbud stage by immunohistochemistry using GFP

antibody. Flognog (GFP) was observed in the anterior neural tube and prospective eye

regions in stage 19 cross embryos (Fig. 4.6I). Flognog (GFP) was not observed in other

cross embryos at stage 19 (Fig. 4.6J). At stage 25, Flognog (GFP) was confined to the

anterior CNS (forebrain and midbrain) and eye in 29/119 (24%) cross embryos (Fig

Figure 4.6 GAL4-dependent Mis-expression of Flognog in a Spatially Restricted Manner in Otx2-gal4 X UAS-flognog Cross Embryos from Gastrula to Tailbud Stages

In all panels, images are anterior views and dorsal is to the top. A-H. *Flognog* mRNA is induced in embryos from *Otx2-gal4* X *UAS-flognog* cross. A, C, E, G *Flognog* is expressed at stage 11 in anterior neuroectoderm (A). At stage 13 *Flognog* is expressed in anterior neural plate (C). *Flognog* is expressed in developing forebrain, midbrain and eye (E, G). B, D, F, H Sibling embryos displaying no *Flognog* expression. Black arrow in G points to forebrain. Red arrow points to two dots of expression, which is likely to be due to *GFP* expression in the hindbrain from the γ-crystallin reporter. I-L Flognog protein is induced in embryos from the *Otx2-gal4* X *UAS-flognog* cross. Immunohistochemistry for α-GFP was used to detect the presence of Flognog (GFP), and monitor the levels and location of Flognog. I Flognog is detected in stage 19, initial neural tube stage embryos in the anterior neural tube and prospective eye region. K At stage 25, Flognog is detected in the developing forebrain, midbrain and eye. M-P Brightfield images of embryos in I-L respectively.

Figure 4.6

Otx2-gal4 X UAS-flognog

	Stage 11		Stage 13	
GFP RNA	A	B	C	D
	Stage 19		Stage 24	
GFP RNA	E	F	G	H
	Stage 19		Stage 25	
α-GFP (Flognog)	I	J	K	L
Brightfield	M	N	O	P

4.6K), suggesting Flognog is translated in all binary embryos. Flognog could not be seen in the anterior CNS in the remaining 76% of embryos at stage 25 (Fig. 4.6 L). Within embryos displaying Flognog there was variation in both the level of Flognog and the location of Flognog. Some embryos displayed less or no Flognog in the anterior CNS, together with a malformation (possible loss of tissue) of the anterior CNS, but still retained Flognog in the eye and midbrain regions in a distorted neural tube. This effect may be due to a transformation, loss or alteration in tissue seen in a phenotype in the *Otx2-gal4;UAS-flognog* double transgenic embryos. These results suggest that GAL4 is sufficient to induce Flognog in double transgenic embryos by transactivation of *UAS-flognog* in *Otx2-gal4 X UAS-flognog* cross embryos.

4.2.5 Decrease of Phospho-Smad-1 in Embryos from a Cross of *Otx2-gal4 X UAS-flognog*

To determine whether BMP signalling was blocked in the *Otx2-gal4 X UAS-flognog* binary cross embryos, progeny from a cross of heterozygous *Otx2-gal4* cross to heterozygous *UAS-flognog* at tailbud stage were analysed by immunohistochemistry for α-phospho-Smad-1.

At early tailbud stage, there was a decrease of phospho-Smad-1 in the anterior neural tube and eye region (Fig. 4.7A-C). This decrease was observed in 9/39 (23%) cross embryos. Within embryos displaying a decrease, there were slight variations in the residual phospho-Smad-1 stain. The remaining embryos displayed no loss of phospho-Smad-1 staining in the anterior neural tube and eye region (Fig. 4.7 D, I-L). Also, WT controls displayed no loss of phospho-Smad-1 staining in these regions. This result suggests that GAL4 is sufficient to reduce phospho-Smad-1, and hence reduce

138
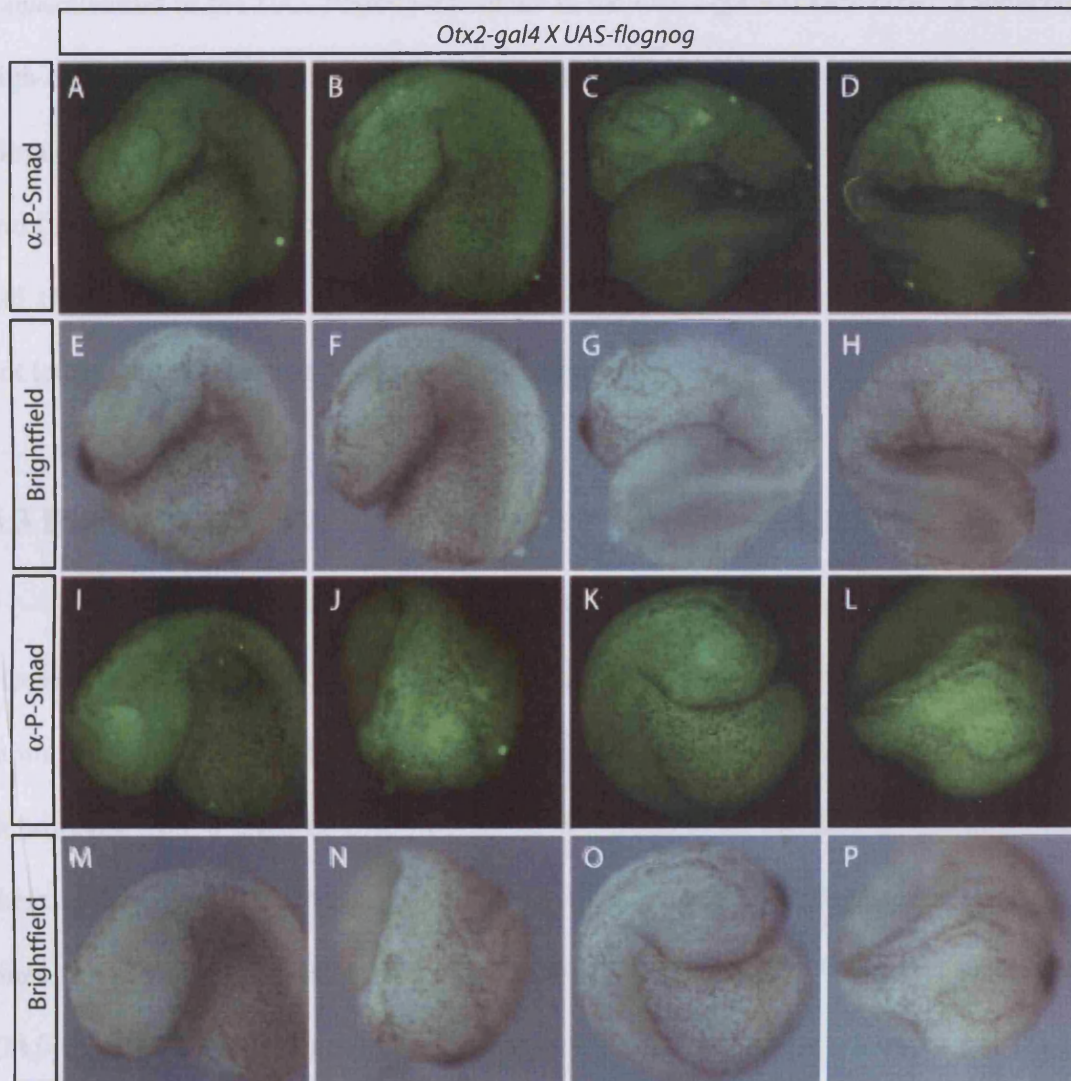
Figure 4.7

Figure 4.7 Decrease of phospho-Smad-1 in *Otx2-gal4 X UAS-flognog* cross embryos
Photos represent fluorescence microscopy or brightfield images of tailbud stage embryos from a cross of *Otx2-gal4 X UAS-flognog* analysed by whole-mount immunohistochemistry for phospho-Smad-1. A-C show a decrease in phospho-Smad-1 staining in the anterior CNS and eye in three representative (based on the reduced staining) *Otx2-gal4 X UAS-flognog* cross embryos. D, I-L show phospho-Smad-1 staining localised to the anterior CNS and eye region in other *Otx2-gal4 X UAS-flognog* cross embryos. E-H, M-P show their respective brightfield images. Images represent data collected from one experiment. There was a decrease of phospho-Smad-1 in 9/39 (23%) embryos.

BMP signalling, by transactivation of *UAS-flognog* transgene in a proportion of *Otx2-gal4 X UAS-flognog* binary cross embryos.

Thus, although tailbud stages are relatively late stages to assess the effects of the *Otx2-gal4 X UAS-flognog* cross, these results indicate that transcription via transactivation of the *UAS-flognog* transgene in the *Otx-2-gal4* binary cross is induced at high enough levels to transcribe *Flognog* transcript, as well as translate Flognog protein. Flognog is, in both *in situ* hybridisation and immunohistochemistry assays, expressed and functioning in ratios close to the expected 25% Mendelian ratio. Hence, illustrating full transgene expression and indicating that the ectopic mis-expression of Flognog is not lethal, but may cause a phenotype in the anterior CNS.

4.3 Discussion

Stable and functional *UAS-flognog* effector transgenic lines were established. Flognog fusion protein is biologically functional as an inducer of neural tissue and as an inhibitor of BMP signalling. The results indicate that, GAL4 transactivation of Flognog in embryos from a cross of *UAS-flognog* to WT leads to an increase in neural tissue in these embryos, and also in a proportion of *Otx2-gal4 X UAS-flognog* cross embryos there is a reduction of BMP signalling (Fig. 4.4A, D; Fig. 4.7). This suggests that the *UAS-flognog* line can be used in conjunction with the *Otx-2-gal4* transactivator (or any other GAL4 transactivator) to reduce BMP signalling in a spatially-restricted manner. The transgene expression and its ability to modify the BMP signalling pathway in *UAS-flognog* lines has been analysed in detail and this is outlined below.

4.3.1 Flognog Transgene Expression

4.3.1.1 Varying Transgene Expression Between *UAS-flognog* Stable Lines

All *UAS-flognog* lines tested were able to respond to GAL4 by activating transcription of their transgene (target gene) (Hartley et al., 2002; Chae et al., 2002). Table III illustrates that *UAS-flognog* T201 line can be transcribed to express *Flognog* and show phenotypic effects at a lower amount of GAL4 than the *UAS-flognog* T139 line. (It does not appear to have a more profound alteration in *Sox3* expression at either amount of GAL4, or compared to the *UAS-flognog* T139 line). Moreover, it appears that there are varying levels of *Flognog* expression between all the *UAS-flognog* stable lines (Fig. 4.3B i, ii, iii, iv). Dose effects of transgenes have being reported, and this can lead to extreme differences in transgene expression levels between lines. It has been previously demonstrated that effector lines vary in their response to GAL4 by transactivating the responsive gene to various degrees (Hartley et al., 2002). The reasons why there are varying levels of responsiveness between *UAS-flognog* lines may be due to positional effects or copy numbers of the transgene for the different founders consistent with findings from Hartley et al. (2002). The copy number may be the reason for the extremely weak expression in one *UAS-flognog* line (Fig. 4.3B iii). There may be a low transgene copy number in embryos from the T14L2 founder, whereas the *UAS-flognog* T201 may have the highest transgene copy number. However, this is unknown and a Southern blot could be carried out to determine this. The generation of the two different *UAS-flognog* lines may allow modulation of the transgene expression level of the *UAS-flognog* effector.

In addition to the differences in the expression level of target gene, there were differences in the germline transmission rate from different *UAS-flognog* founders (assayed by CAR-RFP reporter) (Table II). Transgenic founders are hemizygous for the transgene and transmit it to 50% of their resulting progeny (Kroll and Amaya, 1996; Hirsch et al., 2002). Thus, here there are founders displaying approximately 50% expressors in their progeny, whereas other founders displayed other germline transmission rates. Germline transmission rate can be altered in cases where the founders are half transgenics (indicating that the transgene had integrated after the first cell division) or when the transgene had integrated even later (i.e mosaic integration(s) in the founder), or when the founders contain multiple transgene integrations. This phenomenon has been seen before using the REMI transgenesis method (Hartley et al., 2001). Lines were expanded from founders containing ~50% expressors in their progeny because this suggested that one transgene integration had occured; this allowed easier interpretation of the subsequent genotype ratios from the binary crosses. If there were multiple integrations, the transgene expression pattern in the founder would result from the sum of expression from all of the integrations, which may then segregate in subsequent generations, making crosses difficult to interpret. Lines from founders containing multiple integrations would require, in some cases, the segregation of multiple different insertions at F2 to generate the ideal clonal transgenic populations.

4.3.1.2 Flognog Expression in the *Otx-2-gal4 X UAS-flognog* Binary Cross

The level of GAL4 expression in the *Otx2-gal4 X UAS-flognog* binary cross is sufficient to induce Flognog expression in approximately 25% of cross embryos (Fig. 4.6; section

4.2.4). This may indicate that in the binary cross there is GAL4 transactivation of *UAS-flognog* in all of the embryos containing both transactivator and effector transgenes. However, the transient assays illustrate variations in the amount of *UAS-flognog* X WT cross embryos displaying *Flognog* expression and increased neural tissue (Table III), which is not consistent, in some cases, with the *UAS-flognog* transgenic containing one transgene integration site (Fig. 4.2ii). Considering that the amount of mRNA transcribed from a transgene is usually lower than in microinjection, this raises the question why there are variations in numbers of embryos displaying Flognog transcription or neural expansion. Researchers have recorded expression analysis of UAS-effector gene expression in response to GAL4 activation by a *hsp70* (heatshock) promoter. They showed that the amount of target gene expressed depended on the duration of heatshock (HS); being lower after 5 minutes than after 15 or 30 minutes HS (Scheer et al., 2002). This indicates that the level of target gene expression depends on the duration and hence amount of GAL4 being received. This is further suggested by GAL4 mRNA injection into a *UAS-gfp* reporter transgenic line, which produced a concentration-dependent increase in GFP fluorescence, indicating that the amount of GAL4 can regulate the transgene expression levels (Chae et al., 2002). Other studies show that the level of GAL4 directed expression can be varied, but only by exploiting position effects between lines or by changing copy number (Phelps and Brand, 1998). The threshold amount of GAL4 required to activate *UAS-flognog* transcription is lower than previously reported (Hartley et al., 2002). Thus, the varying responses to GAL4 within embryos from these transient assays may indicate that GAL4 has not reached a threshold amount of GAL4 required to activate transcription in all of the transgenic

embryos. Alternatively, it may be that the transgene position effect is inhibiting expression levels (Hartley et al., 2002). However, this is not a likely explanation for the *UAS-flognog* T139 line as Flognog is induced in approximately 25% cross embryos in the binary cross (Fig. 4.6). Other reasons for the effects seen may include low sample numbers or, for the *Sox3* assay, the criteria being too stringent and therefore not including all embryos displaying an effect.

