Development of the hypothalamic infundibulum in the early chick embryo

Thesis presented for the degree of PhD
by Elizabeth Manning

at
The Centre for Developmental Genetics,
Department of Biomedical Science,
University of Sheffield

February 2004
Acknowledgements

Firstly, I would like to thank Marysia Placzek for all of her help, support and encouragement throughout, for giving me so much time out of her already over-busy schedule, and for making the whole experience a lot less painful than it could have been. I would also like to thank members of the Placzek lab, past and present, Kyoji, Sandrine, Iain, Jo, Liz, Pam and Kathy, for their friendship, patience and help, with additional thanks to Kyoji, for allowing me to use some of his data in this thesis, to Sandrine, for all the help with the PCR, and Jo, Liz and Pam for all their help with plasmids and proteins. Thanks also go to Penny, for all her advice and help (particularly with all things molecular and Mac related), and to the members of the Rashbasslab.

I would like to thank my family and friends for their encouragement and support, for providing distractions and entertainment, and for looking impressed and interested whilst I talked of chickens and hedgehogs. Special thanks go to Mum and Dad for providing a safe haven, the odd loan, and for being there when I need them. Finally I would like to thank Dan, for helping me through the hard times, for being my voice of reason and making me believe I can do it. You are my angel x
Contents
Abstract

1 Introduction

1.0 Development of the nervous system
1.1 Patterning of the neural tube
1.2 Dorso-ventral patterning in the posterior neural tube
1.2.1 The role of Shh in posterior neural tube patterning
1.2.2 The Hh signalling pathway
1.2.3 The Gli genes and dorso-ventral patterning
1.2.4 Studies on Gli3 provide evidence for additional signalling pathways in dorso-ventral neural tube patterning
1.2.5 BMPs act as dorsalising agents to pattern the dorso-ventral axis of the neural tube
1.3 Integration of BMP and Shh signals: antagonism at multiple levels
1.4 Shh and BMP interactions within the forebrain
1.5 Transcriptional control of Shh
1.6 Summary

2 Materials and methods

2.1 In vivo manipulations
2.1.1 Fate mapping
2.1.2 Bead implantation
2.1.3 Prechordal mesoderm ablations
2.2 Explant culture
2.3 In situ hybridisation and immunohistochemistry
2.4 Protein use
2.4.1 Protein production
2.4.2 Proteins in functional assays
2.5 Quantitative RT-PCR

3 Fate Mapping

3.1 Introduction
3.1.1 Complex expression of Shh in the forming hypothalamus
3.2 Results
3.2.1 Expression of Shh in the chick forebrain
3.2.2 Fate-map of diencephalic cells
3.3 Discussion
3.3.1 Shh is down-regulated from the developing infundibulum
3.3.2 Distinct compartments within the forming hypothalamus?
3.3.3 Shh expression within the optic vesicle

4 BMP expression patterns

4.1 Introduction
4.1.1 BMPs in the diencephalon
4.2 Results
4.2.1 BMP2 and BMP7 are both expressed at HH stage 10 and 15 in ventro-medial hypothalamic cells
4.2.2 Expression of the BMP antagonists Chordin and Noggin in axial mesoderm and ventral neural tube
4.2.3 Co-expression of Shh with BMP2 and BMP7
4.3 Discussion
4.3.1 Shh is down-regulated in cells that co-express BMPs
4.3.2 Regulation of BMPs in the hypothalamus
5 BMPs down-regulate Shh in the prospective hypothalamus

5.1 Introduction

5.2 Results

5.2.1 BMP2 and BMP7 can down-regulate Shh in prospective hypothalamic cells

5.2.1a Down-regulation of Shh protein by BMP2 and BMP7

5.2.1b Down-regulation of Shh mRNA by BMP2 and BMP7

5.2.2 BMPs are required in vivo for the down-regulation of Shh in the prospective hypothalamus

5.3 Discussion

5.3.1 BMPs act indirectly to down-regulate Shh in the infundibulum

5.3.2 Blockade of BMP activity does not affect cell migration of M1, M2 or L1 cells

5.3.3 Chordin is an effective antagonist of BMP activity

5.3.4 Regulation of Shh in both M1/M2 and L1 cells by BMPs

6 T-box genes

6.1 Introduction

6.1.1 A T-box gene is involved in the down-regulation of Shh in the infundibulum

6.1.2 T-box genes

6.2 Results

6.2.1 Tbx2 is expressed in the ventral hypothalamus from stage 13

6.2.2 BMPs induce the expression of Tbx2 in explants, in a concentration dependent manner

6.2.3 BMP signalling is required for the expression of Tbx2 in the ventral hypothalamus
1.1 Dorso-ventral and anterior-posterior signals dived the neural tube.
1.2 Dorso-ventral patterning of the spinal cord involved both dorsal and ventral signals.
1.3 Gradient model for the induction of ventral cell types by Shh.
1.4 The homeobox code.
1.5 Hh signalling pathway.
1.6 The role of Gli genes in dorso-ventral patterning in the spinal cord.
1.7 Gli3-/ results in a partial rescue of the Shh-/ spinal cord.
1.8 BMP signalling regulates many aspects of neural tube patterning.
1.9 BMP-Shh antagonism through regulation of Gli3.
1.10 Schematic of Shh locus.

3.1 Shh is expressed dynamically in the ventral forebrain.
3.2 Divisions in the forebrain.
3.3 Fate-mapping technique.
3.4 Shh and BMP7 define M1 and M2 domains in the prospective hypothalamus at stage 10.
3.5 Ventro-medial M1 and M2 cells in the ventral diencephalon can be accurately targeted.
3.6 Progeny of M1a cells occupy both optic vesicles.
3.7 Progeny of M1b cells occupy the ventral infundibulum.
3.8 Progeny of M2b cells occupy the ventro-lateral infundibulum.
3.9 Progeny of L1c cells occupy the lateral hypothalamus.

4.1 BMP7 expression pattern.
4.2 BMP2 expression pattern.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.3 BMP4 expression pattern</td>
<td>85</td>
</tr>
<tr>
<td>4.4 Chordin expression pattern</td>
<td>88</td>
</tr>
<tr>
<td>4.5 Noggin expression pattern</td>
<td>90</td>
</tr>
<tr>
<td>4.6 Co-expression of Shh and BMPs in the prospective hypothalamus</td>
<td>93</td>
</tr>
<tr>
<td>5.1 Explant culture in collagen beds</td>
<td>102</td>
</tr>
<tr>
<td>5.2 Analysis of explants exposed to BMPs for 20 hours by immunohistochemistry</td>
<td>105</td>
</tr>
<tr>
<td>5.3 Analysis of explants exposed to BMPs for 20 hours by in situ hybridisation</td>
<td>107</td>
</tr>
<tr>
<td>5.4 Quantitative RT-PCR</td>
<td>109</td>
</tr>
<tr>
<td>5.5 Analysis of explants exposed to BMPs for 20 or 40 hours by quantitative RT-PCR</td>
<td>112</td>
</tr>
<tr>
<td>5.6 Blocking BMP signalling in the ventral diencephalon results in the maintenance of Shh in the infundibulum at stage 18</td>
<td>115</td>
</tr>
<tr>
<td>6.1 Mutations in the Tbx binding site on the Shh enhancer results in ectopic expression of Shh in the infundibular region of the ventral hypothalamus in mice</td>
<td>122</td>
</tr>
<tr>
<td>6.2 Tbx2 is expressed in the Shh negative infundibulum</td>
<td>128</td>
</tr>
<tr>
<td>6.3 Tbx2 is expressed in the Shh negative infundibulum at st 18</td>
<td>130</td>
</tr>
<tr>
<td>6.4 Tbx2 is up-regulated in vitro in a concentration-dependent manner by both BMP2 and BMP7</td>
<td>132</td>
</tr>
<tr>
<td>6.5 Blocking BMP signalling results in a down-regulation of Tbx2 in the M1/M2 infundibular cells at stage 15</td>
<td>135</td>
</tr>
<tr>
<td>7.1 Expression of BMPs in the stage 10 infundibulum coincides with a decrease in the number of M-phase cells detected</td>
<td>148</td>
</tr>
<tr>
<td>7.2 Cells of the stage 13 infundibulum are in synchronised cell cycle</td>
<td>151</td>
</tr>
</tbody>
</table>
7.3 Cells of the prospective infundibulum no longer express BMP7 and are in cycle at stage 10 following ablation of prechordal mesoderm

8.1 Compartment boundary formation in the fly eye
8.2 Neurons from the hypothalamus project through the infundibulum to the pituitary gland
8.3 Patterning of the pituitary by signals from the infundibulum and oral ectoderm
8.4 Possible mechanisms for Tbx2 regulation in the chick infundibulum

Boxes

Box 1 The homeobox code
Box 2 TGFβ signalling pathway
Box 3 The cell cycle
Box 4 The hypothalamus

Tables

3.1
4.1
6.1
6.2
Abstract
In the posterior neural tube of vertebrate embryos, the secreted signalling molecules Sonic hedgehog (Shh) and bone morphogenetic proteins (BMPs) are expressed at opposite poles of the neural tube, and act antagonistically to pattern the dorso-ventral axis of the neural tube. In contrast, in the ventral diencephalon, in regions that will give rise to the hypothalamus, BMPs and Shh are initially co-expressed. Subsequently, Shh shows a dynamic pattern of expression, first undergoing a medio-lateral expansion and then disappearing from ventro-medial cells that will form the infundibulum.

In this study, I address how Shh is regulated within the hypothalamic infundibulum. Using a combination of fate mapping and gene expression analysis, I demonstrate that cells that initially co-express Shh and BMP7 give rise to the Shh-negative infundibulum. Furthermore, I demonstrate, both in vitro and in vivo, that BMP7 is necessary and sufficient to cause the down-regulation of Shh in the infundibulum.

Recent studies on the mouse Shh promoter (Jeong and Epstein, 2003) have revealed that a T-box binding site is necessary for the down-regulation of Shh in the infundibulum. Through analysis of Tbx genes, I have found that Tbx2 is expressed in the prospective infundibulum. Furthermore, my studies reveal that its expression is dependent on BMP activity.

Finally, my studies show that, in addition to their role in regulating Shh expression, BMPs and Tbx2 may also play a role in regulating the size of the infundibulum. Analysis with the M-phase marker, PH3, reveals that, following exposure to BMPs, prospective infundibular cells are transiently cell cycle arrested, entering a synchronised cell cycle once BMP activity is lost, and Tbx2 is expressed.

Together, my results suggest that BMPs act through Tbx2 in order to control the domain of Shh expression within the forming hypothalamus, whilst simultaneously controlling the size of this progenitor domain.
Chapter 1

Introduction
1.0 Development of the nervous system

The nervous system of vertebrates is the most complex of all biological organs. Millions of cells, composed of thousands of individual cell types, all act in co-ordination to mediate both simple reflexes and the complex behaviours exhibited by higher vertebrates. Such function is dependent on the precise spatial organisation of neuronal cells, and neuronal circuits. The assembly of these neuronal circuits is initiated during embryogenesis, and so studying neural development has, in recent years, proved to be a powerful tool towards understanding both normal neural function, and associated diseases.

In vertebrates, the central nervous system (CNS) arises from the neural plate, a homogenous sheet of epithelial cells that forms dorsally in the gastrula stage embryo. Following a series of morphological movements, the neural plate rolls along its medio-lateral axis to form the neural tube, which is grossly divided, along the anterior-posterior axis into the forebrain, midbrain, hindbrain and spinal cord (figure 1.1a). Within these regions, distinct cell groups arise in a temporally and spatially restricted manner. Strict control of the formation, proliferation and migration of these cells, and the subsequent control of their axonal trajectories, goes on to produce the complex networks seen in the adult nervous system.

1.1 Patterning of the neural tube

In recent years a great deal of progress has been made into understanding the mechanisms by which the neural tube is patterned during embryogenesis. Signals from adjacent tissues establish crude patterns along the anterior-posterior and dorso-ventral axes, which are then progressively refined throughout development. Signals act initially on the anterior-posterior axis and in the absence of caudalising signals, the entire neural tube assumes an
Figure 1.1

Integration of signals along the dorso-ventral and anterior-posterior axis act to divide the neural tube into distinct regions, and generate multiple cell types according to their position.

(a) Schematic representation of the neural tube and its anterior-posterior divisions. Coloured triangles represent graded anterior-posterior (yellow, green) and dorso-ventral (red, blue) signals.