Flognog mRNA is expressed from gastrula stages (Fig 4.5 A), as expected from the timing of onset of the *Otx2* promoter prior to gastrula stages (Fig. 3.2 A). *Flognog* expression is maintained up until tailbud stages (Fig. 4.6 E, G). Flognog protein expression can be seen by immunostaining from stage 19 and is maintained until stage 25, again possibly longer (Fig. 4.6 I, K). Expression analysis of UAS-effector gene expression in response to GAL4 activation by a *hsp70* heatshock promoter showed that target gene RNA was first detectable 1.5h after HS treatment, irrespective of the duration of the HS (Scheer et al., 2002). The amount of target gene RNA remained constant or even continued to increase after 3 and 17hr. Others have found that GAL4 may direct expression with a slight time delay (Brand and Perrimon, 1993). (A GAL4 insertion at the hairy locus can direct expression in a pattern that resembles hairy, an hour or so after the onset of stripes of hairy protein expression). Thus, there may be a slight delay of an hour or less between *Otx2* promoter onset at blastula stages and expression of *Flognog* at gastrula stages (Fig. 3.2; Fig. 4.6A), similar to these previous findings. The *Flognog* transcript may also be more stable than the *Gal4* transcript as suggested by previous research (Brand and Perrimon, 1993; Scheer et al., 2002) and the

persistence of *Flognog* expression may be due to Gal4-directed transcription or due to the stability of the GFP portion of Flognog (Heim et al., 1994).

In the binary cross, *Flognog* was expressed in a spatially restricted manner in some embryos, whereas in other embryos expression was altered, in that there was either less expression or a lack of expression in specific areas. *Flognog* expression was seen in the anterior neuroectoderm, and subsequently throughout the anterior CNS including the forebrain and midbrain, and eyes (Fig. 4.6 A, C, E, G), consistent with the expected location of transactivation from the *Otx2* promoter (Fig. 3.4). Flognog protein expression at late neurula to early tailbud stages likely reflects continued transactivation of target gene expression because the *Flognog* transcript is still present. The variations seen in Flognog expression (decreased level or absence) in these areas in some Flognog-expressing embryos (putative double transgenic embryos) may be due to a phenotype in the binary cross (Fig. 4.6). However, embryos were not genotyped so it is uncertain if these embryos are double transgenic. Nevertheless, the Flognog mRNA and protein is detected in the *Otx2-gal4* X *UAS-flognog* cross at stages and tissues relevant for investigation of early neural differentiation or patterning during morphogenesis of the central nervous system.

4.3.2 Flognog Reduces BMP Signalling

The results show that Flognog can induce neural tissue, similar to Noggin in the *x-Sox3* assay (Fig. 4.4, section 4.2.2, Table III). Fig. 4.4 illustrates an expansion of *Sox3* expression domain, indicating an increase in neural progenitor cells (and a parallel shift of the neural-epidermal boundary), in the GAL4-transactivated *UAS-flognog* embryos.

Studies have shown that Noggin (and blocking BMP signalling) can induce neural tissue (Hawley et al., 1995; Huang et al., 2007; Sasai et al., 1998; Reversade et al., 2005). Over-expressing mutant forms of BMP4 or BMP7, which block the normal function of BMPs in *Xenopus*, lead to a neural fate, indicated by *N-CAM* and *Otx2* expression in animal caps (Hawley et al., 1995). Also, radial injections of BMP4 morpholino into all four blastomeres, in *Xenopus*, can lead to an expanded domain of pan-neural *Sox2* expression in the neural plate (Reversade et al., 2005). Others have shown injection of Noggin mRNA into animal blastomere lineages leads to an expansion of neural plate progenitor cells (altering the number of neural progenitor cells in the neural plate), indicated by *x-Sox2* expression (Huang et al., 2007; Sasai et al., 1998). Huang et al. (2007) suggest that Noggin signalling from *Xenopus* animal blastomere lineages promotes a neural fate in neighbouring vegetal blastomere lineages. They propose that this is due to an early signalling center prior to gastrulation present in early animal-dorsal marginal zone lineages (the BCNE center) that secretes Noggin signals to induce a neural fate in neighboring vegetal equatorial cells. Conversely, other studies have shown that a dominant negative BMP receptor (a truncated BMP receptor lacking the intracellular kinase domain) has no effect on embryo pattern when injected into dorsal blastomeres (Suzuki et al., 1994), whereas injection into the ventral marginal region results in secondary axis formation. The findings here are not consistent with the latter, but are consistent with the studies of Huang et al. (2007) indicating cell fate changes prior to gastrulation that predispose a neural fate. This suggests that the mechanism for the neural expansion is that GAL4-activation and over-expression of Flognog (via its effects on BMP signalling) from animal blastomere lineages promotes a neural fate in

neighbouring vegetal equatorial blastomere lineages. There are most likely other signalling pathways that are leading to the expansion of neural tissue as well (Pera et al., 2003). These results may indicate that BMP 2 and 4 (and GDF6) signalling (the BMP/Smad-1 pathway) are blocked by GAL4 transactivation of the *UAS-flognog* transgene in *UAS-flognog* lines.

Western analysis showed a loss of phosphorylation of Smad-1 in GAL4 transactivated embryos from a cross of *UAS-flognog X WT* (Fig. 4.5C). The embryos displaying a loss of Smad-1 phosphorylation are assumed to correspond to the transgenic embryos, although this has not been demonstrated directly. Blocking different components of the BMP signalling pathway individually or in different combinations can decrease phospho-Smad-1 signal, and hence decrease or block BMP signalling (Reversade et al., 2005; Kuroda et al., 2005; Faure et al., 2000). Blocking BMP signalling via a combination of BMP2/4/7 morpholinos (MO) injected into the marginal zone of all four blastomeres at the 4-cell stage results in a decrease in phospho-Smad-1 signal by western analysis, also, co-injection of MO for BMP4/7 or BMP4 alone leads to decrease in phospho-Smad-1 signal, although to a lesser degree (Reversade et al., 2005; Kuroda et al., 2005). Likewise, expression of a dominant negative BMP type I receptor in animal and vegetal poles of two-cell stage embryos decreases phospho-Smad-1 signal (Faure et al., 2000). Moreover, cross-talk from other signalling pathways is involved in modulating the response of the BMP signalling pathway. FGF8 and IGF2 can induce MAPK to phosphorylate Smad-1 in its linker region, inhibiting Smad-1 activity and decreasing BMP signalling (Pera et al., 2003). Thus, as noggin is known to block BMP signalling (Faure et al., 2000), consistent with

these other studies, the reason for a loss in phospho-Smad-1 signal could be due to Flognog activity, and could indicate that there is a reduction in BMP /Smad-1 signalling in these embryos. However, there could be interferences from other signalling pathways, such as MAPK activity or the degradation of Smad-1 by the ubiquitin ligase, Smurf1 (Alexandrova and Thomsen, 2006), which act to inhibit Smad-1 activity. MAPK activity inhibits Smad-1 activity independent of C-terminal phosphorylation of Smad-1, so this is not a likely explanation for alterations in phospho-Smad-1 signal (Pera et al., 2003). Another observation was that in the embryos displaying a loss of Smad-1 phosphorylation, there was still some residual phospho-Smad-1 signal. A faint phospho-Smad-1 signal is also observed in BMP2/4/7 MO knockdown embryos (Reversade et al., 2005). This suggests that not all BMP/ Smad-1 signalling is blocked. The residual phospho-Smad-1 signal could be due to other BMPs activating the BMP/Smad-1 pathway or could indicate that insufficient levels of Flognog (expression) were achieved to block BMP/Smad1 signalling. This raises the question of whether all BMP/Smad-1 signalling will be knocked down enough in the binary cross to produce a phenotype. Although other reasons for the alterations, such as variations in levels of phospho-Smad-1 signal cannot be ruled out, this data could indicate a reduction in BMP signalling in the UAS-flognog embryos, which is consistent with the increase in neural tissue seen in the UAS-flognog embryos by the Sox3 assay (Fig. 4.4A).

There was a reduction of phospho-Smad-1 staining in the anterior neural tube and eye region in approximately 25% of Otx2-gal4 X UAS-flognog cross embryos (Fig. 4.7). There was a slight variation in reduction of the phospho-Smad-1 staining, with some embryos displaying a loss throughout the embryo, whereas other embryos

displaying the main loss only in the anterior of the embryo. The ratio of loss of phospho-Smad-1 signal is consistent with it occuring in all the presumed double transgenic embryos. Blocking BMP signalling can abolish phospho-Smad-1 staining in wholemount embryos (Kurata et al., 2001; Faure et al., 2000). Co-injection of dominant negative-BMPRIA receptor and BMP4 mRNA into the animal pole blocked phospho-Smad1 staining in the animal hemisphere in wholemount stage 8.5 embryos, whereas in BMP4 mRNA alone injected embryos showed specific nuclear staining in the animal hemisphere (Kurata et al., 2001). Moreover, *Xenopus* embryos injected marginally with Noggin mRNA (100pg/embryo) or BMP4 mRNA (500pg/embryo), abolished endogenous phosphorylation of Smad-1 in noggin-injected embryos, whereas the strength and distribution of phospho-Smad-1 signal was enhanced by ectopic activation of the BMP signalling pathway, in BMP4 injections (Faure et al., 2000). Thus, although *Xenopus* is not the best system to use for immunohistochemistry due to its lack of transparency, as BMP inhibition is known to cause a decrease in phospho-Smad-1 (Kurata et al., 2001; Faure et al., 2000), the results here are consistent with a decrease of phospho-Smad-1 and hence a block of BMP signalling, *in vivo*, in binary cross embryos. However, alternatively, pigmentation may obscure visualisation of fluorescence, and thus may be the reason or contribute to the reason for the reduction in phospho-Smad-1 stain. The possible reasons for variations in the loss of stain may be due to variations in transgene expression levels and /or a phenotype in the double transgenic embryos, or again, a possible lack of visualisation due to pigmentation. It would be interesting to carry out a time-course immunohistochemistry for phospho-

Smad-1 to determine exactly when phospho-Smad-1 staining is reduced in the binary cross.

Combined evidence of increase in neural tissue and a decrease of Smad-1 activation indicate that Flognog from the *UAS-flognog* line is functional at blocking (or knocking down) BMP/ Smad-1 signalling induced by BMP2, BMP4 and GDF6. Therefore, the *UAS-flognog* can be used in combination with a GAL4 transactivator to reduce BMP signalling in GAL4 expressing cells.

CHAPTER 5: ANALYSIS OF NEURAL DEVELOPMENT IN BINARY CROSSES

5.1 Aim and Introduction

It remains unclear whether BMP signalling is required in a concentration-gradient-dependent manner from the dorsal signalling centre for patterning of the dorsal telencephalon. Thus, the aims here were to set up binary crosses to determine if there were any alterations in neural patterning, and subsequently to set up a system to determine if this gradient of BMP signalling is required for patterning of the dorsal telencephalon. This required tools that could manipulate gene expression in neural tissue, ideally within the telencephalon, after the initial induction of neural tissue, and during formation of the secondary source of BMPs. Previous chapters have characterised and established the *Otx2-gal4* transactivator line and demonstrated that it can be used to mis-express target gene in the forebrain. Also, a *UAS-flognog* effector line has been characterised that can block BMP signalling in a tissue-specific manner. Although the *Otx2-gal4* transactivator cannot be used to assess the effects of the late BMP signalling source on dorsal telencephalic patterning, due to its early onset of expression, the *Otx-2-gal4* cross to *UAS-flognog* provided a first system to assess whether there were any forebrain patterning alterations by loss of BMP signalling in the anterior neuroectoderm. In a second approach, a *Pax6-galPR* line was established that can be used to manipulate the expression of a target gene in the anterior neural plate and prospective dorsal forebrain in a hormone-inducible manner. This hormone-inducible transactivator allows the late signalling source of BMPs to be manipulated, and thus is a

more direct way to answer whether a late BMP signalling source acts to pattern the dorsal telencephalon.

5.1.1 Dorsal (and Ventral) Markers Confer Identity and Pattern to the Dorsal (and Ventral) Telencephalon

In *Xenopus*, the dorso-ventral axis becomes apparent from neural tube closure (around stage 21, Nieuwkoop and Faber, 1994) (Eagleson and Harris, 1990, 1995). Hence stages from this time are useful to assess dorsal patterning.