(b) Different cell types induced at the same dorso-ventral level according to their position along the anteroposterior axis. Different colours indicate regional differences in the cell types differentiating in response to different combinations of dorso-ventral and anterior-posterior signals.
Figure 1.1

a

b

F - Forebrain, M - Midbrain, H - Hindbrain, S.Cord - Spinal cord
AE - Anterior ectoderm, PM - Prechordial Mesoderm, NC - Notochord, N - Node
Light blue - Ventral hypothalamic cells, Red - Floor plate, Dark blue - Basal ganglionic cells,
Brown - Dopaminergic neurons, Yellow - Serotonergic neurons, Green - Motor neurons
anterior identity (Briscoe and Ericson, 2001; Doniach, 1995; Harland, 2000). Transplantation studies in chick embryos and in vitro explant experiments have indicated that distinct forebrain, midbrain, hindbrain and spinal cord territories may be defined in response to anteriorising and posteriorising signals (Beddington and Robertson, 1999; Ericson et al., 1995; Simon et al., 1995). Dorso-ventral signals then act on anterior-posterior patterned tissue in order to establish a Cartesian grid-like organisation of positional information, according to which, distinct cell types are induced with precise regional variation (figure 1.1b). Thus, for instance, motor neurons and interneurons are induced in ventral spinal cord regions, serotonergic neurons are induced in the ventral hindbrain, dopaminergic neurons in the fore- and midbrain, and basal ganglionic cells in the forebrain (Figure 1.1b). Signals that act to confer anterior-posterior identity emanate from a number of tissues that lie in proximity to the developing neural tube, and include the anterior visceral endoderm, somites and node (Episkopou et al., 2001; Foley et al., 2000; Gavalas and Krumlauf, 2000; Martinez-Barbera et al., 2000; Muhr et al., 1999; Muhr et al., 1997). Likewise, tissues immediately adjacent to the neural tube, namely notochord, prechordal mesoderm and surface ectoderm, act as the source of signals that initially confer dorso-ventral polarity on the neural tube (Dickinson et al., 1995; Liem et al., 1995a; Patten and Placzek, 2000; Placzek, 1995; Selleck and Bronner-Fraser, 1995)(Figures 1.1 and 1.2).

1.2 Dorso-ventral patterning in the posterior neural tube

The tissue interactions and signalling molecules that operate in development to pattern the dorso-ventral axis of the posterior neural tube, in particular the spinal cord and hindbrain, have been studied in particular depth over the last decade. The dorso-ventral pattern of the spinal cord/hindbrain is initially imposed by two main organising centres, the surface ectoderm and axial mesoderm cells of the notochord. These in turn induce the differentiation of organisers within the neural tube itself, the roof plate and floor plate, which
Dorso-ventral patterning of the spinal cord involves both dorsal and ventral signals.

Signals from the surface ectoderm induce roof plate in the dorsal midline of the neural tube, whilst signals from the notochord induce floor plate in the ventral midline. Signals from these structures act in opposition in order to pattern the dorso-ventral axis of the spinal cord.

Figure 1.2

Dorsal

Ventral

neural tube

notochord

SE - Surface ectoderm, RP - Roof plate, FP - Floor plate
NC - Notochord
are situated at the dorsal and ventral midlines respectively (figure 1.2). Signals from these regions act antagonistically to each other to induce different cell types in a spatio-temporally-restricted fashion (see section 1.3 below).

1.2.1 The role of Shh in posterior neural tube patterning

Ventrally, the notochord synthesises Shh, a secreted signalling molecule, which induces cells at the ventral midline of the overlying neural tube to become floor plate. The floor plate also expresses Shh, and its secretion into the neural tube is believed to create a concentration gradient along the dorso-ventral axis (Figure 1.3a), which then plays a critical role in patterning the entire dorso-ventral axis (Patten and Placzek, 2000). Ectopic Shh signalling from recombinant protein, or ectopically grafted notochord can suppress dorsal cell types and induce floor plate and other ventral cell differentiation in dorso-lateral regions of the neural tube, whereas using antagonists or function blocking antibodies to Shh results in the loss of ventral cells (Briscoe and Ericson, 1999; Briscoe et al., 2000; Patten and Placzek, 2002; Placzek et al., 1990).

The ventral spinal cord contains five main classes of post-mitotic neurons, V3, V2, MN, V1, V0 (figure 1.3b). The generation of these neurons at distinct dorso-ventral positions appears to be controlled by Shh, with ventral-most cell types, such as floor plate and V3 interneurons differentiating at high Shh concentrations, and motor neurons at slightly lower levels. At still lower concentrations, further away from the notochord and floor plate, more dorsal cell types form (Briscoe and Ericson, 1999; Ericson et al., 1997a; Pierani et al., 1999a). Experiments in vitro have shown that exposing naive neural tissue to small increases in extracellular concentrations of Shh results in the generation of these ventral neuronal classes, and the concentration of Shh required to induce them corresponds directly to their dorso-ventral position in
Figure 1.3

Gradient model for the induction of ventral cell types by Shh.

(a) Gradient of Shh signal moving from its sources of expression in the ventral neural tube and notochord.
(b) Distinct ventral cell types differentiate at stereotyped positions in the ventral neural tube. FP – floor plate, MN – motor neurons, V0-V3 – classes of interneurons generated at spinal cord levels.
(c) The concentration of Shh required to induce specific cell types in vitro correlates directly with their dorso-ventral position in vivo.

Diagram taken from Patten and Placzek, 2000
Figure 1.3

a. Gradient of SHH signal, moving ventral to dorsal

b. Increasing [Shh]

VO
V1
V2
MN
V3
FP

In 
creasing [Shh]
vivo (Figure 1.3c) (Ericson et al., 1997a). Experiments have shown that the gradient of Shh controls the expression of homeodomain transcription factors that in turn direct specific neuronal fate (Box 1).

**Box 1**

The homeobox code

How is a graded Shh signal converted into discrete cellular subtypes? A number of homeodomain transcription factors are expressed in discrete domains of the neural tube, and analysis of their expression has led to the homeobox 'code' model (Briscoe and Ericson, 1999; Briscoe and Ericson, 2001; Briscoe et al., 2000). In this model, transcription factors from the Pax, Dbx, Irx and Nkx family are expressed in restricted regions of the neural tube, creating five progenitor cell territories that give rise to different neuronal subtypes (Figure 1.4).

Initially, there is no regional dorso-ventral identity in the neural tube, and Pax and Msx transcription factors are expressed uniformly throughout, later becoming regionally restricted to dorsal regions. A number of studies have shown that Shh can repress these transcription factors from ventral cells (Ericson et al., 1996; Goulding et al., 1993; Liem et al., 1995b). Pax3 and Pax7 are expressed in the dorsal most neural tube as they are sensitive to low concentrations of Shh, whereas Pax6, Dbx1, Dbx2 and Irx3 are repressed only by higher concentrations of Shh, and so their expression boundaries extend further ventrally (Briscoe et al., 2000; Ericson et al., 1997b; Pierani et al., 1999a). These proteins, whose expression is repressed by Shh, have been termed 'class I' genes. The ventral limit of class I genes appears to correlate with the concentration of Shh required to repress them, leading to a graded repression of genes (Briscoe et al., 2000).
A second set of transcription factors has also been identified, whose members include Nkx2.2 and Nkx6.1. These are referred to as the 'class II' genes, as their expression in the ventral neural tube seems to depend on the repression of class I genes by Shh. It is not clear if class II genes are induced directly by Shh or if their expression is wholly a consequence of derepression by class I genes, but the fact that in the pax6 mutant, a dorsal expansion of Nkx6.1 is seen, suggests that its expression is an indirect consequence of Shh signalling (Briscoe et al., 2000; Ericson et al., 1997a).

In the spinal cords of mice lacking Shh, expression of class I proteins becomes expanded ventrally, and class II progenitor proteins are not properly induced. This results in an absence of almost all ventral cell types, including floor plate, V3 interneurons, motor neurons, and V0, V1, and V2 interneurons (Chiang et al., 1996; Litingtung and Chiang, 2000).

The dorsal expression of the class II genes abuts the ventral expression of those in class I, and once these progenitor domains are established, they can be maintained independently of Shh signalling (Briscoe et al., 2000; Briscoe et al., 1999). This, in addition to experiments showing that ectopic Nkx expression in the dorsal neural tube can repress Pax6 expression (Briscoe et al., 1999), indicates that there is a reciprocal inhibition functioning between the class I and class II genes. This would suggest that Shh acts initially to set up progenitor domains but these are then refined and maintained independently. However, there is evidence that Shh is still required at late stages of differentiation for some ventral cell types (Ericson et al., 1996). The reciprocal inhibition demonstrated between class I and class II genes may serve to sharpen the boundaries, and allow for the maintenance of the progenitor domains in the expanding neural tube.
The homeobox code

(a) Shh mediates the repression of class I homeodomain proteins (Pax7, Dbx1, Dbx2, Irx3 and Pax6) at different threshold concentrations and the induction of expression of class II proteins (Nkx6.1 and Nkx2.2) at different threshold concentrations. Class I and class II proteins that abut a common progenitor domain boundary have similar Shh concentration thresholds for repression and activation of protein expression, respectively. Shh signalling defines five progenitor domains in the ventral neural tube.

(b) The pairs of homeodomain proteins that abut a common progenitor domain boundary (Pax6 and Nkx2.2; Dbx2 and Nkx6.1) repress each other's expression.

(c) The relationship between neural progenitor (p) domains and the positions at which post-mitotic neurons are generated along the dorsoventral axis of the ventral spinal cord.

Diagram taken from (Jessell, 2000)
Figure 1.4

a) Pax7, Dbx1, Dbx2, Irx3, Pax6

b) Class I: Pax6, Nkx6.1, Class II: Nkx2.2

Class I Neuronal fate

Class I
Class II
Neuronal fate
1.2.2 The Hh signalling pathway

How are the different extracellular concentrations of Shh interpreted by neural cells to direct different transcriptional responses? Analysis of the Shh signalling pathway has led to an understanding of how Shh is involved in setting up progenitor domains.

Initially, insights into the signalling pathway were discovered by work done in Drosophila. Here, signalling by the homologous molecule Hedgehog (Hh) acts via the transmembrane proteins patched (ptc) and smoothened (smo) to trigger smo activity (figure 1.5). Activation of smo results in the release of the zinc-finger transcription factor Cubitus interruptus (Ci), which is tethered by a cytoskeletal-associated complex that includes the proteins fused, suppressor of fused and costal (Aza-Blanc and Kornberg, 1999; Methot and Basler, 2001). In the absence of Hh signal, Ci is proteolytically cleaved to a 75 kD repressor form (CiR), whereas in the presence of Hh signal this cleavage is prevented and Ci instead functions as a transcriptional activator (Aza-Blanc et al., 1997; Ingham and McMahon, 2001; Methot and Basler, 1999). Hh signalling is also required to potentiate the transcriptional activity of Ci, possibly through increasing its nuclear accumulation (Chen et al., 1999; Ohlmeyer and Kalderon, 1998; Wang and Holmgren, 2000). Loss of Hh function results in all Ci being converted into CiR, thus resulting in a more severe phenotype in Hh mutants than in Ci mutants, which do not have CiR (Methot and Basler, 2001).

Vertebrates have been shown to have three homologues of Ci: Gli1 Gli2 and Gli3, (Hui et al., 1994; Ruppert et al., 1990) all of which are expressed in the neural tube. There is a high degree of conservation of components of the Hh signalling pathway between different species, and all three Gli proteins have been implicated in vertebrate Hh signal transduction. However, the situation in vertebrates is more complicated, not only because there are three Gli genes, but also because these three Gli proteins also appear to have different
Hh signalling pathway

(A) In the absence of Hedgehog (Hh) the Hh receptor Patched (ptc) represses the activity of the transmembrane protein Smoothened (smo). The transcription factor Cubitus interruptus (Ci) is tethered to microtubules by the proteins Cos2 and Fused. This binding allows the cleavage proteins PKA and Slimb to cleave Ci into a transcriptional repressor that blocks transcription of particular genes.

(B) The binding of Hh to ptc results in the release of inhibition of smo. Smo releases Ci from the microtubules (probably by adding more phosphates to cos2 and fused), and inactivates PKA and Slimb. The full length Ci protein can then act as a transcriptional activator of Hh responsive genes.

Figure 1.5 The Hh signalling pathway

(A) Patched protein

Cytoplasm

Microtubule

Fused

Cos2

PKA

Slimb

P

Ci

Patched inhibits smoothened

Repressor

No transcription of Hedgehog-responsive genes

(B) Hedgehog

Smoothened inhibits PKA and Slimb

PKA

Cos2

Slimb

P

Fused

Ci

Activation

Transcription of Hedgehog-response genes
properties. As with Ci, both Gli2 and Gli3 can be cleaved into repressor forms, although only Gli3 is cleaved in a Hh-dependent manner. Gli1 on the other hand is resistant to cleavage (Aza-Blanc et al., 2000; Dai et al., 1999; Litingtung et al., 2002; te Welscher et al., 2002; Wang and Holmgren, 2000).

1.2.3 The Gli genes and dorso-ventral patterning

In recent years, a great deal of progress has been made into deciphering the properties of the three Gli genes and their role in dorso-ventral patterning, through expression analyses, gain-of-function and loss-of-function studies in vivo, and biochemical studies in heterologous systems and cell lines (Jacob and Briscoe, 2003)

Expression of each Gli gene appears to be differentially regulated. Gli1 is directly transcribed in response to Shh signalling, and therefore expressed immediately adjacent to Shh-expressing tissues, whilst Gli2 transcription is independent of Shh signalling, and is expressed throughout the neural tube during development. Likewise, Gli3 is initially expressed throughout the neural tube, but is later restricted to dorsal regions, being suppressed in response to Shh signals (Grindley et al., 1997; Hynes et al., 1997; Lee et al., 1997; Litingtung and Chiang, 2000; Marigo et al., 1996; Rowitch et al., 1999).

Mutations in Gli1 result in mice that are phenotypically normal (Park et al., 2000). However, ectopic expression of Gli1, but not Gli2 or Gli3, can induce expression of the floor plate marker, the basic helix-loop-helix transcription factor Foxa2 (HNF3β) in the dorsal central nervous system (CNS) of mouse and frog embryos (Hynes et al., 1997; Lee et al., 1997; Marine et al., 1997; Park et al., 2000; Sasaki et al., 1999). Mice with mutations in Gli2, however, die at birth and have defects in floor plate and V3 interneuron development (figure 1.6), as well as abnormalities in many other tissues (Bai et al., 2002; Ding et al., 1998; Matise et al., 1998; Mo et al., 1997; Motoyama et al., 1998;
Park et al., 2000). These data indicate that Gli2 is the major mediator of Shh signalling, whereas Gli1 is not required in development. However, studies in which Gli1 was expressed in the same spatial and temporal manner as Gli2 throughout development, by using a gene targeting knock-in approach to replace the Gli2-coding sequences with those of Gli1 (Bai and Joyner, 2001), show that Gli1 can functionally substitute for Gli2 in all developmental processes in mice. This raises the question as to why endogenous Gli1 does not compensate for the loss of Gli2 in mutant mice. One possible explanation is that Shh signalling acts through Gli2 in order to initiate Gli1 transcription. Therefore, in Gli2 mutants, Gli1 transcription would be decreased and unable to compensate for the lack of Gli2. Indeed, in Gli2 mutant embryos, expression of Gli1 is down-regulated (Ding et al., 1998). However, when both copies of Gli2 are replaced with Gli1, lethality occurs due to new gain-of-function defects, despite CNS patterning being normal. These defects are due, at least in part, to Gli1 interfering with Gli3 function.

The differences seen in Gli1 and Gli2 function may be a consequence of post-transcriptional modifications. Gli2 has been shown to have both activator and repressor domains, and can be cleaved in a Shh-independent manner, although only the activator function of Gli2 is required during mouse embryonic development (Bai and Joyner, 2001). Gli1 on the other hand is resistant to cleavage, and can act as a constitutive activator (Bai and Joyner, 2001; Dai et al., 1999; Sasaki et al., 1999; Yoon et al., 1998). It seems that when Gli2 or Gli3 are injected into ectopic sites in the dorsal neural tube, they are converted to their repressor forms, whereas Gli1 is not and so is able to induce ectopic ventral markers. This is supported by experiments where ectopic Gli1 has been expressed together with Gli2 or Gli3, and the ectopic activation function of Gli1 has been inhibited (Ruiz i Altaba, 1998).

There is evidence that, prior to its repression by Shh to the dorsal spinal cord, Gli3 acts as a weak activator of Shh signalling in the ventral spinal cord. In the neural tube of Gli1 -/-; Gli2 -/- mutant mice, the patterning defects are
The role of Gli genes in dorso-ventral patterning of the spinal cord

Schematic representation of the different cell types along the dorso-ventral axis of the spinal cord following null mutations of the Gli genes. In Shh -/- mutants, most ventral cell types fail to form, however, they are present in both Gli1-/- and Gli3-/- mice. Mutations in Gli2 result in the absence of floor plate, and defects in V3 interneuron development.

The status of dorsal cell types that have not been generally assessed is denoted by a question mark, and in the case of the roof plate, by a broken line.

Adapted from Ruiz i Altaba et al, 2003
much milder than those in Shh -/- mutants, for example, in the absence of Gli1 and Gli2, motor neurons are able to form, whilst in Shh -/- mutants no motor neurons are detected. This suggests that either Gli3 or a non-Gli activity induces/promotes the development of ventral cell fates (Park et al., 2000). In Gli2 -/-; Gli3 -/- double mutants, the differentiation of ventral neuronal subtypes is more affected than in Gli2 mutants alone. Additionally, Gli3 activates transcription from the promoters of Gli1 and Ptc in cultured cells (Dai et al., 1999; Shin et al., 1999). Furthermore, it has been shown that Gli3 can induce floor plate and V3 progenitors in the rostral neural tube and only simultaneous inactivation of Gli2 and Gli3 can abolish motor neuron development (Motoyama et al., 2003). Nonetheless, mice with a mutation in Gli3 (extra toes, Gli3 Xtj) have dominant dorsal brain defects and polydactyly, but have a relatively normal spinal cord (figure 1.6) (Ding et al., 1998; Litingtung et al., 2002; Theil et al., 1999). The lack of spinal cord phenotype seen in Gli3 mutants may be due to its weak activator function being compensated by Gli2 (Sasaki et al., 1999).

1.2.4 Studies on Gli3 provide evidence for additional signalling pathways in dorso-ventral neural tube patterning.

As with Gli2, Gli3 has an N-terminal repressor domain, and work by several labs has demonstrated that, in the absence of Shh, Gli3 is processed into a transcriptional repressor amino-terminal fragment (Gli3R) (Aza-Blanc et al., 2000; Dai et al., 1999; Ruiz i Altaba, 1999; Wang and Holmgren, 2000). Activation of the Shh response in the ventral spinal cord prevents formation of this repressor form, allowing the transcription of Shh-response genes (Aza-Blanc et al., 2000; Dai et al., 1999; Marine et al., 1997; Sasaki et al., 1999; Shin et al., 1999; Wang and Holmgren, 2000). The negative role becomes prominent in the ventral spinal cord only if Shh function is absent (Chiang et al., 1996).
Studies using a truncated Gli3 protein, which acts as a constitutive repressor that is insensitive to Shh signals, have demonstrated that the repressor form of Gli3 prevents expression of class II progenitor proteins, while mediating the ectopic expression of class I proteins in the ventral neural tube (Meyer and Roelink, 2003). It seems likely therefore, that the Shh gradient in the ventral neural tube acts directly to set up a gradient of Gli3R activity by preventing Gli3 processing.

Analyses of Gli3-/-; Shh-/- double mutant mice reveals that loss of Gli3 partially restores the Shh -/- phenotype, as evidenced by the restoration of most ventral cell types, including motor neurons, V1, and V2 inter-neurons, which are absent in Shh -/- mice (figure 1.7) (Litingtung and Chiang, 2000). This partial rescue of the Shh-/- phenotype by Gli3-/- suggests that Shh is required to expose a pre-existing pattern, hidden by Gli3, and imposed by a patterning signal other than Shh.

One candidate molecule for the Shh independent patterning of the dorso-ventral axis is retinoic acid (RA). Retinoids are expressed in the paraxial mesoderm and the notochord, and exposure to RA has been shown to induce ventral interneuron differentiation in a Shh independent manner (Pierani et al., 1999a). However, in the absence of Shh signalling, the differentiation of definitive dorsal cell types such as neural crest, roof plate, and dorsal interneurons is still restricted to the dorsal neural tube (Liem et al., 1997) suggesting the additional action of 'dorsalising' inductive signals.

1.2.5 BMPs act as dorsalising agents to pattern the dorso-ventral axis of the neural tube

The most likely candidates for Shh independent patterning signals are members of the TGFβ superfamily (see box 2). The TGFβ superfamily of signalling molecules has two general branches, the BMP/GDF and
**Gli3/- results in a partial rescue of Shh/- spinal cord.**

Schematic representation of the different cell types along the dorso-ventral axis of the spinal cord following null mutations of Shh and Gli3. Loss of Shh results in the loss of most ventral cell types in the spinal cord, however, a partial rescue is observed in Gli3/-;Shh/- double mutants, indicating a Shh independent signal is also acting to pattern the neural tube. The status of dorsal cell types that have not been generally assessed is denoted by a question mark. Multiple neuronal types within a single coloured domain indicates an overlap of cell types.

Adapted from Ruiz et Altaba et al, 2003
TGFβ/Activin/Nodal families, whose members have diverse, yet often complementary effects (Massague, 2000). It is thought that the BMP/ GDF branch is responsible for dorso-ventral patterning as many members of this branch, including BMP4 and BMP7, are expressed in the ectoderm overlying the neural tube and later in the dorsal part of the neural tube, including the roof plate (Figure 1.2) (Lee et al., 1998a; Liem et al., 1997; Liem et al., 1995a). It has been difficult to demonstrate an absolute requirement for BMPs in dorso-ventral patterning and dorsal cell fate specification as mice lacking BMP2 or BMP4 activity die early in embryogenesis. Mice that lack BMP7 functions have defects in growth and morphogenesis of the eye, but abnormalities in neural crest and dorsal spinal cord development have not been detected. The failure to demonstrate essential functions for specific BMPs in early dorso-ventral patterning of the neural plate may reflect functional redundancy between BMP family members (Barth et al., 1999; Dudley and Robertson, 1997; Lee et al., 1998a; Nguyen et al., 2000).

Despite the failure to demonstrate essential functions for specific BMPs in early dorso-ventral patterning of the neural plate, a large body of evidence has accumulated to suggest that they do play a central role. Studies in vitro have shown that the induction of neural crest and roof plate cells by epidermal ectoderm in chick is mimicked by BMP4 and BMP7 (Basler et al., 1993; Liem et al., 1997; Liem et al., 1995a). Additionally, the generation of dorsal neuronal cell types has been shown to be dependent on BMP signals from the roof plate, as genetically ablating the roof plate or using BMP antagonists in vivo, results in the failure of dorsal cell types to form (Lee et al., 2000; Liem et al., 1997). Moreover, mutations in the BMP family member GDF7, which is expressed at slightly later times within the roof plate, result in the elimination of the D1A class of dorsal neurons (Lee et al., 1998b)

Five distinct classes of dorsal interneurons have been described on the basis of their expression of LIM homeodomain factors (Lee et al., 2000; Lee et al., 1999; Pierani et al., 2001). These interneuron populations, D1A, D1B, D2, D3,
and D4, express LH2A, LH2B, Islet1, Lim1/2, Lmx1b, respectively (figure 1.8). Recently, progress has been made in defining progenitor populations in the dorsal neural tube by their expression of bHLH factors (Gowan et al., 2001; Timmer et al., 2002). Cash1 (Chick acheate-scute homologue), Ngn1 (Neurogenin 1), Ngn2 (Neurogenin 2) and Cath1 (Chick atonal homologue1) are expressed in mutually exclusive domains in the dorsal neural tube, and their role in specifying distinct neuronal identities has been shown using Math1 (Mouse atonal homologue1), Ngn1, and Ngn2 mutant mouse strains, as well as through the ectopic expression of these bHLH factors in chick neural tube (Gowan et al., 2001).