Genes conferring positional information are expressed in spatially and temporally restricted patterns throughout the nervous system and may be used as stable markers of regional identity. *Pax6* is a highly conserved transcription factor and is essential for the development of the alar plate of the forebrain (e.g. cerebral cortex) (as well as eye and other regions of the CNS) (Schmahl et al., 1993; Stoykova et al., 1996). *Pax6* can be used as a dorsal or lateral marker of the prosencephalon/ prosencephalic neural plate. In *Xenopus*, *Pax6* expression is initiated at the end of gastrulation/ beginning of neurulation in two lateral stripes, one on either side of the midline, and in a crescent at the anterior of the embryo (Hirsch and Harris, 1996). Expression continues in the anterior neural plate and the neural ridge, spanning most of the neuroectoderm. *Pax6* is expressed in lateral (alar) regions of the prosecencephalic neural plate, and is excluded from the medial region. The two stripes of expression in the posterior neural plate give rise to the ventral-lateral spinal cord and hindbrain. At stage 23, *Pax-6* expression can be seen in the developing telencephalon and diencephalon. Transverse sections through the brain show that *Pax6* expression occurs throughout the dorso-

ventral extent of the telencephalon, but becomes restricted in the diencephalon where it is confined to presumptive dorsal thalamus. There is a gradient of *Pax6* expression throughout the dorsal pallium in a [caudo-medial] [low] to [rostro-lateral] [high] gradient, with highest levels present in the progenitor cells of the ventral pallium. At stage 32 and 35, *Pax6* is expressed in the pallium, in the VP (ventral pallium) and LP (lateral pallium) in the SVZ (sub-ventricular zone) and MZ (mantle zone), whereas in the DP (dorsal pallium) and MP (medial pallium), *Pax6* is expressed in the VZ (ventricular zone)/SVZ, with an area negative for Pax6 expression in the MZ of the DP. In the diencephalon *Pax6* is expressed in P3 zone (Bachy et al., 2002). *Pax6* is not expressed in the dorsal midline itself, possibly due to the high levels of BMPs expressed there (Timmer et al., 2002). There is evidence to support *Pax6* in forebrain regionalisation, where it plays a role in specifying dorsal telencephalic character, as well as subdivision or patterning (cell fate) decisions of the dorsal telencephalon (Toresson et al., 2000; Yun et al., 2001). *Pax6* mutation leads to a down-regulation or dorsal retraction of the expression of pallial markers (*Emx1, Ngn1 and Ngn2, Tbr1, Tbr2*) and the dorsal expansion of subpallial gene expression (*Gsh2, Mash1, Dlx1, Dlx2, Vax1, Six3*) into the pallium. The ventral pallium is partially re-specified to express some molecular characteristics of the dorsal LGE. The expression of *Pax6* in the lateral alar prosencephalic plate and subsequently throughout the developing dorsal forebrain, means that it is co-expressed at least in part with the *Otx2* promoter driven expression. Furthermore, since *Pax6* expression commences after gastrulation (i.e. after the onset of *Otx2-gal4* transactivator driven expression) and the fact that *Pax6* is a key regulator of forebrain regionalization,

Pax6 could be a good candidate marker for analysis of lateral (dorsal) patterning in the *Otx2-ga4 X UAS-flognog* cross.

X-dll3, a ventral forebrain marker (which is a conserved gene, orthologous to *Dlx5* in mouse (Liu et al., 1997)), is expressed from open neural plate stages (stage 16) (Papalopulu and Kintner, 1993). At stage 16, *X-dll3* expression is evident in the anterior transverse rim or border of the neural plate and in the cement gland. At initial neural tube stage, Stage 19 (Nieuwkoop and Faber, 1994), *X-dll3* is expressed in the anterior neural plate boundary in regions fated to become part of the ventral telencephalon, ventral diencephalic structures (which are mostly derived from the alar plate) and olfactory placodes (Eagleson and Harris, 1990). At stage 32 and 35, *x-dll3* has been shown to mark the future pallidum arisen from the sub-pallium (in the SVZ and the MZ), and forms a sharp boundary, at the pallio-subpallial boundary. Its expression also marks the diencephalon (Bachy et al., 2002). The late onset of expression, its distinct expression profile, and the overlap with expression of the *Otx2* promoter suggests that this marker can also be used to assess neural plate/ neural plate boundary (ventral forebrain) regional identity and position.

5.1.2 Timing of BMP Signalling Alteration on Dorsal (and Ventral) Forebrain Patterning

Gain- or loss-of-function studies, increasing or decreasing BMP signalling at various stages throughout nervous system development have been found to cause changes in expression of either dorsal (or ventral) forebrain or dorsal nervous system markers

(Hartley et al., 2001; Hartley et al., 2002; Golden et al., 1999; Hanel et al., 2006). Further to these molecular alterations, there are malformations of the brain or head.

Mis-expressing BMP4 in the anterior neuroectoderm from the *Xenopus Pax6* promoter or by BMP4 mRNA injections targeted to the anterior neuroectoderm in *Xenopus* have been reported to cause different effects on expression of the ventral forebrain marker, *X-dll3* (Hartley et al., 2001). Early BMP4 signalling inhibited the expression of this gene in the anterior region of the embryo, whereas late BMP signalling after gastrulation resulted in a significant increase in expression. As for the dorsal marker, *Pax6*, an increase in BMP signalling in the anterior neuroectoderm resulted in a reduced level of expression and a reduced extent of expression domain of *Pax6*, whereas mis-expressing BMP signalling after gastrulation from the *Pax6* promoter resulted in a slight down-regulation of *Pax6* expression. Thus, there are different responses of these regional markers (implying different competence of the neural tissue) to BMP signalling at different stages of neural development and/or different responses of these regional markers due to the levels of BMP misexpression the embryo received. In the same study morphological alterations were also observed. In the *Pax6-BMP4* transgenic embryos there was a suppression of anterior brain and eye formation in 90.7% of tailbud stage embryos, compared to in 36% of GFP expressing *Pax6-GFP* transgenic embryos. Also, by BMP4 mRNA injection into the anterior neuroectoderm, there was a reduced or entirely absent neural plate. This indicates a similar response of the tissue from these two methods, and that increasing BMP signalling throughout early neural development results in a decreased neural development.

In another cross, mis-expression of a downstream target of BMP signalling, vent-2, by the *Pax6* promoter resulted in a microcephalic phenotype, as well as a severe ventralization phenotype "bauchstuck" by stage 28 (Hartley et al., 2002). The dorsal axis of the embryos was shortened and all head structures were absent. There were variations in the severity of the phenotype between lines. When the same *Pax6-gal4* line was crossed to two different *UAS-vent-2* lines, a cross from one line produced the severe ventralized phenotype, whereas the other line produced the microcephalic phenotype. These results illustrate the variability in phenotypes resulting from the use of different transgenic lines with different transgene expression levels, hence presumably different levels of BMP signalling. Nevertheless, these results show severe malformations of anterior neural structures as a result of increases in BMP signalling after gastrulation.

Furthermore, in the study by Golden et al. (1999), mentioned before, the alterations in dorsal and ventral forebrain markers, as well as the brain and head malformations in response to a late source of BMPs, indicate that, at late developmental stages BMPs are sufficient to specify dorsal forebrain development, in part, as indicated by *Wnt4* expression alterations, and that the nervous system is still competent to respond to dorsal/ ventral patterning signals. Furthermore, BMPs can cause severe brain malformations at these later stages of neural development. However, there was only a reduced *Pax6* expression in a reduced brain size, and *Pax6* expression was maintained. It is interesting that there was a no alteration in level of *Pax6* expression, consistent with only a slight alteration of *Pax6* at late stages of neural development in the studies of Hartley et al. (2001).

In loss of function studies, using a GDF6 morpholino to reduce BMP signalling, there was an overall reduction in size of embryos by neurula stage (stage 20, Nieuwkoop and Faber, 1994), and a reduction in size and shape of the *Pax6* expression domain in the retina and forebrain. By stage 27 there was a reduced or altered shape of *Pax6* expression in the retina and a marked loss of *Pax6* expression in the forebrain (Hanel et al., 2006). Thus, implicating a loss of BMP signalling from early stages of neural development in retina and forebrain specification at later stages.

These studies have assessed the effects of BMP signalling alterations at different stages in forebrain development and have indicated that this can result in different changes in expression of forebrain markers depending on the stage of development.

5.1.3 *Pax6* Promoter and Hormone-inducible GalPR as a Tool to Investigate Dorsal Telencephalon Patterning

The inducible GalPR system allows both temporal and spatial control of gene expression. A chimeric regulator (pGL-VP) comprising the ligand-binding domain of human progesterone receptor hPRB891 fused to the yeast transcriptional activator GAL4 DNA-binding domain and the herpes simplex virus protein VP16 activation domain can activate target genes in response to RU486 (Wang et al., 1994).

The *Xenopus Pax-6* promoter drives expression of a GFP reporter in a similar but not identical domain to that of endogenous *Pax-6* (Hartley et al., 2001). Unlike endogenous *Pax6* expression (Liu et al., 1997), the transgene driven expression does not down-regulate in the medial domain of its anterior-most expression domain during neurulation (Hartley et al., 2001). Also, there is a higher level of transgene expression

in the telencephalon than endogenous *Pax6* expression, and there is a lack of strong transgene expression in the diencephalon and lens. This suggests that separate elements missing from the 3.6kb genomic promoter fragment are required for these expression domains. Upon analysis of the *Pax6* promoter driven expression in *X. tropicalis*, it was established that *Pax6-GFP* transgene expression was similar to the *Pax6* expression profile (Hirsch et al., 2002). Therefore, there are some differences in *Pax6* promoter driven expression compared to that of the endogenous *Pax6* gene (Hirsch and Harris, 1996). However, the strong *Pax6* transgene driven expression in the telencephalon, that is presumably outside the dorsal midline (containing high levels of BMPs), indicates that the *Pax6* promoter in conjunction with other transgene and transgenic tools may be used to a investigate the effects of a BMP signalling gradient on dorsal telencephalon patterning.

A binary system using *Pax6-galPR* would be more useful than the *Otx-2* promoter-based approach because its inactivity in the absence of inducer may avoid the early effects associated with alterations of BMP signalling during gastrulation. Moreover, the *Pax6* promoter has restricted expression in the dorsal central nervous system in the rostral CNS, allowing more targeted mis-expression to the dorsal telencephalon (whereas *Otx2* promoter targets expression to ventral domains as well). *Pax6-galPR* induction at late stages of telencephalon development could be used to dissect out whether BMP signalling is required for specification of dorsal fate and pattern formation in the dorsal telencephalon. Another advantage of using the GalPR is that it contains the VP16 transcriptional activation domain, which is capable of driving higher levels of transcription compared to GAL4 alone.

5.2 Results

5.2.1 Open Neural Tube Defects in *Otx2-gal4* X *UAS-flognog* Cross Embryos

To determine whether there was any morphological effects caused by inhibiting BMP signalling in the prospective anterior CNS (anterior neuroectoderm), embryos from an *Otx2-gal4* X *UAS-flognog* cross (both heterozygotes) were analysed over early development until tadpole stages for abnormalities. All frog crosses were heterozygous, unless otherwise stated.

At neural tube stage a partial open neural tube was observed in *Otx2-gal4* X *UAS-flognog* cross embryos, whereas sibling control embryos did not display these alterations (Fig. 5.1A, B alteration, C control). *Otx2-gal4* X *UAS-gfp* cross (*n*=95) and WT (*n*=54) (Fig. 5.1D) control embryos did not display these alterations either. The altered morphology was observed in 68/349 (19%) *Otx2-gal4* X *UAS-flognog* cross embryos. Within these embryos displaying the phenotype there were variations in the severity of the open neural tube and embryo size. At stage 19 there was a failure of the neural tube to fully close (or a partial closure), with some embryos being more open than others, as well as some embryos being smaller compared to others. The opening focused around the prospective midbrain area of the anterior CNS, with the propective anterior CNS area of the neural tube closed. This data could suggest that BMP signalling in the anterior neuroectoderm, including neural plate, is required for proper closure of the neural tube in a proportion of *Otx2-gal4* X *UAS-flognog* cross embryos.

Double trangenics were viable at least up until stage 42. A proportion of these had small heads with eyes closer together (16/115 (14%)). Some *Otx2-gal4;UAS-flognog* double transgenic tadpoles displayed an extended pigmented retinal epithelium

Stage 19

Otx2-gal4 X UAS-flognog (or WT)

WT
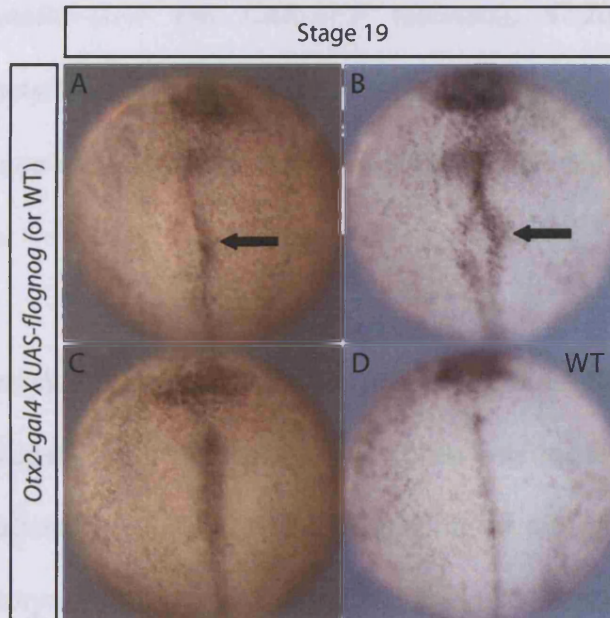
Figure 5.1 Partial open neural tube in *Otx2-gal4 X UAS-flognog* cross embryos

Images represent fixed stage 19 embryos from a cross of *Otx2-gal4 X UAS-flognog* (A-C) or a stage 19 WT embryo (D). A, B illustrate embryos with a partially open neural tube, with the anterior of the neural tube closed. C shows a sibling embryo with a normal closed neural tube. D shows a WT embryo with a normal closed neural tube. *n*=349

(making the eyes appear as an hourglass shape). This could result from an interference

of the bi-lateralisation signal from the (ventral) midline structures of the neural plate and

tube (Reichenbach et al., 1997). The transgenic identity of embryos from the *Otx2-gal4*

X UAS-flognog cross were observed as, 65/200 (32.5%) double transgenics (containing

both the γ-*crystallin-GFP* and *CAR-RFP* reporters), 47/200 (23.5%) *Otx2-gal4*

(containing γ-*crystallin-GFP*), 43/200 (21.5%) *UAS-flognog* (containing *CAR-RFP*) and

45/200 (22.5%) non-transgenic, and did not significantly (P>0.05, P=0.10) deviate from

Mendelian ratios.