Recent work has demonstrated a direct role for BMPs in regulating the expression patterns of these bHLH genes in the dorsal neural tube (Timmer et al., 2002). BMPs were found to set the dorsal border of Cash1, as high levels of BMP repress its expression. Low levels of BMP signalling were found to induce the expression of Ngn1, again, setting up the expression of this gene at a specific distance from the roof plate (figure 1.8b). In addition to BMPs regulating the expression borders of these genes, the bHLH genes in this area have been shown to inter-regulate (Gowan et al., 2001). Therefore, the expression of the bHLH genes are set at specific thresholds of BMP signalling activity, which ultimately results in populations of mature neurons differentiating along a gradient of BMP activity. However, any direct relationship between bHLH protein expression and cell fate is restricted to dorsal cells, as most of these bHLH proteins are also broadly expressed in ventral cells.

Although BMPs do not appear to regulate intermediate cell types through the regulation of bHLH genes, they do play a role in regulating homeodomain proteins in this region. Specifically, activation of the BMP response causes a ventral shift in the boundary of the dorsally expressed homeodomain transcription factors Pax6, Pax7, Msx1, and Msx2, and represses expression of intermediate and ventral homeodomain proteins, such as Dbx1 and Dbx2 (figure 1.8 a, c) (Pierani et al., 1999b; Timmer et al., 2002). These results
Figure 1.8

**BMP signalling regulates many aspects of neural tube patterning.**

(A) BMP signalling regulates homeobox gene expression to define dorsal and intermediate cell fates. In co-operation with Shh singling, BMPs set the expression domain boundaries of Pax6 and Pax7. The border between dorsal and intermediate cell fates, marked by the dorsal border of high level Pax6 expression is refined by the BMP-mediated activation of Msx1, which represses Dbx2 expression.

(B) BMP signalling regulates the dorsal expression of bHLH proteins along a gradient of activity. bHLH protein expression boundaries are set by thresholds of BMP signalling. bHLH expression domains give rise to a limited number of types of terminally differentiated neurons.

(C) BMP signalling promotes a diversity of intermediate cell fates. BMP regulation of Pax7 sets a dorsal limit on the generation of Evx1-expressing neurons. BMP regulation of the dorsal border of Dbx1-expressing cells may help divide the Pax2 + , Lim1/2 + cells into two distinct progenitor populations.

Diagram taken from Timmer et al, 2002
Figure 1.8

**A.**
- BMPs
- Shh
- BMPs
  - Pax7
  - PAX6
- Shh

**B.**
- Dorsal Cell Population
- Intermediate Cell Population
- Post-mitotic neurons
  - Lh2A/B
  - Lim-1/2
  - Isl-1
  - Lim-1/2
  - Lmx1b
  - D1A
  - D1B
  - D2
  - D3
  - D4

**C.**
- Pax7
- Dbx1
- Dbx2
- Pax2+ and Lim1/2+
demonstrate that the proteins which comprise the progenitor domains can be both Shh- and BMP-responsive.

Regulation of both bHLH and homeodomain transcription factors by BMPs have been shown to be concentration dependent, suggesting that BMPs can also act as morphogens in generating patterning information (Lee et al., 1998b; Liem et al., 1997; Pierani et al., 1999b; Timmer et al., 2002). Increasing evidence indicates that dorsally derived BMPs influence patterning in ventral regions of the neural tube. BMP antagonists expressed in the notochord are able to diffuse through the neural tube and regulate the activity of BMPs in ventral cells (Liem et al., 2000; McMahon et al., 1998; Patten and Placzek, 2002). Additionally, mutations in the BMP signalling pathway in zebrafish show an expansion of ventral neural fates, indicating that appropriate BMP levels are required for normal neural fate specification at all dorso-ventral levels of the neural tube (Barth et al., 1999). Thus, Shh/Gli3 independent patterning may rely, at least in part, on a gradient of BMP activity set up by BMP expression in the dorsal ectoderm and roof plate of the neural tube, and the expression of BMP antagonists in the notochord.

Box 2

TGF-β signalling pathway.

The TGF-β family of signalling molecules has two general branches (the BMP/GDF and TGF-β/Activin/Nodal branches) whose members have diverse, yet often complementary, effects. TGF-β signalling is mediated by a family of serine/threonine receptor kinases that are classed either type I or type II. Ligand binding leads to the association of these two receptor types, resulting in the phosphorylation of the type I receptor by the type II receptor. This in turn activates Smad transcription factors and propagates the intracellular signal (Massague, 2000).
Smad proteins activated through receptor activation are collectively referred to as receptor-phosphorylated Smads (R-Smads). These fall into two distinct categories, Smad1 and its two close homologues Smad5 and Smad8 are BMP receptor substrates, whereas Smad2 and Smad3 are substrates of the related TGF-β and activin receptors in vertebrates (Kretzschmar and Massague, 1998; Massague, 2000). Phosphorlyation increases the affinity of R-Smads for Smad4, which is required for active transcriptional complexes to assemble, and to unmask its nuclear import function. As both BMP and TGF-β/Activin pathways compete for the same Smad4 protein, this can result in a mutual antagonism of the two general branches.

The response of cells to TGF-β signalling is wide ranging and often context dependant. This is due to tight regulation at every level of the pathway. Firstly, there are many extracellular molecules which are able to bind the ligands, such as noggin, chordin, follistatin and cerberus, which prevent interaction with the receptors. Additionally, the TGF-β superfamily are active as dimers, and so co-expression of different family members can result in heterodimer formation, with the composition of these molecules dramatically affecting the signalling activity. Recent reports have suggested that BMP7/Nodal heterodimers can form, resulting in an antagonism of BMP signalling (Soubes et al., in preparation). Each ligand may have a choice of several type I and/or type II receptors and a given cell may express different receptor forms. At the level of the Smads, numerous repressive and activational molecules have been found which dictate the transcription of specific target genes (Massague, 2000).

Seven type I or activin-like kinases (ALKs) and five type II receptors have been identified in vertebrates, yet only BMPR-1a (ALK-2), BMPR-1b (ALK-3), and ALK-6 appear to specifically transduce BMP signals (Solloway and Robertson, 1999), and the majority of responses to BMP signals are covered through the actions of BMPR-1a and BMPR-1b (Panchision et al., 2001).
BMPR-1a is expressed throughout the neural tube and its activation results in the up-regulation of BMPR1b. BMPR1b is therefore expressed in and next to areas of BMP expression. It is thought that the serial action, first of BMPR1a, and then of both R1a and R1b, provides a mechanism for concentration and temporal differences in BMP signalling, as distinct roles have been suggested for the two receptors (Panchision et al 2001).

The complexities in the TGF-β signal transduction pathway, and the many ways in which the competence of the cell to respond to these signals can be altered by other, external signals and developmental history, mean that the outcome of TGF-β signalling is highly dependent upon the cellular context. Thus it seems that the cell itself determines the outcome of the signal, as a result of inductive and repressive cues, received both at the time of the signal transduction, and at earlier time points throughout development.
1.3 Integration of BMP and Shh signals: antagonism at multiple levels

The weight of evidence, then, currently suggests that the integration of Shh and BMP signalling patterns the dorso-ventral axis of the neural tube in the normal embryo, and suggests largely that Shh and BMP7 exert antagonistic effects in dorso-ventral neural tube patterning. Indeed, numerous studies have shown that BMPs and Shh have antagonistic effects on one another, both at the level of protein interactions, and at the transcriptional level (Liem et al., 2000; Monsoro-Burq and Le Douarin, 2001; Panchision et al., 2001; Soubes et al., in preparation; Watanabe et al., 1998).

Explants exposed to a fixed concentration of Shh will give rise to neurons and progenitor populations with a more dorsal identity when cultured with BMPs, than when cultured with Shh alone. Conversely, exposure of neural explants to BMP antagonists results in a dorsal-to-ventral switch in progenitor cell identity and neuronal fate (Arkell and Beddington, 1997; Basler et al., 1993; Liem et al., 2000). This indicates that there is an interaction down-stream of the protein, which modifies the response to Shh signalling. Moreover, the antagonism seen at the protein level also appears to work in the opposite direction, with Shh protein modulating the response of cells to BMP signals (Liem et al., 1995a).

There are a number of mechanisms by which the mutual modulation of these two signalling pathways may occur. Firstly, Gli proteins have been shown to form part of a complex which is involved in sequestering Smads (Liu et al., 1998), the transcriptional effectors of BMP signalling (see box 2). Thus, Shh and BMP signalling may converge in neural cells at the level of a transcriptional regulatory complex that contains both Smad and Gli proteins.

Another way in which the two pathways can interact is through the regulation of BMP receptors by Shh. BMP signals are thought to act initially through
BMPR1a, which then causes the up-regulation of BMPR1b. However, Shh has been shown to antagonise this response, preventing the expression of BMPR1b (Panchision et al., 2001).

Recent studies have indicated that BMPs are able to maintain the expression of Gli3 in explants (Meyer and Roelink, 2003), and it is likely that BMPs are also responsible for maintaining Gli3 expression in the ventral neural tube in the absence of Shh. Therefore BMP-mediated antagonism of the Shh signal may be through maintenance of Gli3 transcription (Figure 1.9). The action of BMPs in controlling intermediate cell fate appears to be through the regulation of homeodomain transcription factors. Signalling by BMPs appears to simultaneously induce expression of class I transcription factors, whilst promoting the expression of Gli3, which in turn represses the expression of class II proteins, creating dorsalised progenitor domains. Shh on the other hand promotes the expression of class II transcription factors whilst antagonising the repressive action of Gli3 on their expression.

Additionally, Shh and BMPs can act antagonistically at the mRNA level. Beads soaked in BMP4 can down-regulate Shh mRNA expression lateral to the Node (Monsoro-Burq and Le Douarin, 2001). Similarly, explants of axial mesoderm cultured in BMPs show a decrease in Shh transcription (Soubes et al., in preparation).

Together, then, in most of the neuraxis, Shh and BMPs are expressed in cells at opposite poles along the dorso-ventral axis of the developing neural tube and exert mutually antagonistic effects, both at the level of mRNA transcription and by interfering with one another's downstream signalling pathways.
Figure 1.9

BMP - Shh antagonism through regulation of Gli3.
Activation of the BMP response results in expression of class I genes and Gli3, which are thought to mediate dorsal differentiation. Activation of the Shh response represses Gli3 expression and conversion to Gli3R, whilst promoting expression of class II genes, thought to promote ventral cell fates.

Diagram taken from Meyer and Roelink, 2003
1.4 Shh and BMP interactions within the forebrain

Although, as outlined in section 1.1, region-specific signals can alter the outcome of Shh and BMP signalling at different anterior-posterior levels, the basic model, whereby an antagonism between the two molecules results in the dorso-ventral patterning of the neural tube, appears to operate throughout most of the neuraxis, including the midbrain, hindbrain and spinal cord. Likewise, in the forebrain, loss of Shh signalling results in the loss of ventral cell types, as well as cyclopia and holoprosencephaly. Conversely, overexpression of BMPs through addition of protein-soaked beads or loss of BMP antagonists produces a very similar phenotype, which indicates that BMP-Shh signalling antagonism is required for correct morphogenesis and patterning (Anderson et al., 2002; Golden et al., 1999).

Implantation of BMP-soaked beads into the telencephalon results in a down-regulation of Shh mRNA in ventral cells that extend from the telencephalon through to the midbrain (Ohkubo et al., 2002). Interestingly, following the use of cycloheximide to block protein synthesis, Shh is still down-regulated from the retrochiasmatic (ventral telencephalon) area, but its expression remains in regions posterior to this. This indicates that the manner in which BMP antagonises Shh mRNA occurs very distinctly in different forebrain regions: BMPs down-regulate Shh directly in the retrochiasmatic region, but down-regulate Shh indirectly in more posterior regions, including those that will form the hypothalamus.

The ability of BMPs to down-regulate Shh, albeit indirectly, within the hypothalamus, under experimental conditions, presents something of a paradox, given earlier work. These earlier studies had shown that one area of the neuraxis in which the dorso-ventral antagonism of Shh and BMPs does not appear to behave in the same manner as in the rest of the neural tube, is the developing hypothalamus. Studies in both chick and rodent embryos have shown that, in contrast to other regions of the neural tube where Shh
and BMPs are expressed at opposite dorso-ventral poles, in ventral-most regions of the hypothalamus that will form the hypothalamic infundibulum, and in underlying prechordal mesoderm, BMP7 is co-expressed with Shh. Moreover, the combined activity of Shh and BMP7 is required to induce a ventro-lateral hypothalamic cell phenotype (Dale et al., 1997; Dale et al., 1999; Ohyama et al., in preparation). As yet, the mechanism by which Shh and BMP7 come to be co-expressed, and act co-operatively, rather than antagonistically, remains unclear. However, one possibility is suggested by studies in cell lines that show that, at high concentrations, Shh may initially activate, rather than suppress BMP transcription. In the mouse anterior pituitary, Shh is thought to induce ventral BMP2 expression (Burgess et al., 2002). Additionally, several Gli protein binding motifs have been found on the promoters for BMP4 and BMP7, and these promoters can be stimulated on co-transfection of Gli1 or Gli3 into COS-7 cells (Kawai and Sugiura, 2001). No such stimulation was detected when Gli proteins were transfected into Hos cells, indicating a cell context-dependent stimulation. However, these observations raise the possibility that, as has been observed with other signalling molecules (e.g. Nodal-Lefty), a signalling molecule may, in some cases, induce expression of a repressor that subsequently limits the initial signal-activity.

These observations, then, show an unusual co-expression of Shh and BMP7 in prechordal mesoderm and prospective hypothalamic infundibular cells, at least at early stages of development. Further support for an unusual ventral action of BMPs within this region derives through analyses of the Gli2/-mutant mouse. In these mice, Gli3 is able to induce ventral character, including Shh expression, in diencephalic cells that will form the ventral hypothalamus, but is not able to induce Shh in posterior regions of the neuraxis (Motoyama et al., 2003). Thus, the control of ventral character in the forming hypothalamus appears to be very different to that in posterior regions of the neural tube. A likely explanation is that the ventral expression of BMP7 within the prechordal mesoderm and prospective infundibulum maintains Gli3
expression ventrally, where it is able to act as a positive regulator of Shh expression (see section 1.3).

1.5 Transcriptional control of Shh

The ability of BMPs to down-regulate expression of Shh, both directly and indirectly (see section 1.3) raises the question of how Shh expression is regulated at the transcriptional level. Direct analyses of cis-acting sequences that regulate Shh expression in zebrafish and mouse embryos has revealed discrete regulatory enhancers within the Shh promoter/introns that direct Shh expression in the notochord and ventral regions of the neural tube (figure 1.10) (Epstein et al., 1999; Jeong and Epstein, 2003)(Strahle et al, 2004).

In the mouse, three separate enhancer regions regulate Shh expression: Shh brain enhancer 1 (Sbe1), drives Shh expression in the ventral midline of the midbrain and caudal diencephalon, Shh floor plate enhancer 1 (Sfpe1), drives floor plate expression caudal to the midbrain, and Shh floor plate enhancer 2 (Sfpe2), acts in conjunction with Sbe1 to drive expression in the floor plate, the diencephalic ventral midline and notochord (Epstein et al., 1999). No combination of these enhancer regions resulted in the reporter construct being expressed in the telencephalon, indicating that the control of Shh expression in this region is through other areas of the promoter not looked at in these studies.

Further analysis of the Sfpe2 region has revealed a number of conserved sequences which correspond to binding sites for various transcription factors expressed within the neural tube, including Foxa2 (HNF3β), Foxh1 (Fast1, a component of the Nodal signalling pathway), and a homeobox domain. Deletion/mutation studies show that these binding sites are required for the positive regulation of Shh expression (Jeong and Epstein, 2003). In addition, within the 88 bp sequence within Sfpe2 is a highly conserved binding site that
Schematic of the Shh locus in zebrafish (top) and mouse (bottom), showing coding exons (green boxes), non-coding sequences (solid line) and intronic enhancers (red ovals). Homologous enhancer regions between species are shown by fading black lines. Areas in which individual enhancer regions have been shown to drive Shh expression are indicated next to the enhancers.

Diagram from Strahle et al, 2004
matches the consensus for homeodomain Tbx transcription factors. Mutational analysis reveals that, in contrast to Foxa2, Foxh1 and the homeodomain consensus binding sites, the Tbx binding site is required for the repression of Shh in the hypothalamic infundibulum.

Together, these mutational analyses point to the ability of complex signalling networks to control Shh mRNA levels, both positively and negatively. Of particular interest to my studies is the observation that Shh can be negatively regulated within the mouse infundibulum (see section 3).

1.8 Summary

Together, the studies outlined in the Introduction suggest that in the posterior neural tube, Shh and BMPs act antagonistically. However, in the hypothalamus, at early stages of development, BMP7 and Shh are initially co-expressed within prechordal mesoderm and within ventral midline hypothalamic cells and can co-operate to induce ventro-lateral hypothalamic cells. The co-expression and co-operation of BMP7 and Shh are highly unusual, and raise the question of whether this is a transient event, followed by a more predictable antagonism. The major aim of my thesis was to clarify this, in the developing chick embryo by:

- Constructing a fate map of the early hypothalamus
- Assessing the expression patterns of Shh and BMPs at various stages of hypothalamic development
- Addressing whether, and how, BMPs act antagonistically to Shh at later stages of hypothalamic development.

My studies show that:

- Early hypothalamic cells that initially co-express Shh and BMPs and lie
adjacent to Shh+ BMP7+ prechordal mesoderm give rise to Shh-hypothalamic cells.

- BMP2 and BMP7 are expressed in and beneath hypothalamic infundibular cells that will down-regulate Shh.
- BMPs can down-regulate Shh in prospective hypothalamic infundibular cells, and are required to down-regulate Shh in vivo.
- BMPs govern expression of the T-box homeodomain transcription factor, Tbx2. Expression of Tbx2 is reciprocal to that of Shh, both normally and after experimental manipulation, suggesting a mechanism for the BMP-mediated down-regulation of Shh.
- In addition to regulating expression of Shh, BMPs/Tbx may govern cell cycle within the forming infundibulum.

Together, these studies suggest that BMPs/Tbx2 exert a dual effect on Shh-expressing cells of the ventral hypothalamus, simultaneously regulating the character and spatial extent of a distinct progenitor domain, with cells within the domain synchronised in their ability to respond to further signals.
Chapter 2

Materials and Methods
2.1 In vivo manipulations

2.1.1 Fate Mapping

HH stage 9 embryos were accessed in ovo by opening a small window in the eggshell and removing the membranes overlying the embryo. Black Indian ink diluted in Leibowitz's L15-Air medium (1:100) was then injected underneath the embryo in order to visualise it. In order to access the ventral neural tube, a small hole was made in the vitelline membrane above the forebrain, then an incision made in the dorsal neural tube (see fig 3.2). Prospective hypothalamic cells were identified by reference to their position relative to the underlying axial mesoderm, and focal injections were made using a picospirtzer II microinjection system (General valve corporation), with a solution of Dil, 5mg/ml in absolute ethanol (Molecular Probes). After injecting, the embryos were removed and fixed immediately for analysis by in situ hybridisation, or the eggs were resealed and incubated for 24 hours until HH stage 16-18 prior to fixation and analysis by immunohistochemistry.

2.1.2 Bead implantation

Affigel blue beads (Biorad) were soaked in Chordin protein for 24 hours at 4°C prior to implantation. HH stage 5-6 embryos were accessed in ovo through a small window in the eggshell, and visualised using Indian ink. Beads were then inserted under the vitelline membrane and positioned adjacent to the neural plate, above the prechordal mesoderm. The eggs were resealed and incubated for 30 hours until HH stage 16-18, prior to fixation and analysis by immunohistochemistry or in situ hybridisation.
2.1.3 Prechordal mesoderm ablations

Embryos were prepared and maintained in New culture (New 1955; Stern and Ireland 1981) using Pannett-Compton saline (Pannett and Compton 1924), such that the ventral surface of the embryo faced uppermost. The prechordal mesendoderm at HH stage 4/5, identified by morphology and comparison with gsc expression (not shown) was removed using a microsurgery knife. A cut was made horizontally through the endodermal and mesodermal layers at the anterior limit of Hensen’s node prior to gentle scraping away of both endodermal and mesodermal tissue anterior to the node. Any embryos in which the epiblast layer was damaged were discarded. Following surgery, embryos were maintained in New culture for around 20 hours until stage 10.

2.2 Explant culture

All embryos were staged and dissected in cold L15 medium (Gibco-BRL) Explants of anterior ventral midline from stage 7 were prepared by isolating the neurectoderm from the axial mesoderm using Dispase (1mg/ml). Parallel cuts were made either side of the midline, and the section from the prospective forebrain removed, identifiable by its morphology. Explants were then cultured in collagen beds according to published techniques established in the lab (Placzek and Dale 1999).

2.3 In situ hybridisation and immunohistochemistry.

Embryos and explants were analysed by immunohistochemistry according to standard techniques (Placzek et al. 1993). Following cryostat sectioning (15μm sections), the following monoclonal antibodies were used (dilutions in parentheses): 68.5E1, anti-Shh (1:50) (Ericson et al. 1996); anti-Phosphorylated Histone H3 (anti-PH3) (Upstate; Hendzel et al, 1997)
Secondary antibodies (Jackson Immunoresearch) were conjugated to Cy3 or fluorescein isothiocyanate (FITC).

Embryos were processed for in situ hybridisation as described previously (Vesque et al. 2000). The following template DNAs were used to generate digoxigenin labelled antisense RNA probes (Vesque et al. 2000); Plasmid pcBMP2 containing a cDNA fragment encoding chick BMP2 was linearised with Hind III and transcribed with T3 RNA polymerase. Plasmid pBH2 containing a cDNA fragment encoding chick BMP7 was linearised with Xho I and transcribed with T3 polymerase. Plasmid pcBMP4 containing a cDNA fragment encoding chick BMP4 was linearised with BamH1 and transcribed with T3 RNA polymerase. Plasmid pCRII containing a cDNA fragment encoding chick BMP6 was linearised with XbaI and transcribed with SP6 polymerase. Plasmid pMT21 containing a cDNA fragment encoding chick Noggin was linearised with Xho I and transcribed with T3 polymerase. Plasmid pMT23 containing a cDNA fragment encoding chick Chordin was linearised with Eco R1 and transcribed with SP6 polymerase. Plasmid pcvhh containing a cDNA encoding chick sonic hedgehog was linearised with Sal-I and transcribed with SP6 polymerase. Plasmid PBS Sk containing a cDNA fragment encoding chick Tbx2 was linearised with Sal 1 and transcribed with T7 polymerase. Plasmid PBS Sk containing a cDNA fragment encoding chick Tbx3 was linearised with Not 1 and transcribed with T3 polymerase. Plasmid PBS Sk containing a cDNA fragment encoding chick Tbx4 was linearised with EcoR1and transcribed with T3 polymerase. Plasmid PBS Sk containing a cDNA fragment encoding chick Tbx5 was linearised with Not 1 and transcribed with T3 polymerase. Plasmid PBS Sk containing a cDNA fragment encoding chick Tbx15 was linearised with Not 1 and transcribed with T7 polymerase. Plasmid PBS Sk containing a cDNA fragment encoding chick Tbx18 was linearised with Bam H1 and transcribed with T7 polymerase. (Tbx genes courtesy of Malcolm Logan).

Following development, embryos or explants were analysed as whole-mount preparations. In some cases, 15µm serial frozen sections were taken for detailed analysis of expression patterns.
2.4 Protein use

2.4.1 Protein production

Proteins were either obtained in purified form (hu BMP2, hu BMP7/OP1: Creative Biomolecules) were produced from transient transfections (chick chordin, chick BMP7,), or produced from baculovirus (chick chordin).

Chick BMP7 and Chordin were produced after transfection of expression constructs into COS 7 cells. Cells were grown in DMEM containing 10% fetal calf serum (FCS), and transfections performed using lipofectamine (Gibco BRL). Following transfection, cells were grown in serum-free optimem medium (Gibco-BRL) for 24hrs prior to collection of supernatants.

Chick Chordin was also generated by infection of S2 cells with baculovirus containing the VL1E chordin expression construct (see (Dale et al. 1999).

2.4.2 Proteins in functional assays

Proteins were tested for function in one of two ways: i) by addition to culture medium in in vitro assays; ii) after soaking to a bead, and bead implantation.

i) Addition of proteins to culture supernatant.

Purified proteins were used at defined concentrations – established in independent assays (Liem et al) and after Western blotting (Soubes et al). In all experiments using proteins produced through transient transfections, supernatants were concentrated ten-fold using Centri-plus columns (Amicon) and then diluted 1:10 in explant culture medium. In explant experiments, proteins were added to cultures at the start of incubation. BMP7 and BMP2 were used at concentrations of 10-100nM respectively, unless otherwise stated (as in chapter 6).
ii) Attachment of proteins to beads

Affigel Blue beads (Biorad) were washed 5 times in PBS and then soaked in protein overnight at 4°C prior to grafting.