5.2.2 *Pax6* Expression is Disrupted in the *Otx2-gal4 X UAS-flognog* Cross Embryos

To further investigate the phenotype, the next step was to determine whether dorsal

patterning was altered by a loss of BMP signalling in the prospective anterior CNS

(including in embryos displaying the variably altered open neural tube). Embryos were

therefore analysed for the dorsal neural marker, *Pax6*, by whole-mount *in situ*

hybridisation. Embryos were analysed both in whole-mount and by vibratome

sectioning to reveal the dorso-ventral (transverse) axis within the (prospective) forebrain

tissue. Neural tube and tailbud stages were taken for analysis, as these stages occur

during and after dorsal patterning has occured, and the dorso-ventral axis is established

(Eagleson and Harris, 1990). Furthermore, at stage 23 to late tailbud, the dorsal

telencephalon is already formed and the brain sub-divisions are more distinct by

morphology, as well as gene expression.

At stage 19, in whole-mount embryos, there was an altered shape, a reduced size

of domain and a closer together or fused anterior domain of *Pax6* expression in 8/54

(15%) *Otx2-gal4* X *UAS-flognog* cross embryos (Fig. 5.2I-K, sibling displaying no alterations M-O). Although WT embryos did not display any alterations in *Pax6* expression (Fig. 5.2A-H), a proportion of *Otx2-gal4* X *UAS-gfp* cross embryos did display a reduced size of *Pax6* expression domain. The same alterations in *Pax6* expression were observed in *Otx2-gal4* X *UAS-flognogT201* cross embryos (Fig. 5.2K, sibling control displaying no alteration O) as in the *Otx2-gal4* X *UAS-flognogT139* cross embryos, defending that the effects are consistent with the *Otx2-gal4* X *UAS-flognog* cross. There were variations in the size of the expression domains. The reduced expression domain was more pronounced in the *Otx2-gal4* X *UAS-flognog* T201 cross, possibly reflecting a higher level of *Flognog* transgene expression in this cross. This is consistent with the varying severity of phenotypes observed from binary crosses employing two different effector lines in previous studies (Hartley et al., 2002). At tailbud stage alterations in *Pax6* expression were observed in 4/111 (~4%) of *Otx2-gal4* X *UAS-flognog* cross embryos (Fig. 5.2L, sibling embryo displaying no alteration P). In whole-mount embryo analysis, there appeared to be a reduced and/or altered shape of *Pax6* expression domain in the eye, and transverse sections revealed that there was reduced *Pax6* expression in the eye. There was normal *Pax6* expression throughout the forebrain and eye in an *Otx2-gal4* sibling single transgenic control embryo. This data could suggest that BMP signalling in the anterior neuroectoderm, including neural plate, is required for correct specification and patterning of the anterior neural tube in a proportion of *Otx2-gal4* X *UAS-flognog* cross embryos. However, there are transgene-mediated alterations in *Pax6* expression as well, which may contribute to the effects seen.

Figure 5.2 *Pax6* **is Expressed in the Developing Forebrain of WT Embryos and in an Altered Shape in Whole-mount** *Otx2-gal4 X UAS-flognog* **Cross Embryos**

Images show embryos analysed by whole-mount *in situ* for *Pax6* expression. Images are anterior views. *Pax6* expression in WT embryos (A-H). *Pax6* is expressed in two lateral stripes one on either side of the midline, and in a crescent at the anterior of the embryo at the end of gastrulation/ beginning of neurulation (stage 12.5) (A). *Pax-6* is expressed in the anterior neural plate in cells that will form the telencephalon and parts of the diencephalon, spanning most of the neuroectoderm at stage 14 (B), stage 18 (C), stage 19 (D), stage 19 to 20 (E, F) and stage 21 (G). *Pax-6* is also expressed in the posterior neural plate in two stripes that give rise to the ventral-lateral spinal cord and hindbrain. At stage 23, *Pax-6* expression can be seen in the developing telencephalon and diencephalon, between the prominent domains of expression in the eye (H). There is a characteristic lack of expression in the midbrain. I-P illustrates *Otx2-gal4 X UAS-flognog* cross embryos analysed by *in situ* hybridisation for *Pax6*. I-K illustrate embryos displaying the altered shape and fusion of expression domain in stage 19 *Otx2-gal4 X UAS-flognog* cross embryos. J represents an embryo with a partially closed neural tube. I, J show a wider *Pax6* expression domain in the neural tube in comparison to sibling controls M, N and WT control D. M-O illustrates normal *Pax6* expression in the prospective dorsal forebrain and eyes. L represents an altered expression domain of *Pax6* in a tailbud stage *Otx2-gal4 X UAS-flognog* cross embryo. P represents normal expression of *Pax6* in the eyes and forebrain in a sibling *Otx2-gal4 X UAS-flognog* cross embryo.

Figure 5.2
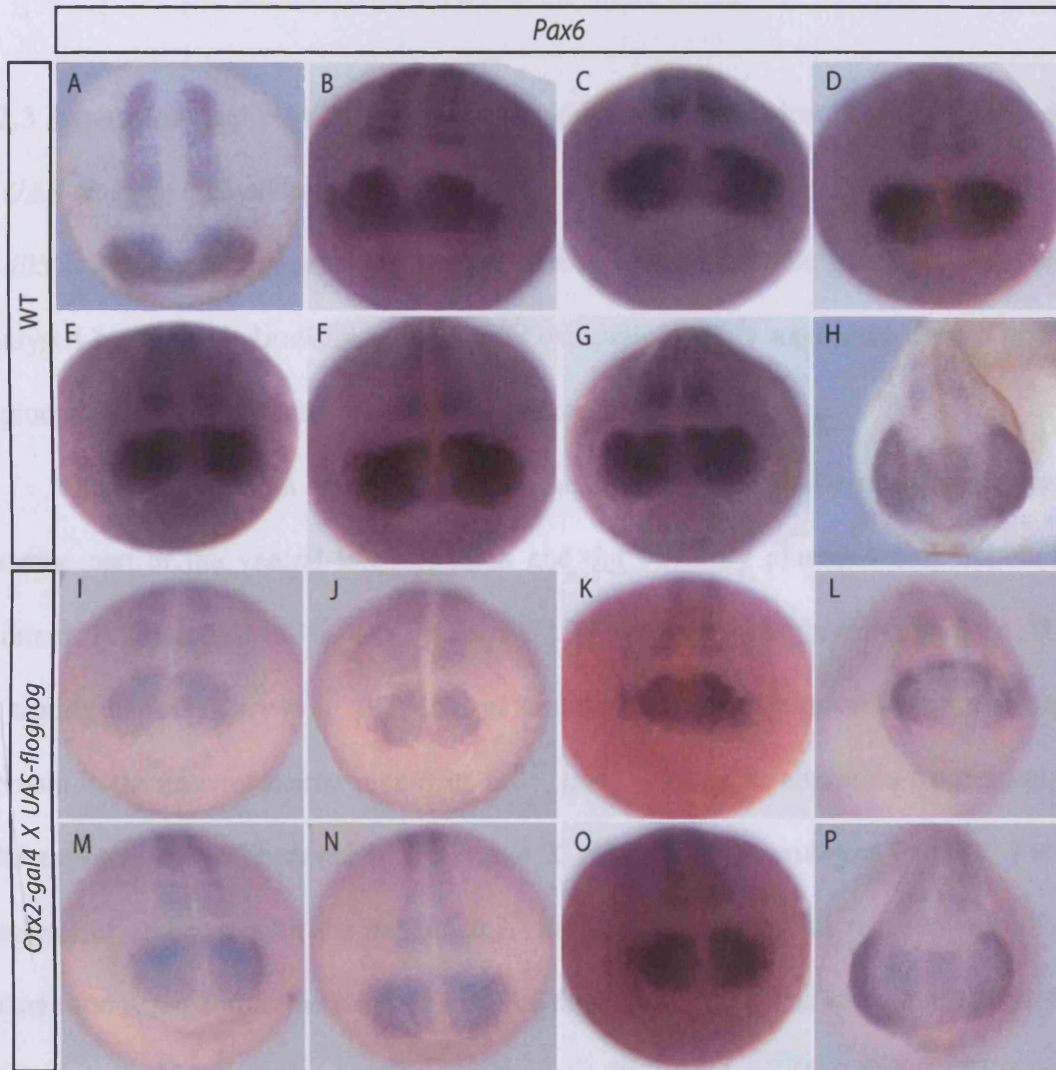
The alterations in *Pax6* expression suggest that there may be transformation in the identity of other anterior CNS tissue. To understand the alterations in more detail, I next asked if there were any other alterations in anterior regionalised neural gene markers in the *Otx2-gal4 X UAS-flognog* mutant embryos.

The alterations in *Pax6* expression suggest that there may be transformation in the identity of other anterior CNS tissue. To understand the alterations in more detail, I next asked if there were any other alterations in another regionalised neural gene marker in the *Otx2-gal4* X *UAS-flognog* cross embryos.

5.2.3 Expansion and Fusion of Ventral Telencephalon Marker X-*dll3* in *Otx2-gal4* X *UAS-flognog* Cross Embryos

X-*dll3* is a ventral telencephalon marker across vertebrates. Stage 19 embryos were analysed by *in situ* hybridisation for X-*dll3* expression. This stage was chosen because regionalized patterning markers are more distinct in neurulae.

X-*dll3* expression was expanded in the anterior neural tube in an area fated to become part of the ventral telencephalon and the olfactory placodes (Papalopulu and Kintner, 1993) in 6/45 (13%) *Otx2-gal4* X *UAS-flognog* cross embryos (Fig. 5.3A, B (C-E sibling controls), n=45). WT control embryos displayed expression in a compact domain in the anterior neural tube (Fig. 5.3F, n=45). Also, no alteration from normal x-*dll3* expression was observed in *Otx2-gal4* X *UAS-gfp* cross embryos (n=11). Further analysis of embryos with the expanded X-*dll3* expression domain revealed that X-*dll3* expression fused across the anterior ridge of the neural tube. The same alterations were observed in 11/72 (15%) embryos from an *Otx2-gal4* X *UAS-flognogT201* cross confirming the effects are specific to the *Otx2-gal4* X *UAS-flognog* cross. This result could suggest that BMP signalling in the anterior neuroectoderm, including neural plate, is required for correct specification and patterning of prospective ventral forebrain tissue.
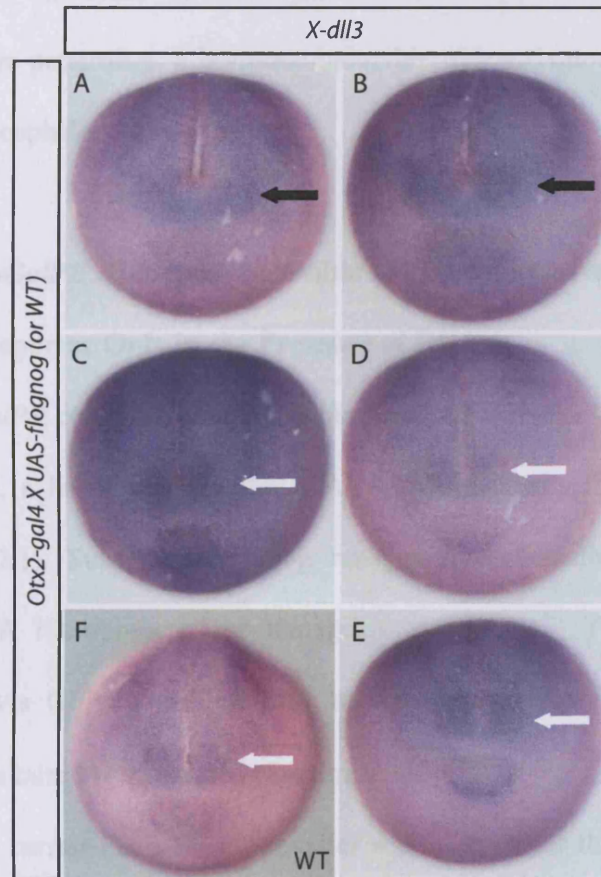
Figure 5.3 Expansion of *X-dll3* expression domain, which marks the prospective ventral telencephalon and olfactory placodes, in *Otx2-gal4 X UAS-flognog* cross embryos

Images display anterior views of *Otx2-gal4 X UAS-flognog* cross embryos analysed by whole-mount *in situ* hybridisation for *X-dll3* (at stage 19). A,B there is an expansion in the domain of expression of *X-dll3* in the anterior neural tube (see black arrows) in an area fated to become the olfactory placodes and part of the ventral telencephalon in *Otx2-gal4 X UAS-flognog* cross embryos. C-F displaying compact *X-dll3* expression in the anterior neural tube (see white arrows) in *Otx2-gal4 X UAS-flognog* sibling control embryos (C, D, E) or WT embryos (F). *n*=45.

The resulting morphological and molecular alterations in the *Otx2-gal4 X UAS-flognog* cross may be due to early roles of BMP signalling during gastrulation and/or neurulation. In order to investigate the later roles of BMP signalling on dorsal telencephalic patterning, a hormone-inducible transactivator, which is expressed in the dorsal telencephalon, was generated.

5.2.4 *Pax6-GalPR*, Hormone-inducible GAL4 transactivator Activates GFP from a *UAS-gfp* Reporter Only in the Presence of RU486

A *Pax6-GalPR* construct containing the *Xenopus Pax-6* promoter (Hartley at al., 2001) and GalPR, a hormone–inducible GAL4 (Wang et al., 1994) was made (Fig. 5.4O) (Section 2.2.1). Subsequently, using transgenesis as described by Hirsch et al. (2002), a *Pax6-GalPR* F0 transactivator transgenic was made. The transgenic founder was identified via GFP in the lens due to the reporter γ-*crystallin-gfp*. The transgenic tadpole was raised to adulthood (Section 2.1).