2.5 Quantitative RT-PCR

Total RNA was isolated from single pieces of tissue (anterior medial neural plate; see figure 5.1) after in vitro culture (see section 2.2), with Strataprep Total RNA microprep kit (Stratagene, La Jolla, CA). RNA was reverse transcribed with Superscript II (Gibco-BRL,) using random primers. For each sample, half of the RNA was used for reverse transcription and the other half for a minus reverse transcriptase control.

Real time quantitative RT-PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems). Primers and probes were designed with the Primer Express software (Perkin-Elmer). Primers were purchased from Gibco-Brl and the TaqMan fluorogenic probes from Applied Biosystems. The quantification of Shh was achieved with the forward primer 5’-CGGCTTTGACTGGGTCTACT-3’, reverse primer 5’CGCTGCCACTGAGTITTCTG-3’ (generating an amplicon of 76 bp) and the Taqman probe 5’-CGAGTCCAAGGCGCACATCCACT-3’ (labelled with the reporter dye FAM on the 5’ nucleotide and the quenching dye TAMRA on the 3’ nucleotide). Amplification of β-actin as an endogenous control was used to standardise the amount of RNA in each sample. The quantification of β-actin was achieved with the forward primer 5’-GGTCATCACCATTGGCAATG-3’, reverse primer 5’-CCCAAGGAAGATGGCTGGAA-3’ (generating an amplicon of 66 bp) and the Taqman fluorogenic probe 5’-TTCAGGTGCCCGAGGCCTCCT-3’ (labelled with the reporter dye VIC on the 5’ nucleotide and the quenching dye TAMRA on the 3’ nucleotide).
Primer and probe optimisation for Shh and actin amplification were performed on notochord RNA isolated from HH st 15 chicks. Amplification of Shh was optimised with 50 nM forward primer, 900 nM reverse primer and 100 nM Taqman probe. Amplification of β-actin was optimised with 50 nM forward primer, 300 nM reverse primer and 100 nM Taqman probe. PCR amplification of Shh and β-actin was done in separate tubes in a 25 μl reaction volume with 1/8 of the cDNA obtained from the reverse transcription reaction. PCR conditions were as suggested by Applied Biosystems. Samples were run in triplicate. The minus reverse transcriptase controls were run at the same time to verify the lack of DNA contamination.

Initially a validation experiment was performed to determine that amplification of Shh and β-actin was equally efficient: Shh and β-actin expression were quantified on serial dilutions of reverse transcribed products from stage 15 notochord RNA, and the log of cDNA input versus the difference in Ct values of Shh and actin (Dct) plotted. The absolute value of the slope was inferior to 0.1 indicating that amplification of Shh and β-actin was approximately equal. Thereafter relative quantification of Shh mRNA was calculated using the comparative Dct method. Ct values refer to the cycle number, during the exponential phase of PCR amplification, at which the fluorescence, and hence the PCR product, reaches a certain threshold – i.e. the higher the Ct value, the lower the amount of mRNA extracted from the explant. The quantification of Shh was normalized to β-actin; i.e. Dct = Ct Shh - Ct Actin. In some cases, the relative amount of Shh mRNA in control explants was directly compared to that in treated explants. In this case, the amounts were calculated using the formula: $2^{-\text{DD} \text{Ct}}$ where DDct = Dct (control) - Dct (treated). In several cases, we converted the Dct levels, to express levels of Shh as a percentage of expression in explants exposed compared to unexposed explants. This was achieved using the calculation $(2^{-\text{Dct(exposed)}}/2^{-\text{Dct(unexposed)}}) \times 100$. Robust rank order tests (Siegel and Castellan, 1988) were used to determine statistical significance.
Chapter 3

Fate Mapping
3.1 Introduction

3.1.1 Complex expression of Shh in the forming hypothalamus

As outlined in the Introduction (sections 1.2 and 1.4), Shh acts as a ventralising agent throughout most of the neuraxis (Ericson et al., 1995). The idea that Shh acts as a general ventralising factor is supported by its expression profile. In most regions of the neuraxis, Shh is expressed in axial mesoderm underlying the neural tube, and then in neural tube cells that occupy ventral-most positions (the floor plate in the posterior neuraxis). Thus, in the telencephalon, midbrain, hindbrain and spinal cord, Shh is expressed in ventro-medial cells at early stages of embryogenesis, and is then maintained in these cells at later stages. In contrast, in the diencephalon, Shh is expressed in ventro-medial cells at early stages of embryogenesis, but at later stages, is no longer expressed in ventral-most regions, and is now expressed more laterally — i.e. within ventro-lateral regions of the diencephalon (Jeong and Epstein, 2003; Mathieu et al., 2002, and see section 3.2.1). The ventral diencephalon, which expresses Shh, will give rise to the hypothalamus, and so will be referred to as the hypothalamus/hypothalamic region hereafter (see figure 3.2).

The relationship between the early population of ventral-most Shh-expressing cells of the hypothalamus, and the later lateral Shh-expressing cells is unclear. One possibility is that cells initially located ventrally maintain Shh expression, but then migrate, to occupy a more lateral position. A second possibility is that the ventral cells remain at this ventro-medial position, but down-regulate Shh, while a second, more lateral population, comes to express Shh at a slightly later stage of development. As a first step in understanding the relationship between Shh and BMPs within the forming hypothalamus, I set out to distinguish between these possibilities by performing fate mapping experiments in the prospective hypothalamus of the chick embryo.
3.2 Results

3.2.1 Expression of Shh in the chick forebrain

Previous studies in the chick embryo have revealed that the expression pattern of Shh mRNA is far more complex within the hypothalamus than in other regions of the neuraxis. To extend these analyses, I examined expression of Shh protein within the forming hypothalamus, and compared expression of Shh mRNA with that of Shh protein. As shown in figure 3.1, Shh mRNA and protein show an identical distribution within the ventral diencephalon/ hypothalamus (in situ performed by MP). However, there are striking differences between expression of Shh in the spinal cord with that in the hypothalamus. In the spinal cord, Shh is consistently expressed throughout early development in a narrow group of cells which form the ventro-medial floor plate, whereas expression in the hypothalamus is much more dynamic. At stage 7, Shh expression is fairly constant along the anterior-posterior axis, so that anteriorly, within the hypothalamus, expression is confined to the medial-most cells (figure 3.1 A, D). However, by stage 10, the expression domain in the hypothalamus has begun to expand, forming an oval shape (figure 3.1 B, E). Previous studies have suggested that the expansion of Shh, which begins at HH stage 9 (not shown), reflects a vertical induction from the underlying prechordal mesoderm, which appears itself to be broad (see figure 3.3 d). As the embryo develops further, Shh expression is lost in these ventro-medial cells, the prospective infundibular region of the hypothalamus, but is now expressed in the lateral cells of the forming hypothalamus (figure 3.1 C, F).

In the interests of clarity, I have divided the forebrain of the stage 18 embryo into distinct regions, depending on morphology and/or Shh expression. Hereafter, the different regions of the forebrain will be referred to as in figure 3.2. The diencephalon is composed of two main sections, the dorsal diencephalon, which will form the thalamus, and the ventral diencephalon,
Figure 3.1

Shh is expressed dynamically in the ventral forebrain.

(A-C) Whole-mount in situ hybridisation of Shh mRNA expression on isolated chick neuroepithelium at stages 7, 10 and 18. (D-F) Transverse sections taken through embryos of the same stages, at the level of the diencephalon, the ventral region of which will give rise to the hypothalamus and infundibulum. Sections were analysed for Shh protein by immunohistochemistry (red). Shh is expressed in a narrow group of cells at stage 7 (A, D). The expression pattern is similar along the entire anterior-posterior axis. At stage 10 (B, E), there is a marked expansion of Shh expression in the ventral diencephalon (prospective hypothalamus and infundibulum; arrow), whilst in more posterior regions, Shh expression remains in a narrow domain (arrowhead). At stage 18, there is a loss of Shh in ventral most cells of the diencephalon (I), which will form the infundibulum (C, F), but it is expressed in lateral cells at this level (lateral hypothalamus; LH). Shh expression begins to be lost from the ventral diencephalic cells at around stage 15 (data not shown). (See also figure 3.2)

Divisions in the forebrain

In order to clarify areas of the forebrain subsequently referred to, divisions between different regions of the stage 18 embryo have been made, according to morphology and Shh expression. The dorsal diencephalon (DD) is composed of those cells between the midbrain (M) and telencephalon (T), dorsal to the region of Shh expression. The ventral diencephalon (VD) forms the hypothalamic area, and is composed of the following sections: the caudal hypothalamus (CH), which comprises the Shh positive diencephalic cells caudal to the zii; the lateral hypothalamus (LH), which forms the medial hypothalamic region and is composed of the Shh positive cells dorsal to the infundibulum; the infundibulum (I), which lies in the ventral medial hypothalamus and can be identified through its lack of Shh expression; the preoptic area (PO), which forms the rostral most point of the hypothalamus.

The most important areas for this study are the lateral hypothalamus and the infundibulum. These areas are seen in cross-section in figure 3.1F.
Figure 3.2

T - Telencephalon, DD - Dorsal Diencephalon, M - Midbrain, H - Hindbrain, ZII - Zona limitans intrathalamica, ON - Optic nerve, VD - Ventral Diencephalon

VD is further subdivided into: CH - Caudal hypothalamus, LH - Lateral hypothalamus, PO - Preoptic area, I - Infundibulum
which will give rise to the hypothalamus. The hypothalamic region has been further divided into the caudal hypothalamus (the Shh positive cells between the midbrain-forebrain boundary and the zona limitans intrathalamica (Zli)), the infundibulum (the Shh negative region of the ventral hypothalamus), the lateral hypothalamus (the Shh positive cells dorsal to the infundibulum), and the preoptic area (the rostral-most Shh positive region) (figure 3.2). The regions that I will refer to most in this study are the lateral hypothalamus and the infundibulum. These areas are seen in cross-section in figure 3.1F.

### 3.2.2 Fate-map of diencephalic cells

Immediate questions raised by the pattern of expression of Shh in the forebrain are a) what causes the loss of Shh in the infundibulum? and b) is the expression of Shh seen in the lateral hypothalamus at HH stage 18 due to an expansion of the ventral Shh-positive population, to a migration of the ventral Shh-positive population or to the de novo induction of Shh in the lateral hypothalamic cells?

In order to assess this, I performed fate-mapping studies of the area. Cells of the prospective hypothalamus were labelled at stage 9 or 10 using the lipophilic dye, Dil. As shown in figure 3.3, the embryo was first exposed by windowing the egg and removing the membranes. Indian ink was then injected underneath the embryo in order to visualise it, and a dorsal incision made in the neural tube so as to access the ventral cells. Dil was then injected into a small group of cells (10-30 cells) using compressed air at a controlled pressure and duration. The eggs were then re-sealed and the embryos developed to the required stage, HH stage 16-18.

The prospective hypothalamic cells were identified according to the position of the axial mesoderm, which lies directly beneath the ventral neural tube. Previous studies have shown that hypothalamic cells are induced and patterned by prechordal mesoderm, which forms a triangular shape that can...
Figure 3.3

(A) The embryo was accessed by making a small window in the egg and removing the overlying membranes. Black Indian ink was then injected underneath the embryo in order to visualise it. (B) Surrounding the yolk of the egg is the vitelline membrane which had to be cut in order to access the embryo. (C) An incision along the dorsal midline of the neural tube was then made in order to access the ventral cells. (D) The hypothalamic cells could be identified by the underlying prechordal mesoderm, which forms a triangle shape that can be seen through the neuroepithelium.

Fate-mapping technique
be seen through the neuroepithelium, as shown in schematic in figure 3.3 D. The junction of the prechordal mesoderm and notochord was judged to be the caudal-most point of the prospective hypothalamus, and measurements taken in relation to this point were used in order to target other cell groups.