The carrier *Pax6-GalPR* founder was assayed for the germline transmission and transactivation potential of *Pax6-galPR* line by crossing to a homozygous *UAS-gfp* reporter transgenic line. The embryos from this cross were incubated in either 0.5μM RU486 or DMSO control from the 4 to 8 cell stage. Embryos were raised in individual well dishes to monitor each embryo over time, and assess the transgenic identity at tadpole stages. At early neurula stages, GFP was detected via fluorescence microscopy in the anterior neural plate in a *Pax6-GalPR X UAS-gfp* cross embryo incubated in 0.5μM RU486 (Fig. 5.4A), that at later stages contained ECFP in its lens from the γ-*crystallin* promoter in the transactivator transgene and RFP in the somites from the *CAR*

Figure 5.4 *Pax6-GalPR* **Hormone-inducible GAL4 Transactivator Activates GFP from a** *UAS-gfp* **Reporter Only in the Presence of RU486**
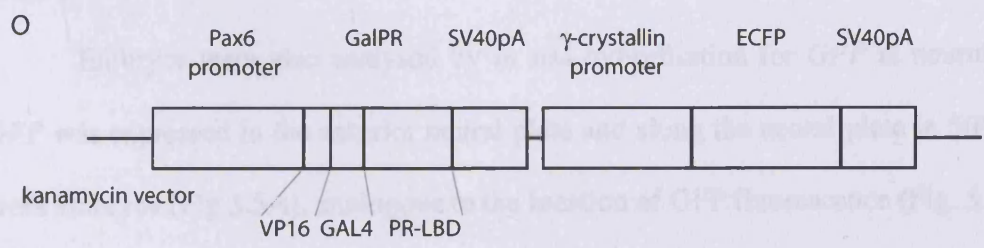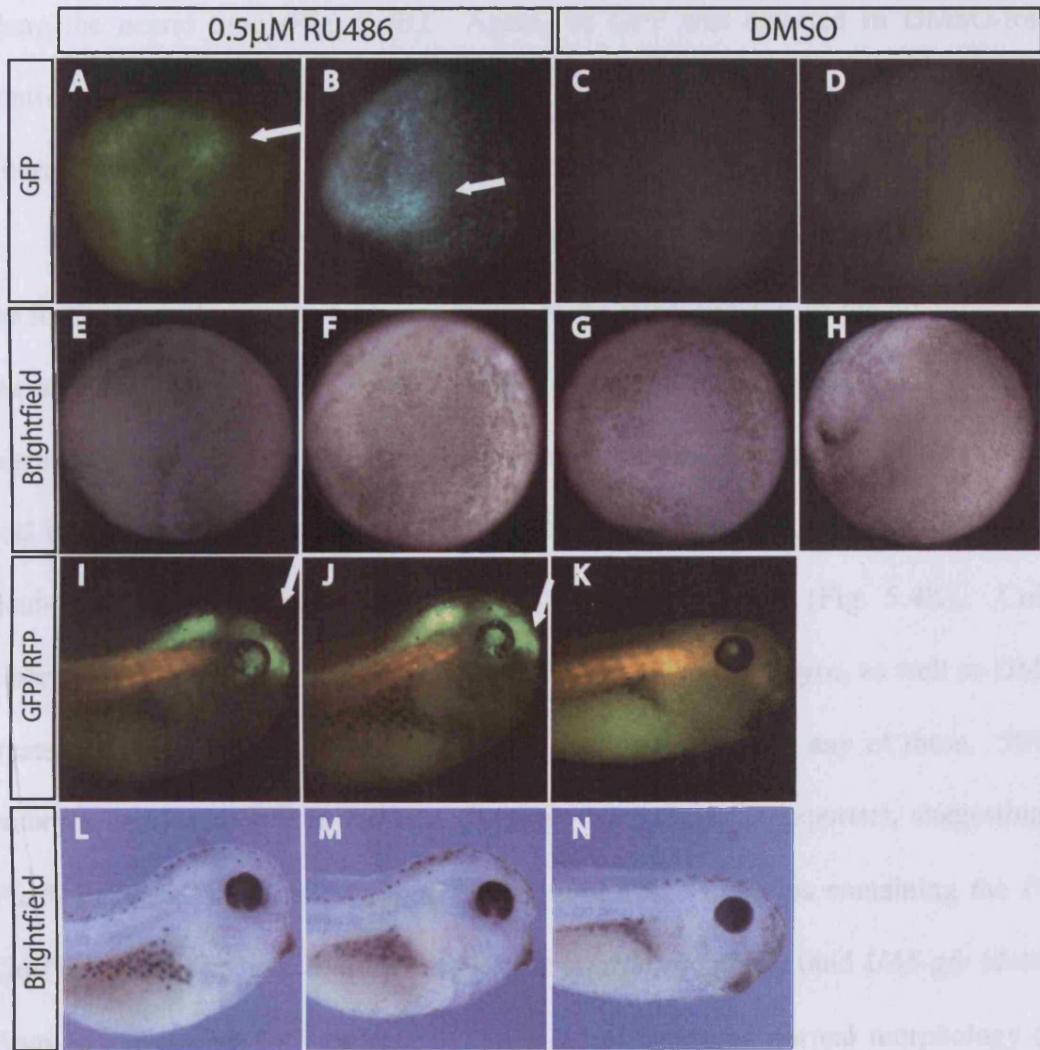
All images are either GFP fluorescence microscopy (A-D, I-K) or brightfield (E-H, I-N) of embryos from a cross of *Pax6-GalPR* transgenic founder X *UAS-gfp* reporter frog, incubated in either 0.5μM RU486 (A, B, E, F, I, J, L & M) or DMSO alone (C, D, G, H, K & N). GFP is throughout the anterior neural plate and along the posterior neural plate at stage 13 in double transgenic embryos (expressing RFP in their somites and GFP in their lens) incubated in RU486 (A, E, I & L). No GFP is detected in stage 13 double transgenic embryos incubated in DMSO alone (C, G, K & N). At stage 17, GFP is detected in anterior neural tube, and along the dorsal neural tube in double transgenic embryos incubated in RU486 (B, F, J & M). No GFP is detected in stage 17 double transgenic embryos incubated in DMSO alone (D, H, K & N). At tadpole stage, GFP can be seen in the forebrain (white arrow), hindbrain and retina in double transgenic embryos incubated in RU486 (I, L, J & M). No GFP is detected in double transgenic embryos incubated in DMSO alone (K & N). A, E, I, L represents an RU486-treated embryo. B, F, J, M represents another RU486-treated embryo. C-D, G-H, K, N represents the DMSO control embryo. O represents a diagram of the Pax6-GalPR construct used to make the *Pax6-GalPR* F0. It contains GalPR, which is a chimera consisting of the ligand binding domain of the human progesterone receptor hPRB891 (PR-LBD), the DNA-binding domain of yeast activator GAL4, and the activation domain of herpes simplex virus VP16, downstream from the *Xenopus Pax6* promoter (Hartley et al., 2001), and upstream of SV40pA. Also, there is a secondary cassette, the reporter cassette, which contains GFP downstream of the γ-*crystallin* promoter, and upstream of the SV40pA.

Figure 5.4

	0.5µM RU486		DMSO	
GFP	A	B	C	D
Brightfield	E	F	G	H
GFP/RFP	I	J	K	
Brightfield	L	M	N	

O

Pax6 promoter GalPR SV40pA γ-crystallin promoter ECFP SV40pA

kanamycin vector

VP16 GAL4 PR-LBD

promoter in the effector transgene (Fig. 5.4 I). No GFP was detected via fluorescence in DMSO-treated control embryo (Fig. 5.4C) that, subsequently, contained expression from both reporter cassettes at later stages (Fig. 5.4 K). By stage 17, GFP detection was more pronounced in the anterior neural plate, and expression was also more evident along the neural tube (Fig. 5.4B). Again, no GFP was detected in DMSO-treated control embryos (Fig. 5.4D). 50% of the RU486-treated cross embryos displayed GFP fluorescence.

Embryos were then allowed to develop to tadpole stages, GFP was detected in the forebrain, including the olfactory region of the telencephalon, in the hindbrain, along the spinal cord and in the ventral retina (part of the retina that is not covered by pigmented epithelium) in the double transgenic embryos (indicated by ECFP in the lens and RFP in the somites from the reporter cassettes) (Fig. 5.4I, J). In DMSO-treated double transgenic embryos, there was no GFP fluorescence (Fig. 5.4K). Control embryos included sibling heterozygous and non-transgenic embryos, as well as DMSO-treated embryos, and no GFP was detected via fluorescence in any of these. 50% of embryos expressed GFP in the lens (through the *γ-crystallin* reporter), suggesting the *Pax6-GalPR* founder could have one integration site. Embryos containing the *Pax6-GalPR* (detected by lens ECFP from the *γ-crystallin* promoter) (and *UAS-gfp* (detected from RFP from the *CAR* promoter)) transgene(s) displayed normal morphology (Fig. 5.4L-N).

Embryos were also analysed by *in situ* hybridisation for *GFP* at neurula stage. *GFP* was expressed in the anterior neural plate and along the neural plate in 50% of the cross embryos (Fig 5.5A), analogous to the location of GFP fluorescence (Fig. 5.4A, B).
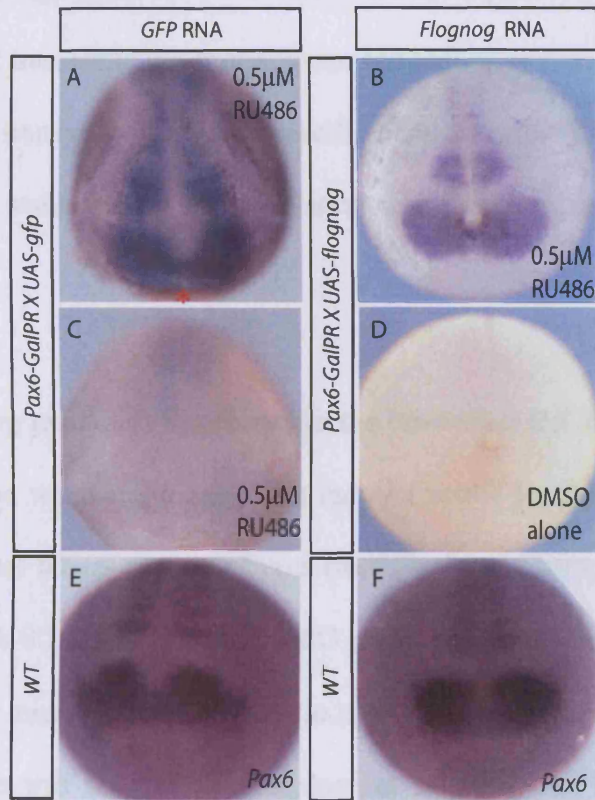
Figure 5.5 *GFP* **is expressed in the anterior and posterior neural plate in** *Pax6-GalPR X UAS-gfp* **cross embryos induced with RU486;** *Flognog* **is expressed in** *Pax6-GalPR X UAS-flognog* **cross embryos induced with RU486**

Images show anterior views of embryos from a cross of *Pax6-GalPR X UAS-gfp* (A, C) or *UAS-flognog* (B, D), either treated from the 4-8-cell stage with 0.5μM RU486 (A, B, C) or DMSO (D), or WT embryos (E, F). Embryos were analysed by *in situ* hybridisation for *GFP* (A, C), *Flognog* (B, D) or *Pax6* (E, F). There is anterior and posterior neural plate-specific expression of *GFP* in the *Pax6-GalPR X UAS-gfp* cross embryos induced with RU486, image depicts a stage 17 embryo (A) (red asterisk illustrates strong medial expression), background expression in stage 17 sibling control (C). *Flognog* expression is induced in the anterior neural tube and hindbrain, whereas it is reduced in the posterior neural tube in *Pax6-galPR X UAS-flognog* stage 19 cross embryos induced with 0.5μM RU486 (B), control embryo from stage 19 cross of *Pax6-galPR X UAS-flognog* incubated in DMSO alone (D). E, F illustrate *Pax6* expression in the prospective eye and forebrain regions in WT embryos at stage 14 (E) or stage 19 (F).

The *GFP* expression domain in the anterior medial neural plate and in prospective

hindbrain regions of the neural plate was expanded compared to *Pax6* expression in WT

embryos (Fig. 5.5E). RU486-induced sibling (Fig. 5.5C) and DMSO-treated *Pax6-*

GalPR X UAS-gfp cross control embryos did not express *GFP*. This result suggests that

Pax6-GalPR transgenic can transactivate and induce transcription (mRNA) from a *UAS-*

gfp reporter transgene in a tissue-specific manner, in the CNS, only in the presence of

RU486. A stable viable F1 population was therefore raised from the *Pax6-GalPR*

founder.

5.2.5 *Flognog* mRNA is Expressed in the *Pax6-GalPR X UAS-flognog* Cross

To determine whether *Flognog* was induced in the *Pax6-GalPR* (F0) *X UAS-flognog*

(heterozygote) binary cross, embryos from the *Pax6-GalPR X UAS-flognog* cross were

cultured with $0.5\mu M$ RU486 or DMSO alone, and were subsequently analysed both by

fluorescence microscopy and by *in situ* hybridisation for *GFP*.

There was no GFP fluorescence at stages 14 to 19 in *Pax6-galPR* X *UAS-*

flognog cross embryos. However, at neural tube stage, *Flognog* was expressed across

the anterior neural tube, and in two patches in the prospective hindbrain region of the

neural tube, whereas there was less expression in posterior regions of the neural tube in

Pax6-GalPR X UAS-flognog cross embryos induced with $0.5\mu M$ RU486 (Fig. 5.5B, D).

In WT embryos, *Pax6* is expressed in two uniform stripes along the neural tube

(Fig5.5F). *Flognog* expression was evident in 25% of cross embryos. Although

embryos were not genotyped, it is assumed that the embryos expressing *Flognog* are

Pax6-galPR; UAS-flognog double transgenic.

5.3 Discussion

The establishment of sub-divisions is an essential step in the formation of neuronal patterns and ultimately the functioning of the vertebrate forebrain. It is possible that levels of BMP signalling determine distinct sub-divisions along the D-V axis of the telencephalon. Gain-of-function studies have demonstrated that BMP signalling is sufficient to specify (and pattern) the dorsal forebrain (including the telencephalon) (Golden et al., 1999; Monuki et al., 2001), whereas loss-of-function studies have not yet established directly whether graded BMP signalling in the dorsal forebrain is required for dorsal telencephalic patterning.

Here, the *Otx2-gal4* transactivator was crossed to the *UAS-flognog* effector to test if the binary approach can result in alterations of development and developmental regulatory genes. The findings here show that BMP signalling is required in the anterior neuroectoderm for correct specification of prospective dorsal (or lateral) cell fates (patterning) within the prospective anterior CNS. It has been shown that BMP signalling can regulate genes that encode homeodomain proteins, which are essential for the development and patterning of forebrain structures. Interestingly, the findings here also reveal that, in the *Otx2-gal4 X UAS-flognog* cross, there is an increased prospective ventral telencephalon/ olfactory placode/ diencephalic identity; as well as open neural tube defects. BMPs have been implicated in both dorsal and ventral specification, dorsal patterning, and growth and apoptosis of the forebrain. How BMP signalling (via molecular, cellular and tissue morphogenesis processes) regulates the expression and hence function of these developmental regulatory genes, and what causes these profound morphological alterations here, is discussed below.

5.3.1 Open Neural Tube Defects

The data indicates that a loss of BMP signalling in the anterior neuroectoderm results in a partial open neural tube (Fig. 5.1A-D). This result is consistent with many studies, which have implicated alterations of BMP signalling in alterations in morphogenesis of the nervous system. Previous studies have described either a failure or delay of the neural tube to close as a result of a loss of BMP signalling (Lim et al., 2005; Solloway and Robertson, 1999). There was a failure of the neural tube to close, as well as defects in eye and craniofacial development, resulting from a loss of BMP signalling by *dnBmpr1* (*a* and *b*) electroporations into the chick neural tube (Lim et al., 2005). They suggest that the reason for the neural tube closure defect could be due to neural crest depletion in the midline. Delayed closure of the neural tube has also been reported as a result of loss of BMP signalling (Solloway and Robertson, 1999). In mice lacking *Bmp5* and *Bmp7* there is a delayed closure of the rostral neural tube, hypoplasia of the telencephalic vesicles and reduced apoptosis in the telencephalic roof. The open neural folds of normal embryos initiate fusion at multiple sites, including the fore/midbrain boundary and anterior extremity of the forebrain (Geelen and Langman, 1977). *Bmp5;Bmp7* double mutants also show closure defects from the hindbrain to anterior-most forebrain (Solloway and Robertson, 1999). By 10.5dpc mutants had initiated closure of these regions, however, the hindbrain roof plate was reduced in size. The growth of the lobes of the forebrain (telencephalon) is severely compromised in *Bmp5;Bmp7* mutants. The phenotype correlates with the overlapping expression of *Bmp5* and *Bmp7* in the hindbrain and dorsal telencephalon. Both these studies suggest that a loss of BMP signalling could be the underlying cause of the open neural tube/

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brain phenotype. Other studies using transgenic mice overexpressing Bmpr1a or Bmpr1b have indicated that constitutively active BMP signalling can cause an open neural tube phenotype (Panchision et al., 2001). These studies together indicate that it is the level of BMP signalling that is critical in neural tube closure, as both an increase and decrease in BMP signalling can cause an open neural tube. Thus, in a similar manner to the above studies the direct inhibition of BMP signalling within the anterior neuroectoderm in the *Otx2-gal4* X *UAS-flognog* cross may inhibit BMP signalling throughout neurulation, resulting in neural tube closure being blocked or suppressed or delayed to varying degrees depending on the level of intercellular BMP signalling. The viability of *Otx-2-gal4;UAS-flognog* double transgenic tadpoles suggests that there may be a delayed closure. The reason why there is a transient open neural tube may be due to the knockdown approach via the *Otx2* promoter. It may be interesting to perform histological analysis to uncover possible reasons for the open neural tube defects seen here.

Furthermore, other studies have implicated synergistic actions of BMP4, SHH and FGF8 in the rostral prosencephalon in regulating morphogenesis of the telencephalic and optic vesicles (Ohkubo et al., 2002) or found that inhibition of BMPs results in decreases in proliferation as a result of decreased Wnt (Chesnutt et al., 2004). Increasing BMP signalling by implantation of BMP2, BMP4 or BMP7 beads into the anterior neuropore of HH stage 10 chick embryos during neurulation results in a loss of Shh and Fgf8 expression, decreased proliferation, increased cell death, and hypoplasia of the telencephalic and optic vesicles (Ohkubo et al., 2002). However, decreased BMP signalling, through ectopic electroporation of Noggin in the telencephalon of stage 14

chick embryo results in decreased proliferation and hypoplasia of the telencephalic and optic vesicles. Also, *Emx2* expression is weakened and recedes caudally, and there is a maintanance of *Shh* and *Fgf8* expression. They suggest that optimum growth and patterning of the telencephalon depends on the combined effects of BMP, SHH and FGF expression. Also, it has been found that inhibition of BMPs by Noggin decreases *Wnt1/3a* expression in the roof plate and decreases proliferation. They show that it is Wnts that are responsible for the alterations in proliferation, as Wnts act as mitogens (Chesnutt et al., 2004). These studies indicate neural tissue growth is reduced by increases or decreases in BMP signalling, suggesting that growth malformation results from interactions between other signalling molecules. Thus, the absence of BMP signalling within the anterior neuroectoderm may affect several tissues involved in neural tube morphogenesis. As BMPs in co-ordination with SHH and FGF8 have being implicated in growth and morphogenesis of the telencephalic vesicles. It may be that there is a de-regulation of the balance between these signalling factors, hence resulting in the morphological alterations observed in the *Otx-2gal4 X UAS-flognog* cross. However, this is not known and further molecular characterisation may dissect out the underlying cause.

5.3.2 Alterations in *Pax6* Expression in the Anterior Neural Tube

The loss of BMP signalling in the anterior neuroectoderm results in an altered shape or pattern, a reduced domain size, and closer and/or fused bi-lateral domains of *Pax6* expression in *Otx2-gal4 X UAS-flognog* cross embryos (Fig. 5.2).

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Explanations for these findings may include regulation of regionally restricted markers and morphogenesis as a result of alterations in BMP signalling from altered formation of the roof plate and/or a decreased BMP signalling gradient throughout early neural development. Studies diminishing BMP signalling in whole embryo or ablating the roof plate (hence blocking BMP signalling) have reported altered expression of dorsal telencephalon markers (Monuki et al., 2001; Cheng et al., 2006; Hanel et al., 2006). A conditional allele of the toxin DTA driven from the Gdf7 locus has been used to ablate the roof plate in mouse embryos. This reduced *Lhx2* expression in the cortical neuroepithelium and the normal graded pattern of *Lhx2* was no longer apparent. There was also a reduction of the *Lhx2*-negative domain at the dorsal midline. Therefore, due to the non-cell-autonomous actions of the roof plate, this implicated BMP signalling in dorsal telencephalic development and patterning (Monuki et al., 2001). The phenotype was difficult to interpret due to severe open forebrain defects. In an attempt to further elucidate forebrain roof plate functions they modified the Gdf7-mediated ablation to generate embryos with a closed forebrain phenotype. In *ACTBCre;Gdf7-DTA* roof plate ablated mice (Currle et al., 2005), at E11.5 to E12.5, *Pax6* was expressed in its normal high ventral to low dorsal gradient in the cortex (Cheng et al., 2006), whereas there was a decrease and inversion of the normal high dorsal to low ventral expression for both *Lhx2* and *Emx2* markers, again implicating and extending the BMP signalling function in dorsal cortical patterning. They suggest that these roof plate patterning functions are mediated via a Bmp activity gradient which depends on the roof plate in a non-cell-autonomous manner. They also found that roof plate ablation results in both a flatterning and reduction of a BMP signalling gradient as opposed to a dimished

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gradient, as assessed by P-Smad-1 levels in dorsal telencephalic tissue sections. They

suggest that some BMP signalling, particularly in the ventro-lateral domain, is also

regulated by a nondorsal source of BMPs or other factors that promote nuclear P-Smad1

accumulation, such as the ventral midline. It is known that the maximum that BMP

signalling can act is 10 cell diameters away or is poorly diffusible from its source of

expressing cells (Niehrs, 2001; Jones et al., 1996; Nikaido et al., 1999). Thus, the lack

of alteration in *Pax6* expression in ventro-lateral domains of the dorsal telencephalon

may be due to being too far away from the BMP signalling source. However, this

depends on the mechanism of the BMP signalling gradient. These studies implicate

BMP signalling in alterations of regionally restricted telencephalon markers, however

they analyse (dorsal) patterning defects at late stages of neural development. As, there

were no alterations in *Pax6* expression in the above studies, the alterations in *Pax6*

expression in the *Otx2-gal4 X UAS-flognog* cross embryos are not likely to be due to

similar mechanisms.

Further to these findings, other studies have indicated that early Bmp activity

establishes a gradient of positional information throughout the entire neural plate. Bmp

activity is required for establishing fates at the margin of the neural plate and patterning

neurons at all D-V levels of the CNS (Barth et al., 1999). Analysis of zebrafish null

mutant *swirl/bmp2b-* *(swr-)* revealed a neuralised embryo with an expansion of *flh* (a

dorsal diencephalon marker) into ventral ectoderm, a loss of *Emx-1* (a dorsal

telencephalic gene) and expansion and radialised domain of *Pax6* expression in the

diencephalon and hindbrain. There is an absence of sensory neurons and an expansion

of interneuron populations. Furthermore, they find a loss of marginal neural plate

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expression of *flh*, *emx1* and *fkd6* in noggin-injected embryos. In conclusion, they show

that the underlying mechanism of the alterations is due to an early gradient of BMP-

dependent positional information (medial and lateral extent of ectodermal gene

expression domains) extending throughout the entire neural and non-neural ectoderm.

Due to the early onset of the *Otx2-gal4* transactivator, resulting in mis-expression of

Flognog in the anterior neuroectoderm, consistent with the early BMP activity gradient

affecting latero-medial cell fate (Barth et al., 1999), the roles of inhibition of a BMP

signalling gradient during initial neural plate patterning and neurulation are likely to be

the underlying cause of the alterations in *Pax6* expression. As mentioned previously in

the study by Hanel et al. (2006), loss of BMP signalling by GDF6 (Bmp13) morpholino

results in an altered expression domain of *Pax6* in the retina. Furthermore, many studies

have implicated a loss or gain of BMP signalling, by *BMP4* and *BMP7* mutant mice,

dominant negative Bmpr1, noggin overexpression or constitutively active Bmpr1, in eye

defects, such as microphthalmia (small eye), anophthalmia (no eye) or eye cell fate

decisions (Alder and Belecky-Adams, 2002; Furuta and Hogan, 1998; Wawersik et al.,

1999; Lim et al., 2005). Dominant negative or constitutively active Bmpr1

electroporated into chick neural tube resulted in microphthalmia and anophthalmia, and

furthermore constitutively active Bmpr1 resulted in a transformation of forebrain tissue

to retinal tissue (Lim et al., 2005). In a similar manner to the neural tube defects they

speculate that the level of BMP signalling in the eye is critical for the phenotype

observed. Hence, the reasons for the altered shape of *Pax6* expression could indicate an

altered retinal expression resulting from altered positional information (Barth et al.,

1999) as a result of the loss of BMP signalling mediated via different BMPs (such as

GDF6), which are expressed and function throughout early neurula development (Hanel et al., 2006).

Other data shows that an increase or decrease in BMP signalling results in a fusion of the cerebral hemispheres (Golden et al., 1999; Bachiller et al., 2000; Cheng et al., 2006; Panchision et al., 2001). Beads soaked in recombinant BMP4 or 5 were implanted into chick rostral neural tube and the resulting embryos displayed a single forebrain vesicle (holoprosencephaly HPE), as well as cyclopia and other craniofacial defects (Golden et al., 1999). Whereas, roof plate ablation in conditional *ACTB-Cre;Gdf7-DTA* mice resulted in failed separation of the cortical primordia at the dorsal midline, a HPE phenotype (Cheng et al., 2006), and transgenic mice expressing activated BMPR1A receptor mutant under the control of the Nestin promoter caused HPE due to specification of the entire forebrain to choroid plexus epithelium (Panchision et al., 2001). A disruption of ventral forebrain induction underlies most HPE cases, however, there are some cases in HPE where there is a dysgenesis of forebrain dorsal midline structures. Thus, HPE can result from defective midline induction, and hence the failure to separate the cerebral cortex and other bi-lateral forebrain structures. Importantly, Golden et al., 1999 suggests that interrupting dorso-ventral patterning independent of disruptions in ventral Shh signalling can cause a holoprosencephaly phenotype too. Although, they speculate that HPE in their study is a secondary consequence of the loss of basal telencephalon. Also, compound mutant mice for both Chordin and Noggin, display a HPE phenotype, indicating a requirement of Chordin and Noggin for forebrain development (Bachiller et al., 2000). These studies implicate either increases or decreases of BMP signalling in the HPE phenotype. Thus,

the altered shape and closer bi-lateral expression domains seen here could be due to a secondary effect from altered midline induction (Panchision et al., 2001; Cheng et al., 2006).

Also, other studies have indicated prechordal plate affects medial-lateral markers in the forebrain (Shimamura et al., 1997). A culture mouse explant from 3-somite stage embryos in which the axial mesendoderm was removed displayed a medially expanded and bi-laterally fused *Pax6* expression domain. Conversely, in an explant whereby additional axial mesendoderm was transplanted to lateral neural plate, there was a reduced *Pax6* expression domain around the site that the transplanted tissue was added. These results indicate that prechordal plate regulates ML patterning in prosencephalic neural plate explants.

Finally, the results indicated a smaller expression domain compared to siblings was observed in *Otx2-gal4 X UAS-gfp* cross embryos, implying that the transgene expression may contribute to the reduced size in *Pax6* expression domain seen in the *Otx2-gal4 X UAS-flognog* cross embryos. Thus it is not clear whether the alterations in *Pax6* expression in the *Otx2-gal4 X UAS-flognog* cross are as a result of a loss of BMP signalling in anterior neuroectoderm, and this transgene-mediated artifact needs to be taken into account for any further analysis of the phenotype.

To further investigate the reason for the alterations in *Pax6* expression, one possibility was that there was an alteration in other anterior regionalised identity markers.