Before carrying out the fate mapping, I first confirmed that distinct cell groups could be targeted with fidelity and precision. This was done by injecting specific groups of cells, then analysing their position using a genetic marker. Previous studies have shown that BMP7 is expressed in the ventro-medial cells of the forebrain at stages 8-10 (Dale et al., 1999), and in contrast to Shh, the expression of BMP7 remains in ventral-most cells over this period and does not expand. Therefore, BMP7 was used as a marker of ventro-medial cells. Figure 3.4 shows the expression of BMP7 compared to that of Shh at stage 10. Superimposing the two images reveals that BMP7 is expressed in a medial subset of Shh-expressing cells at this stage (Fig 3.4C). Specific groups of cells were identified by dividing the ventral forebrain into grid-like domains, the ventro-medial most BMP7 expressing cells, termed M1, the ventro-medial Shh+ BMP7- cells, termed M2, and a region located lateral to the Shh-positive domain, termed L1 (figure 3.4D). Divisions were also made along the anterior-posterior axis, termed a, b and c (figure 3.4C, E). Morphologically, group a cells are defined as those at the widest part the neural tube, at the point where the prechordal mesoderm ends, group b cells lie just posterior to these, and lie over the main area of prechordal mesoderm, and group c cells are those at the 'neck' of the neural tube.

Once these distinct cell groups had been identified, embryos were injected in the M1a, M1b, M2a and M2b domains, and then fixed immediately and processed for BMP7 expression by in situ hybridisation. Figure 3.5 shows stage 9-10 embryos which have been injected with Dil, then processed for BMP7 expression. When injections have targeted the M1a (n=2) and M2a (n=2) populations, the Dil is seen to label cells anterior to the BMP7-expressing region (figure 3.5 A, B), whilst when targeting the M1b population, Dil is expressed in the BMP7 positive midline (n=3; figure 3.5 C, E). When
Shh and BMP7 define M1 and M2 domains in the prospective hypothalamus at stage 10.

(A + B) Whole-mount in situ hybridisation on isolated neuroepithelium of stage 10 embryos with probes against Shh (A) and BMP7 (B). BMP7 is expressed in the ventral-most cells of the diencephalon and midbrain, whilst Shh is expressed throughout the ventral midline and in an expanded domain in the diencephalon.

(C) Schematic representation of the superimposed expression patterns of Shh (red) and BMP7 (blue), revealing BMP7 expression in a subset of the Shh positive cells. For the purpose of fate mapping, the diencephalon has been divided along the anterior-posterior axis into regions a, b and c, as defined by the ‘neck’ of the neural tube (c), the area overlying the main body of the prechordal mesoderm (b), and the end of the prechordal mesoderm (a).

(D) Schematic representation of a transverse section through the diencephalon. The expression of Shh (red) and Shh/BMP7 (blue) divide the ventral diencephalon into distinct populations, which express both BMP7 and Shh (M1), Shh alone (M2) or neither (L1). Combining these medio-lateral divisions with those along the anterior-posterior axis, as shown in (C), results in a grid-like division of the ventral diencephalon/ prospective hypothalamus at this stage (E).
Figure 3.4

Shh

BMP7
Fig. 3.5

Ventro-medial and M1 and M2 cells of the ventral diencephalon can be accurately targeted.

Stage 9-10 embryos were prepared as described in figure 3.3, and Dil (pink) was injected into the M1 (A, C), or M2 (B, D) populations at levels a or b of the ventral diencephalon (see figure 3.4). The embryos were then fixed immediately and processed for BMP7 (blue) by in situ hybridisation. In both cases, the injection into the M1 cells is in the medial-most cells. When level a has been targeted, the Dil is anterior to the limit of BMP7 expression (A), whilst when targeting level b, the Dil is contained wholly within the BMP7 positive midline (C). When the M2a cells were targeted, the Dil is again seen anterior to the BMP7 expression, and slightly to the right (B), whilst the M2b injection is seen within the anterior-posterior limit of BMP7 expression, but immediately adjacent to it (D). (E-F) Schematic representation of the level b injection sites in transverse sections (red = Shh, blue = Shh and BMP7).
Figure 3.5

M1 injection

Region a

A

Region b

C

M2 injection

B

D

E

F
injections were targeted to the M2b cells, the Dil is lateral to the BMP7 positive cells (n=2; figure 3.5D, F). These experiments show that the correct cells can be targeted accurately and consistently.

Once accurate targeting of cells had been confirmed, the experiment was then repeated, this time allowing the embryos to develop to HH stage 16-18. Once developed, the embryos were sectioned and processed for Shh expression by immunohistochemistry.

Initially, cells were targeted in the M1 domain. Surprisingly, cells targeted in the M1a region (n=1) did not appear to populate the ventral hypothalamus, but progeny of the labelled cells were found in both eyes, and the pre-optic area (figure 3.6). This result suggests that cells in region M1a of the forebrain are composed of retinal precursors, but further analysis is required to confirm this as only one embryo was analysed.

As cells of region M1a do not appear to populate the ventral hypothalamus at HH stage 16-18, the M1b population was targeted next (n=4). Figure 3.7 shows a stage 9-10 embryo with the M1b population of cells labelled with Dil. Transverse sections through the same embryo at stage 18 reveal that the progeny of the cell injected in the M1b region now populate the ventro-medial most cells of the Shh-negative infundibulum. Reconstruction of serial sections, as shown in the schematic in figure 3.7C, indicates that these cells contribute exclusively to the ventro-medial infundibulum. Dil labelled progeny were found at the most rostral end of the infundibulum but were no longer detected at the point where Shh is re-expressed in the pre-optic area.

As the cells labelled in M1b populated only the ventro-medial most infundibulum, the M2b population was labelled in order to see whether these cells gave rise to more laterally located cells within the Shh-negative infundibulum, or whether they expanded in order to populate the lateral Shh positive rostral hypothalamus. The progeny of the M2b cells also appear to populate the Shh-negative infundibulum, but occupy a more lateral position.
Progeny of M1a cells occupy both optic vesicles.
(A) Stage 9 embryo with Dil injection in the M1a domain (pink). (B + C) Wholemount views of left- and right-hand sides of the same embryo following development to stage 17. Dil labelling can clearly be seen in the optic vesicles at this stage (arrows).
Figure 3.7

*Progeny of M1b cells occupy the ventral infundibulum.*

(A) Stage 9 embryo with Dil injection (pink) in the M1b domain.

(B) Transverse sections taken through the same embryo at the level of the medial hypothalamus/infundibulum, after development to stage 18. Shh (green) is shown in the lateral hypothalamus, whilst Dil labelling (pink) is seen in the ventro-medial most cells of the infundibulum. Cell nuclei are labelled with DAPI (blue).

(C) Schematic representation of reconstructed whole mount neuroepithelium showing region of Dil labelling (pink). Progeny of the labelled cells appear to contribute exclusively to the ventro-medial infundibulum (region M1) and are not seen within Shh (red) expressing regions.
compared to the progeny of M1b cells (n=3; figure 3.8). Therefore, the progeny of the stage 10 M1/M2b cells divide the stage 18 infundibulum into two domains, the medial-most M1 cells, and the more lateral M2 cells. This then leads to the question of which cells give rise to the Shh-positive lateral hypothalamus?

To test this, I injected Dil into L1b of HH stage 9-10 embryos. Cells labelled in the L1b domain did not populate the lateral hypothalamus, but instead the progeny of this area were found in the eye (not shown). Surprisingly, the region that was found to populate the lateral, Shh positive hypothalamus were laterally-located cells immediately posterior to domain b, in a domain termed L1c (n=2), and occupying the 'neck' of the neural tube. The progeny of these cells populated not only the Shh-positive lateral hypothalamus, but also the caudal Shh-positive region of the hypothalamus and Shh-negative regions of the dorsal diencephalon (figure 3.9). As depicted in the schematic in figure 3.9 C, labelled L1c progeny occupy the Shh-negative dorsal diencephalon, the Shh positive lateral hypothalamus, and have an anterior limit at the optic chiasm. Cells in the pre-optic area are not labelled. The fact that the L1c cells give rise to both Shh positive and Shh negative cells indicates that their fate has not been specified by stage 10. It is also interesting to note that progeny of the L1c cells are at no time observed within the infundibulum, and that whilst these cells are initially situated caudally to the M1 and M2b cells which will form the infundibulum, their progeny occupy a far larger domain, covering the entire rostro-caudal extent of the diencephalon.
Progeny of the M2b cells occupy the ventro-lateral infundibulum

Transverse section taken at the level of the hypothalamus/infundibulum of a stage 18 embryo, labelled in the M2b domain at stage 9 (not shown). Shh (green) is seen in the lateral hypothalamus, whilst Dil labelling (pink) is detected in the ventro-lateral cells of the infundibulum (M2 region). Dil is not detected in the Shh positive domain.

Experiment done by Kyoji Ohyama
Progeny of the L1c cells occupy the lateral hypothalamus

(A) Stage 9-10 embryo with Dil injection in L1c domain on both sides of the neural tube (arrows).

(B) Transverse sections taken through the same embryo at the level of the medial hypothalamus/infundibulum, after development to stage 18. The lateral hypothalamus is co-labelled with Shh (green) and Dil (pink). Dil is also seen in regions dorsal to the Shh positive lateral hypothalamus. Cell nuclei are labelled with DAPI (blue).

(C) Schematic representation of reconstructed whole mount neuroepithelium showing region of Dil labelling (pink). Progeny of the labelled cells appear to contribute to the entire anterior-posterior axis of the diencephalon, including the Shh negative dorsal diencephalon, Shh positive caudal hypothalamus, the Shh positive lateral hypothalamus, and have an anterior limit at the optic chiasm (see also figure 3.2). However, no Dil labelling is detected in the Shh negative infundibulum.
In summary, the fate mapping experiments yielded the following conclusions:

1. The most ventro-medial parts of the Shh-negative infundibulum at HH stage 18 derive from ventro-medial M1 cells (region M1b) of the HH stage 9/10 embryo, which co-express Shh and BMP7.
2. More lateral regions of the Shh-negative infundibulum derive from ventro-medial M2 cells (region M2b), which express Shh, but not BMP7, at HH stage 9/10.
3. The lateral, Shh-positive hypothalamic cells at stage 18 are derived from cells which are initially Shh negative, and which lie caudal to the prospective infundibulum at HH stage 9/10.

These results are summarised in table 3.1.

Together these analyses show that the Shh-negative infundibulum derives from cells that expressed Shh at an earlier time in embryogenesis.
<table>
<thead>
<tr>
<th>Stage 10 region</th>
<th>Properties</th>
<th>Fate-maps to (stage 18)</th>
<th>Properties</th>
<th>Fate-maps to (stage 18)</th>
<th>Properties</th>
<th>Fate-maps to (stage 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BMP7</td>
<td>Shh</td>
<td>BMP7</td>
<td>Shh</td>
<td>BMP7</td>
<td>Shh</td>
</tr>
<tr>
<td><strong>M1</strong></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>M2</strong></td>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><strong>L1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>a</strong></td>
<td></td>
<td>Optic vesicle/Pre-optic area</td>
<td>-</td>
<td>+</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td><strong>b</strong></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Shh -ve ventro-lateral infundibulum (M2)</td>
<td>-</td>
</tr>
<tr>
<td><strong>c</strong></td>
<td></td>
<td>?</td>
<td>-</td>
<td>+</td>
<td>?</td>
<td>-</td>
</tr>
</tbody>
</table>
3.3. Discussion

3.3.1 Shh is down-regulated from the developing infundibulum

Analysis of Shh expression in the forebrain shows that there is an initial expansion along the medio-lateral/ dorso-ventral axis that is thought to reflect a vertical induction from the broadening prechordal mesoderm (Vesque et al., 2000). This is followed by a loss of Shh in the broad ventro-medial cells, and a concomitant novel expression of Shh in lateral cells. My fate mapping studies reveal that the ventro-medial cells (M1 and M2) remain in a ventro-medial position, and down-regulate Shh. My results also demonstrate that the lateral expression of Shh is not due to an expansion and/or migration of ventro-medial Shh-positive cells, but to the de novo expression of Shh in lateral cells. Together, these studies show that, although Shh and BMPs are initially co-expressed in prechordal mesoderm and in the ventro-medial hypothalamus (at least region M1), at later stages of development, Shh is down-regulated from ventro-medial hypothalamic cells (M1 and M2 populations).

3.3.2 Distinct compartments within the forming hypothalamus?

My fate mapping studies reveal, therefore, that the Shh-negative infundibulum and the Shh-positive lateral hypothalamus initially derive from very different cells located at different anterior-posterior regions. Thus, their differential expression of Shh at HH stage 18 may reflect distinct intrinsic properties, set up during early patterning. However, as the progeny of the L1c population of cells populate both Shh positive and Shh negative regions of the stage 18 diencephalon, this suggests that these cells are not committed to a particular ‘Shh-expressing’ state by stage 10. The early expression of BMP7 within the M1 population, however, does imply an early specification. This is further supported by studies which suggest that cells of the ventral diencephalon are specified at early stages of development as they
are derived from more posterior regions of the early embryo, close to the node, which then move rostrally to occupy their position in the forebrain (Dale et al., 1999; Varga et al., 1999; Woo and Fraser, 1995).