5.3.3 Alterations in *X-dll3* Expression in the Anterior Neural Tube/ Neural Tube Border

An expansion and fusion of *X-dll3* expression domain in the anterior neural tube/ neural tube border in *Otx2-gal4 X UAS-flognog* cross embryos was observed (Fig. 5.3). The findings here suggest that a loss of BMP/Smad-1 signalling in the anterior neuroectoderm causes an expansion of prospective ventral telencephalon or diencephalon or olfactory placode tissue, indicated by *X-dll3* expression (Papalopulu and Kintner, 1993; Eagleson and Harris, 1990).

The reason for the increase in expression domain of *X-dll3* could be due to alterations in pattern (and cell fate) as a result of an altered gradient of BMP signalling from adjacent tissues (Hartley et al., 2001; Dale et al., 1997, 1999; Luo et al., 2001; Anderson et al., 2002; Barth et al., 1999). Alternatively, transgene-mediated expression may affect marker expression (Hartley et al., 2001). BMP4 over-expression by targeted mRNA injection into one dorsal cell of a 4- to 8- cell stage embryo produced a reduced level and circumference of *X-dll3* expression in the boundary of the anterior neural plate (Hartley et al., 2001). This suggests that the reason for the down-regulation of neural gene expression by BMP4 misexpression is because antagonism of BMP signalling is needed for anterior neural development. However, in contrast, they found that BMP4 misexpression after gastrulation from the *Pax6* promoter using the *Pax6-BMP4* transgenic resulted in an up-regulation of *X-dll3* expression. They suggest that the expression of this neural gene is maintained by the expression of localised inhibitors of BMP expression and signalling which are in the anterior neural plate. In line with this finding that BMP signalling modulation differentially alters *X-dll3* expression, it has

been found that a BMP morphogenetic gradient differentially regulates *Dlx* expression (Luo et al., 2001). In northern blot assays on animal caps from *Xenopus* embryos injected with Chordin RNA (a BMP antagonist), there is a concentration-dependent modification of *Dlx5* expression (which corresponds to *X-dll3*). *Dlx5* expression is stimulated by a low chordin dose, whereas it is inhibited by higher levels of chordin. This suggests that this finding is evidence to support the conclusion that BMP-based morphogenetic gradients can control the differential expression of *Dlx* homodomain genes, suggesting a possible mechanism for linking the BMP gradient to regionalised tissue specification.

It is also known that mesendodermal tissue is implicated in the induction and patterning of the forebrain (Reviewed by Kiecker and Niehrs, 2001). *BMPs* (*Bmp4 and Bmp7*) are expressed in the prechordal mesoderm tissue at neurula stages onwards underlying the rostral diencephalon (Dale et al., 1997, 1999; Hartley et al., 2001). Bmp7 has been shown to be required in conjunction with Shh for induction of ventral midline cells of the rostral diencephalon identity, for induction of a hypothalamic rather than a floorplate fate (Dale et al., 1997, 1999). This indicates that BMPs are needed for induction of the ventral midline. In contrast, Chordin and Noggin from the prechordal plate have been implicated in promoting SHH function from the prechordal plate, and hence ventral forebrain identity (Anderson et al., 2002). Also, it has been found that inhibition of BMP/GDF ligands by Noggin and normal SHH signaling is also required for proper formation of ventral cell types (McMahon et al., 1998). The opposing functions may be attributed to different stages of development, as *chordin*, *noggin* and *Bmp7* are expressed in these regions at different times.

Thus, due to the persisting expression from the *Xenopus Otx2* promoter in the prechordal mesoderm (Hirsch et al., 2002), *Flognog* over-expression in this region may therefore be the reason for the expansion in the ventral forebrain identity (Anderson et al., 2002). Moreover, this expansion in *X-dll3* expression may be due to low *Flognog* transgene expression levels in these regions causing a graded BMP signal and consequently acting to specify and expand regionalized marker, *X-dll3* (Luo et al., 2001). However evidence for this explanation comes from *in vitro* animal cap assays, thus it is different to the *in vivo*/ whole-embryo system employed here and may not be the reason for the alterations. Furthermore, as mis-expression of *Flognog* in the *Otx2-gal4 X UAS-flognog* cross partially overlaps with *X-dll3* expression in the neuroectoderm, these alterations in *X-dll3* expression could be due to cell-autonomous or non cell-autonomous effects. In addition, the similarity of fusion in X-*dll3* expression to the fusion of *Pax6* expression indicates that this fusion may be due to the same underlying cause, such as the effects of BMP signalling during neurulation and the secondary effects on brain morphogenesis. It may be that alterations in induction of the roof plate results in a HPE phenotype in the *Otx2-gal4 X UAS-flognog* cross, similar to the roof plate ablation studies (Cheng et al., 2006).

The morphological and molecular alterations in the *Otx2-gal4 X UAS-flognog* cross are evident in percentages from 9-19%. The reason for the low percentages displaying a phenotype is not due to a viability problem because cross embryos (including double transgenic embryos) were evident in Mendelian ratios. Instead, the numbers of embryos observed displaying alterations are consistent with previous studies whereby either a microcephalic or ventralised phenotype was observed in 13-16% of

either *Pax6-gal4* or *CMV-gal4* cross to *UAS-vent2* embryos (Hartley et al., 2002), and may be attributed to GAL4 variability (*GAL4* expression) (Brand and Perrimon, 1993). This may also be the reason for variations in the severity of the alterations in morphology and molecular marker expression as well. For this reason, it may be a good idea to breed the effector lines to homozygosity to increase sample number of the binary transgenics in the cross to transactivator. To ascertain whether all the alterations seen are real, PCR analysis should be carried out to confirm that embryos displaying these effects are double transgenic.

It should be noted that mis-expression of transgenes can alter development and molecular patterning (Hartley et al., 2001). The mis-expression of BMP4 by the *Xenopus Pax6* promoter suppressed anterior brain and eye formation in 36% of GFP expessing embryos. Moreover, upon analysis of patterning markers, most neural markers displayed a decrease in expression in a proportion of the *GFP*-expressing *Pax6-GFP* embryos, except *X-dll3*, which displayed slight decreases and increases in expression in the *GFP* expressing embryos. Thus, although I did not observe alterations in *X-dll3* expression in the *Otx2-gal4* X *UAS-gfp* cross, the sample number was small, and there is still as possibility that the alterations in *X-dll3* expression may due to transgene-mediated over-expression.

To ascertain whether all these above reasons apply to the *Otx2-gal4* X *UAS-flognog* cross, it will be interesting to do further characterisation of the phenotype, such as characterise the gross morphology of the phenotype by sectioning. This will determine if there is any transformation from one tissue type to another, or if there is a reduction or expansion in tissue. Also, to verify the molecular alterations observed here,

it may be interesting to phenocopy the expansion in domain of *X-dll3* expression, for example, by targeted *Flognog* mRNA injections into the anterior CNS region. Additionally, if the expansion in *X-dll3* expression domain results from an expansion of ventral identity, it may be possible to rescue this effect with exogenous recombinant BMP4 or BMP2 (or BMP7). If the increase in *X-dll3* expression is due to a change in cell fate, it may be interesting to analyse the expression of other identity markers (ventral markers, dorsal markers, as well as other tissue specificity markers) in this region. In addition, it may be interesting to determine whether the respective downstream regulators (i.e. bHLH proteins) and subsequently the neuron populations of *Pax6* and *X-dll3* domains are altered in the *Otx2-gal4* X *UAS-flognog* cross. Furthermore, it may be interesting to determine whether there are alterations in the migration of cells from the ganglionic eminences into the cortex in the *Otx2-gal4* X *UAS-flognog* cross (Chapouton et al., 1999). Other signalling pathways, such as Retinoid, Hedgehog and FGF signals are involved in (dorso-ventral) latero-medial (and anterior) patterning of the rostral CNS (Lupo et al., 2005), and this should be taken into account when assessing any phenotype in *in vivo/* whole embryo models as well. How these diverse signalling pathways interact both temporally and spatially to generate the complex adult nervous system is not understood. The use of the *Xenopus* transgenic lines established here, together with other SLT lines, such as UAS-HIP (UAS-hedgehog interacting protein) may shed some light on these processes.

5.3.4 *Pax6-GalPR* Hormone-inducible Transactivation of Transgene Expression

There are some problems or issues to be aware of when using the GalPR based-system. Difficulties in controlling the level of expression and alterations in expression from a transgenic promoters' normal expression has been previously demonstrated using (inducible) binary transactivation transgene-mediated expression, resulting in uncontrolled transgene expression, amplification of expression, or even alterations in development (Chae et al., 2002; Govindarajan et al., 2005; Gill and Ptashne, 1988; Argenton et al., 1996; Luan et al., 2006). Solubility problems have been reported for RU486, making it difficult to control the amount of RU486 that is delivered to the animal (Das and Brown, 2004). Additionally, RU486 antagonises endogenous hormones at high micromolar concentrations (Philbert et al., 1985; Henderson et al., 1987), which can result in abnormal development. This indicates that caution must be taken to deliver the right amount of RU486. Furthermore, in the absence of RU486, basal transactivation of the promoter can occur by endogenous hormones, at tadpole and post-embryonic stages (Osterwalder et al., 2001; Chae et al., 2002; Das and Brown, 2004). Also, strong transcriptional activators have been found to cause unspecific promoter squelching (Gill and Ptashne, 1988) resulting in retardation of embryogenesis (Argenton et al., 1996). In the mouse, it has been found that a GAL4/VP16 transactivator itself is sufficient to alter ocular development, indicating a dose-dependent intolerance and toxicity from GAL4/VP16 (Govindarajan et al., 2005). As a result of this it was suggested that phenotypes of bi-genic embryos generated using this transactivator need to be interpreted with caution. Also, in crosses using the VP16 activation domain (AD), target gene expression was observed outside the promoter

driven area, and this was due to ectopic VP16 AD expression from an enhancer in the transgene construct (Luan et al., 2006). Hence, indicating that care must be taken to ensure the specificity of target gene expression is within promoter driven areas. Nevertheless, in RU486-treated *Rx-GalPR Xenopus* embryos there was a concentration-dependent (nanomolar range, 5-25nM) increase in transgene expression levels at low amounts of RU486 (below the amount required to antagonise progesterone) (Chae et al., 2002). There were no apparent adverse effects on development from the hormone or the regulatory elements in GalPR, and no observable basal expression in the absence of RU486 using their assays.

Thus, although the concentration used to induce transactivation of *Pax6-GalPR* was relatively high for the system (Fig. 5.4, 5.5), it was below the RU486 amount (1-5µM) required to antagonise endogenous hormones (Philbert et al., 1985; Henderson et al., 1987) and no observable adverse effects on development or viability were observed in the *Pax6-galPR X UAS-gfp* cross. Although I did not notice developmental defects, further analysis of the system needs to be carried out to ensure this problem is not associated with the *Pax6-GalPR* transgenic.

The difference in reporter expression from endogenous *Pax6* expression (Fig. 5.5A, E) may be due to a time-lag in reporter expression (resulting in embryos at a later developmental stage with the earlier expression profile compared to the endogenous *Pax6* expression profile). They may also result from altered or missing promoter elements in the *Pax6* promoter fragment (Hartley et al, 2001).

Furthermore, although I did not fully characterise the system (including determining the time lag from addition of RU486 to functional target protein) it will be

important to determine the spatial expression profile, kinetics and dose-responsiveness of the system to RU486 at a full concentration range down to low nanomolar range 0.1-25nM, for further use (Chae et al., 2002; Wang et al., 1994). Perhaps a time-course whole-mount *in situ* hybridisation analysis of *GalPR* expression, as well as *GFP* expression (in a reporter cross) could be carried out at a full RU486 concentration range. Once the *Pax6-GalPR* system is fully chracterised, it will allow spatial, temporal (or conditional) and quantitative control over gene expression *in vivo* in the dorsal forebrain, allowing many questions to be asked regarding the biological processes involved in forebrain patterning and other developmental processes. By using GalPR system, early effects can be avoided, which permits addressing the effects of transgenes in later embryonic development or adults. Furthermore, the strong transactivation by the *Pax6-GalPR* transactivator seen here (Fig. 5.4, 5.5), which may be due to the VP16 activation domain in GalPR, may allow the expression of dominant negative variants, opening up avenues for loss of function studies using this GalPR-binary transactivation system. In addition, the GalPR system may be able to be adapted to other species, such as zebrafish. Most importantly, this *Pax6-galPR* transactivator can be used together with the *UAS-flognog* effector to analyse the effect of block or suppression of BMP signalling on dorsal telencephalon patterning.

5.3.5 Further Uses of the Binary Crosses

Firstly, using the inducible *Pax6-GalPR* X *UAS-flognog* cross, it is possible to analyse the effects of loss of BMP signalling before, during and after neural tube closure on dorsal telencephalic patterning. In addition, *Pax6-GalPR* driven *Flognog* expression in

the alar (lateral) plate, adjacent to the high levels of BMPs in the dorsal midline, allows

analysis of whether a global concentration gradient-dependent mechanism of BMP

signalling is required to specify pattern of the dorsal telencephalon (pallium), in a

similar manner to the spinal cord. In the spinal cord, the inductive interactions of TGF-

β signals on neuronal patterning involve both qualitative and quantitative differences in

signalling by TGFβ-related factors and temporal changes in the response of neural

progenitor cells (Liem et al., 1997). The main BMP ligands involved in dorsal

telencephalic patterning include BMP2 and BMP4 (and BMP5 in chick) (Furuta et al.,

1997; Golden et al., 1999; Monuki et al., 2001). The use of the *Pax6-GalPR X UAS-*

flognog cross will allow the dissection of the roles of BMP4 and BMP2 in this proposed

model. Importantly, these experiments will allow assessment of patterning of the dorsal

telencephalon independently from the early effects of BMP signalling. After

characterisation of the kinetics of the *Pax6-GalPR* transactivator (including the time lag

from addition of RU486 to functional gene product), in initial experiments, it is

necessary to determine whether BMP signalling is blocked in the RU486-induced *Pax6-*

GalPR X UAS-flognog cross. Again, a decrease in phospho-Smad-1 immunostaining

may be used to indicate a decrease in BMP signalling. Once this is established, it may

be interesting to determine whether BMP signalling is required for dorsal patterning of

the telencephalon (i.e. the pallium, the dorsal telencephalon). In a cross of *Pax6-GalPR*

to UAS-flognog, addition of RU486 at a pre-determined time-point to induce *Flognog*

expression before, during and just after neural tube closure, may decrease BMP

signalling in the developing telencephalon. Then, the effects of this decrease of BMP

signalling on dorsal patterning of the pallium can be assessed by whole-mount *in situ*

hybridisation for a panel of forebrain markers. These could include *eomes* and *Tbr1*, which both mark the whole of the pallium, and *opl-1*, a dorsal forebrain marker. To determine if there are any alterations in D-V patterning as a result of altered dorsal patterning, the pattern of ventral forebrain markers, such as *X-dll3* and *Nkx2.1* on cross embryos could be assessed.

Furthermore, phenotypes resulting from these binary crosses may be used as *in vivo* animal models of diseases, such as HPE or exencephaly to study the pathogenesis of these disorders.

CHAPTER 6: GENERAL DISCUSSION

In conclusion, the utility of the GAL4/UAS binary system in *Xenopus tropicalis* has been demonstrated by analysis of the roles of BMP signalling during early neural development. Use of the transgenic "Ligand-trap" loss-of-function technique works to block BMP signalling and produce an expected phenotype in the *Otx2-gal4 X UAS-flognog* cross. The phenotype in the *Otx2-gal4 X UAS-flognog* cross and embryos from the *Pax6-GalPR X UAS-flognog* cross that are induced with RU486 are evident as viable double transgenic embryos. The viability of the double transgenics from these crosses illustrates the usefulness of the GAL4/UAS system to avoid lethality associated with early functions of BMPs. Here, the effects of mis-expression of Flognog, a ligand trap for BMPs, during early neural development have been demonstrated. The *Pax6-GalPR* transgenic was generated to be able to assess the initial question about the effects of BMP signalling during late CNS development, e.g. to analyse the late BMP signalling source hypothesised to be involved in patterning of the dorsal telencephalon.

6.1 Usefulness of the GAL4/UAS Transcriptional Binary Approach

Consistent with previous studies by Hartley et al. (2002), the binary approach tested here allows spatial and temporal manipulation of gene expression *in vivo*. Lines were identified that induced high enough levels of GAL4 to induce target gene expression in all presumed double transgenics (e.g. Figs. 3.3; 3.4; 4.5). However, phenotypes were displayed with variable severity (eg. Fig. 5.1), making them hard to interpret. In agreement with the system observations by Brand and Perrimon (1993), this variability could be attributed to GAL4 expression directed by the *Otx2* promoter. Thus, although

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phenotypes can be observed, due to problems with phenotype variability resulting in low numbers displaying effects, the GAL4 binary system may not be an optimum approach for gene function analysis. However, the possibility to increase the level of transcription with the *Pax6-GalPR* transgenic line, may allow more embryos to display phenotypes in crosses of this inducible transactivator to a UAS effector.

6.2 Binary Transgenics to Analyse Late CNS Development

The use of the *Pax6-GalPR X UAS-flognog* cross will allow manipulation of gene function, and hence BMP signalling, at later stages of neural development, at least from after gastrulation and at later time points. This will be particularly useful because, primarily, it will allow the analysis of whether a gradient of BMP signalling is required for dorsal patterning of the telencephalon. Furthermore, as RU486 has been found to induce GalPR driven expression into late tadpole stages (Chae et al., 2002), even later roles of BMP signalling during late CNS organogenesis can be assessed. Thus, roles of BMP signalling in D-V patterning in forebrain development and other areas of forebrain development, which still remains unclear, could be elucidated.

6.3 Other Binary Cross Uses of the *UAS-flognog* Transgenic

Firstly, *UAS-flognog* lines have been made that can be used together with *Otx2-gal4* transactivator in a binary cross to down-regulate BMP signalling in the anterior neuroectoderm to investigate neural differentiation and patterning, and the utility of the binary approach.

Recently, by using a *Xenopus* DNA microarray approach, over-expression of a dominant negative BMP receptor in ectodermal cells, has allowed the identification of new targets of BMP signalling (Shin et al., 2005). However, there are BMP target genes that are not known. Therefore, another potential use of the *UAS-flognog* line is in DNA microarrays to search for BMP signalling target genes. Since, it has been demonstrated that BMP signalling is down-regulated in the *Otx2-gal4 X UAS-flognog* cross (Fig. 4.7), it is feasible to seach for BMP signalling target genes in the anterior neuroectoderm using the *Otx2-gal4 X UAS-flognog* binary cross. Furthermore, the loss of BMP signalling by BMP2, BMP4 and GDF5-7 BMPs by Flognog may allow more targets of BMP signalling during early neural differentiation and patterning to be identified.

Furthermore, *UAS-flognog* can be used in crosses to other promoter-gal4 transactivator lines to block BMP signalling in a tissue-specific manner to ask questions about other biological processes.

6.4 Use of Different Types of Stable *Xenopus* Lines to Analyse Development in other Tissues

Due to the many biological processes that BMP signalling is involved in (Hogan, 1996), crosses of the different tissue-specific transactivator lines generated here *N-tubulin-gal4*, *N-tubulin-galPR* (see Appendix 11) and *Rx-gal4* to the *UAS-flognog* line can be used to block BMP signalling in different tissues and unravel the role of BMP signalling in these tissues. For example, BMP has a role in D-V patterning of the eye (Koshiba-Takeeuchi et al., 2000; Sasagawa et al., 2002; Murali et al., 2005; Sakuta et al., 2001). BMP overexpression in the retina has a strong dorsalising effect (Koshiba-Takeeuchi et

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al., 2000; Sasagawa et al., 2002), while BMP inhibition ventralises the eye (Murali et al., 2005; Sakuta et al., 2001; Sasagawa et al., 2002). Others have found that Bmp receptor 1b is required for axon guidance and cell survival in the developing retina (Liu et al. 2003). Therefore, use of *Rx-gal4*, *N-tubulin–gal4* and *N-tubulin-galPR* transactivator crosses to *UAS-flognog* will be a valuable model to expand on the mechanisms of these processes and clarify the roles BMPs play in dorso-ventral patterning of these other tissues.

6.5 Limitations of *Xenopus* Transgenic Lines

Finally, although the trangenesis method used here together with the production of stable lines offers a valuable technique to analyse gene function, it is time- and space-consuming to grow many lines. Transgenesis in other organisms, such as mouse or zebrafish cannot be used as an alternative model system because it produces mosaic animals. There have been some further advances in the *Xenopus* transgenesis technique to improve the integration efficiency of transgenes into the genome by using *I-SceI meganuclease* or ϕC31 integrase-mediated integration (Ogino et al., 2006; Allen and Weeks, 2005). These new techniques result in a high amount of founder embryos displaying non-mosaic transgene reporter expression, and allow founder transgenics to be used for reporter assays or misexpression experiments. Although this does not make the GAL4/UAS approach easier because GAL4/UAS depends on the use of two independent lines, it will allow quicker analysis of gene function in developmental processes.

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Another limitation using the binary approach together with the lines developed here is that the transgene expression pattern is constrained by the limited specificity of the available promoters, and this may not allow refined spatial manipulation of gene function. Recently, new ternary techniques have been implemented that place binary gene activation under the control of a third component. The combinatorial "Split Gal4" system can limit transgene expression to the intersection of two distinct, but overlapping expression patterns from two different promoters (Luan et al., 2006). The system takes advantage of the modular nature of the GAL4 transcription factor, and puts the DNA-binding domain and the activation domain of GAL4 under the control of different promoters. Consequently, only in cells co-expressing both domains of GAL4 can the two domains heterodimerise and become transcriptionally competent, thus activating expression from a *UAS-target* gene in tissues where expression from the two promoters overlap. Although, this system has been implemented in *Drosophila*, there is no reason it cannot be used in genetic model organisms, such as *Xenopus*, where it has been demonstrated here that similar transcriptional systems for controlled gene expression do exist.

APPENDIX I: Characterisation of *N-tubulin-GalPR*

Hormone-Inducible Transactivator

I.1 Generating and Testing the *N-tubulin-GalPR* Transactivator

An *N-tubulin-GalPR* construct containing the *Xenopus N-tubulin* promoter (Richter at al., 1998) and GalPR, a hormone–inducible *GAL4* (Wang et al., 1994) was made (Section 2.2.1). Subsequently, using transgenesis as described by Hirsch et al. (2002), *N-tubulin-GalPR* F0 transactivator transgenics were made. In one transgenesis experiment, there were 12/37 embryos containing GFP in the lens due to the γ-*crystallin-gfp* reporter, indicating that the transgenesis rate was 32%. Of the 12 founder transgenic embryos identified, 11 contained GFP in both the lens, and one embryo displayed GFP in one of the lens, suggesting that it was a half transgenic. The *N-tubulin-GalPR* transgenic tadpoles were raised to adulthood (Section 2.1).

An *N-tubulin-GalPR* transactivator was tested for its transactivation potential by crossing to a homozygous *UAS-gfp* reporter transgenic line. The embryos from this cross were incubated in 0.5μM RU486 from the 4 to 8 cell stage. At tadpole stages, GFP was detected in the brain, spinal cord, and lens in *N-tubulin-GalPR X UAS-gfp* cross embryos (Fig. I1 A), that contained RFP in the somites from the *CAR-RFP* reporter from the effector transgene (Fig. I1 C). No GFP was detected in sibling *N-tubulin-GalPR X UAS-gfp* cross embryos (Fig. I1 B), that, again, contained RFP in the somites (Fig. I1 D). This result suggests that the *N-tubulin-GalPR* transactivator can transactivate GFP from a *UAS-gfp* reporter line in the presence of RU486.
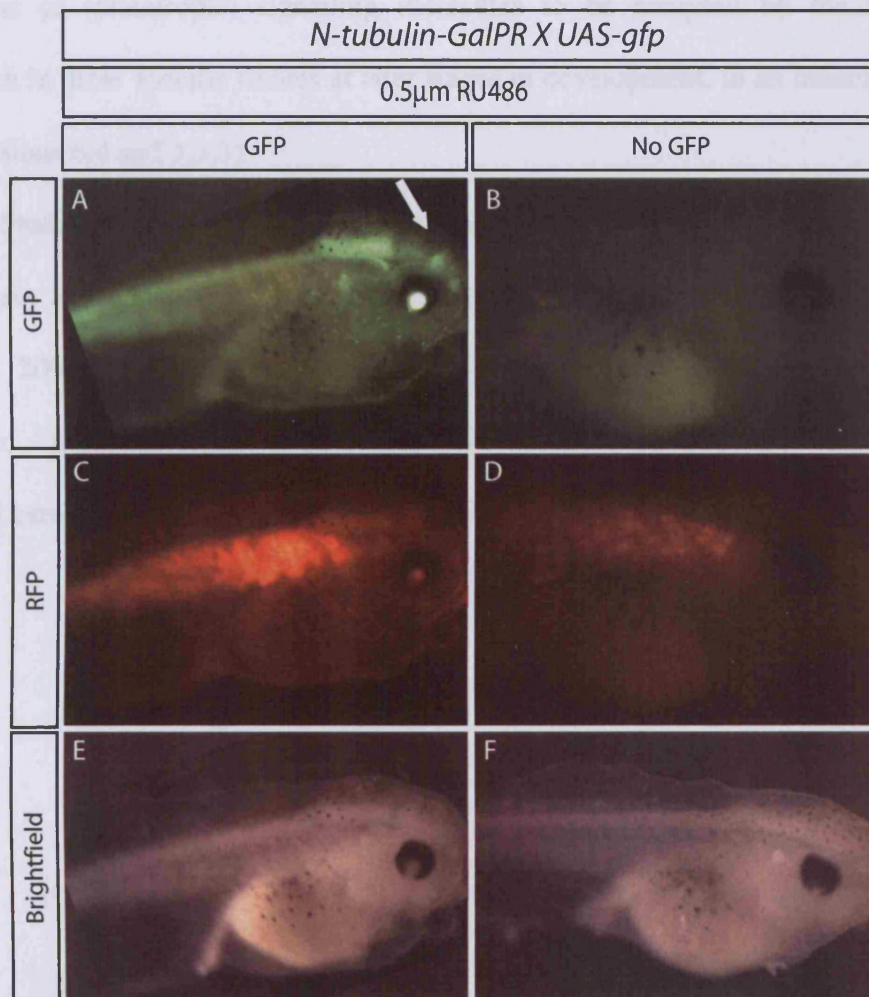
Figure I1 *N-Tubulin-GalPR* **Hormone-inducible GAL4 Transactivator Activates GFP from a *UAS-gfp* reporter in the Presence of RU486** Images are either GFP (or RFP) fluorescence microscopy (A, B or C, D) or brightfield (E, F) of tadpoles from a cross of *N-tubulin-GalPR* transgenic founder X *UAS-gfp* reporter frog, incubated in 0.5μM RU486. GFP is detected in primary neurons in the brain and spinal cord (and in the lens) (A) in an *N-tubulin-GalPR* X *UAS-gfp* cross embryo incubated in 0.5μM RU486 which contained RFP in the somites (C). The white arrow illustrates GFP in the anterior brain (A). No GFP was detected in a sibling *N-tubulin-GalPR* X *UAS-gfp* cross embryo (B), which contained RFP in the somites (D), and was also incubated in 0.5μM RU486. E, F represent brightfield images of A, C, and B, D respectively.

I.2 Discussion

An *N-tubulin-GalPR* transactivator has been established that can be used to drive expression in the primary neurons, in a RU486-inducible manner. This will allow the functions of (pleiotropic) signalling molecules to be analysed by the ligand trap approach in these specific tissues at later stages in development, in an inducible manner (see sections 6.4 and 3.3.3).

Studies have reported that basal transactivation by endogenous hormones can be a problem at tadpole stages (Osterwalder et al., 2001; Chae et al., 2002; Das and Brown, 2004). It was not determined whether basal transactivation of *UAS-gfp* occurred. Also, the timing of gene expression in response to a dose-range of RU486 was not carried out, so this should be established for further use of this system.

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