Another indication that the Shh-negative ventro-medial cells that will form the infundibulum and the Shh-positive lateral hypothalamic cells may have distinct properties comes from the observation that cells labelled in the M1b domain were never seen outside of the Shh negative infundibulum at stage 18, but remained within the confines of the Shh negative area at all dorso-ventral and rostro-caudal levels. Progeny from the L1c domain on the other hand, were never seen inside the Shh negative infundibulum at stage 18, although they did populate both Shh-positive and Shh-negative areas in other regions of the diencephalon. The finding that these two cell types contributed to very distinct populations may simply be explained by a tendency of cells to maintain close cohorts, and undergo little mixing/migration. Alternatively, it may indicate a difference between the cell adhesion properties of the two areas, resulting in the establishment of distinct compartments within the hypothalamus, similar to the compartments seen in the hindbrain (Dahmann and Basler, 1999; Fraser et al., 1990).

Although my experiments cannot distinguish between these two hypotheses, this idea of compartmentalisation within the hypothalamus is supported by recent experiments carried out in zebrafish. Here chimaeric fish were made in which cells of the ventral hypothalamus were unable to transduce signals by Nodal, a member of the TGFβ family of signalling molecules, which is also expressed in the developing forebrain. Cells which contained mutations in the Nodal pathway, and hence were unable to transduce Nodal signals, were found to be excluded from their normal territory in the Shh-negative infundibular area, but populated the Shh positive ventro-lateral hypothalamus (Mathieu et al., 2002). This suggests differences in cell surface properties between wild-type infundibular cells and those unable to transduce Nodal. Indeed, analysis of Nodal signalling targets has indicated a role in the expression of cell adhesion proteins such as cadherins and ephrins, both of
which have been implicated in the establishment/maintenance of compartmental boundaries within the hindbrain (Wizenmann and Lumsden, 1997). Together these analyses suggest that Nodal may regulate specific cell surface properties in infundibular cells.

An unknown question is whether BMP7 likewise plays a role in establishing cell surface properties within the hypothalamus. Targets of BMPs in the dorsal neural tube are proteins such as Slug1 and Msx1, which are thought to play a role in cell adhesion and guidance in neural crest cells. Future studies are required to analyse the expression patterns of such markers, and of other specialised cell surface markers, within the hypothalamus.

3.3.3 Shh expression within the optic vesicle

Finally, experiments in zebrafish have indicated that ventral diencephalic cells are required to separate the two eye fields (Varga et al., 1999). Initially, retinal precursors are thought to lie in a single domain in the anterior ventral neural tube. In this study Varga et al showed that the rostral movements of ventro-medial diencephalic precursors are responsible for separating the single eye field into the left and right optic vesicles. This supports my data, where cells labelled in the M1a domain at stage 10 go on to populate both eyes at stage 18. Additionally, in the model proposed by Varga et al, the rostral movement of the ventral hypothalamic cells through the eye field causes the medial-most cells to lie rostrally to the lateral diencephalic precursors. This would support my findings in chick, indicating some continuity between species. Moreover, my observation that the M1a domain expresses Shh at HH stage 10 suggests that the migration of these Shh positive cells may provide a mechanism for how Shh comes to be expressed within the eye.
Chapter 4

BMP expression patterns
4.1 Introduction

4.1.1 BMPs in the diencephalon

Previous studies in the chick embryo have revealed that BMP7 may play an active role in the development of the hypothalamus, specifically in ventrolateral hypothalamic identity (Dale et al., 1997; Ohyama et al., in preparation). Moreover, as suggested in the Introduction (section 1.4) studies in the Gli2−/− mouse provide evidence, albeit indirect, for a ventral role of BMPs in maintaining Gli3 expression in the hypothalamus.

However, despite these suggestions that BMPs play an important role in cell fate specification within the hypothalamus, studies on mutant embryos provide no support for this view. In zebrafish that are mutant for BMP2 or BMP7, no hypothalamic defects are observed (Schmid et al., 2000). Likewise, in the BMP7 knockout mouse, defects are seen in the eye and kidney, but no abnormalities have been reported in the hypothalamus (Zhao, 2003). Why might this be?

As discussed in section 1.6, analysis of BMP function using mouse knockouts has proved difficult owing to the large number of BMP family members expressed during development, and the degree to which they can be functionally interchangeable. The extent to which different ligands can substitute for one another is dependent on the cell type, with some embryonic cells being able to distinguish between ligands sharing a 90% identity, such as BMP6 and BMP7, whilst others respond identically to ligands which are only 60% identical, such as BMP7 and BMP2 or BMP4 (Solloway and Robertson, 1999). Thus, the most likely explanation for the lack of hypothalamic phenotype observed in BMP mutants is due to a functional redundancy. As yet, combinatorial knockout mice, such as a BMP7/BMP2 double mutant have not been generated, and so it is possible that when both signalling pathways are removed at the same time, this may
result in a hypothalamic phenotype.

The lack of phenotype observed in the mouse and zebrafish mutants, therefore, together with the finding that additional BMPs are expressed in mouse ventro-medial cells, raises the possibility that BMPs additional to BMP7 are expressed in hypothalamic regions of the chick embryo. In the previous chapter, I have shown that ventro-medial (M1b) cells of the HH stage 9-10 hypothalamus, which are positive for both Shh and BMP7, later give rise to ventro-medial most regions of the Shh-negative infundibulum. Likewise, ventro-medial (M2b) cells that initially express Shh, and that lie adjacent to BMP7-expressing cells later give rise to more lateral regions of the Shh-negative infundibulum. Given the finding that, in experimental situations, BMPs can antagonise Shh by transcriptional repression (Monsoro-Burq and Le Douarin, 2001; Soubes et al., in preparation), this raises the possibility that expression of BMP7, either on prechordal mesoderm or in ventro-medial M1 cells (or both) is responsible for down-regulating Shh in the forming infundibulum. However, before beginning to address this, I first performed a more widespread analysis of the expression patterns of other BMP molecules and BMP antagonists with a view to defining the extent of BMP expression in the hypothalamic area, and to indicate areas in which BMPs are likely to be active.
4.2 Results

4.2.1 BMP2 and BMP7 are both expressed at HH stage 10 and 15 in ventro-medial hypothalamic cells.

To begin to examine the extent of BMP expression, and BMP activity within the forming hypothalamus, I initially set out to identify whether other BMPs are expressed there. Embryos were analysed by in situ hybridisation at stages 8, 10, 13, 15 and 18 for expression of BMP2, BMP4, BMP6 and BMP7. Expression patterns were analysed using both whole mounts and sections through the neural tube at different anterior-posterior levels.

Figure 4.1 shows the expression pattern of BMP7 at stages 8-18. At stage 8, BMP7 is expressed in the prechordal mesoderm and notochord. In addition it is weakly expressed in ventro-medial neurectoderm cells at the level of the mid- and hindbrain (not shown), but not the forebrain or spinal cord. Expression of BMP7 within prechordal mesoderm and anterior-most notochord is maintained throughout stage 10, then down-regulated (not shown; Dale et al 1999). By stage 9, BMP7 is up-regulated in the forebrain, and comes to be expressed in the M1b population (see figure 3.4), as well as showing continued expression in medial-most cells (occupying an M1 region) in the midbrain and hindbrain. No expression is detected in M1a cells (Dale et al, 1999; and not shown). BMP7 is also expressed dorsally in the surface ectoderm of the spinal cord at this stage. By stage 13, BMP7 appears to be down-regulated in the M1b cells (arrow, fig 4.1 E), but expression is now detected in M2b cells. Expression remains in the ventral-most (M1) population of the midbrain and in the surface ectoderm more posteriorly. At stage 15, BMP7 is once again expressed in the M1b cells, while expression in M2b cells is maintained. However, expression is completely lost by stage 18.
Figure 4.1

**BMP7 expression pattern**

Transverse sections through the neural tube of embryos processed for in situ hybridisation with a probe against BMP7. Sections are taken at the level of the diencephalon (A, C, E, G, I) and hindbrain or spinal cord (B, D, F, H, J) at stages 8-18.

At stage 8, BMP7 is expressed in the prechordal mesoderm underlying the prospective hypothalamus (A) but is not expressed at any point within the neuroepithelium, or notochord (B). At stage 10, BMP7 is still expressed in the prechordal mesoderm but is also expressed in the M1 population of prospective hypothalamic cells of the ventral diencephalon (C). In more posterior regions, BMP7 is expressed in the surface ectoderm (D). At stage 13, BMP7 appears to be down regulated in the M1 population of ventral hypothalamic cells (arrow), but is expressed in the M2 cells (E), whilst there is no expression in the neuroepithelium in posterior regions, but the surface ectoderm expression remains (F). By stage 15, BMP7 is once again expressed in the ventral-most M1 population of the diencephalon as well as the M2 population (G), and expression in the dorsal spinal cord is detected (H). No BMP7 is detected in any part of the diencephalic neuroepithelium at stage 18 (I), although the dorsal expression remains posteriorly (J).
Figure 4.1

BMP7

Diencephalon

Hindbrain/ Spinal Cord

Stage 8

A

Stage 10

C

B

Stage 13

E

F

Stage 15

G

H

Stage 18

I

J

Surface ectoderm

These data suggest that BMP7 and noggin are expressed in the diencephalon at stages 8 and 10, while noggin is expressed in the spinal cord at stages 13 and 15. BMP7 is also expressed in the hindbrain at stage 18. The expression patterns of BMP7 and noggin are consistent with the known roles of these genes in axial development.
Analysis with BMP2 reveals that it is not expressed in any region of the axial mesoderm at any stage analysed. Likewise, no expression of BMP2 is detected within the neural tube at stage 8 (figure 4.2 A-B). However, BMP2 is expressed in the ventral diencephalon at stage 10. Expression is limited to a short domain which appears to coincide precisely with M1b and M2b, as defined in chapter 3 (see figure 3.4). No expression is detected in the neural tube caudal to this. At stage 13, BMP2 is still expressed in the diencephalon, but it is expressed in dorso-lateral regions, dorsal to L1, and not in the ventral M1 or 2 domains (figure 4.2 E). Again, no expression is seen caudal to this (figure 4.2 F). As with BMP7, expression of BMP2 is detected once more at stage 15 within M1b and M2b. By stage 18, no BMP2 is detected anywhere in the diencephalon.

No BMP4 or BMP6 were detected in the axial mesoderm or within the diencephalon at any of the stages analysed (figure 4.3 and not shown). Furthermore, the only place BMP4 was detected was the dorsal spinal cord at stage 10 and 18 (fig 4.3 D, H) whilst BMP6 was not detected in any part of the neural tube.

These results show that BMP7 is expressed in prechordal mesoderm at stages 8-10. At HH stage 10, BMP2 and 7 are co-expressed in M1b and BMP2 is expressed alone in M2b. At stage 13, expression of both BMP2 and BMP7 shift laterally, so that BMP7 now marks M2b cells, and BMP2 is expressed in the thalamus. By stage 15, both BMP2 and BMP7 are re-expressed in the M1b and M2b cells that form the infundibular region.

4.2.2 Expression of the BMP antagonists Chordin and Noggin in axial mesoderm and ventral neural tube

Previous studies have suggested that Chordin acts as a high affinity extracellular antagonist of BMP7, while Noggin acts as a high affinity extracellular antagonist of BMP2 (Dale et al, 1999). Recent studies have
Figure 4.2

**BMP2 expression pattern**

Transverse sections through the neural tube of embryos processed for in situ hybridisation with a probe against BMP2. Sections are taken at the level of the diencephalon and hindbrain or spinal cord at stages 8-18.

At stage 8, no BMP2 is detected at any level of the neural tube (A, B). At stage 10, BMP2 expression is seen in the M1 and M2 domains of the prospective hypothalamic cells of the ventral diencephalon (C), but not in the prechordal mesoderm, or at any level posterior to the diencephalon (D). At stage 13, BMP2 is no longer expressed in M1 or M2 cells, but expression has moved dorsally to occupy the dorso-lateral cells of the diencephalon at this level (E). Again, no expression is detected posterior to the diencephalon (F). By stage 15, BMP2 is no longer expressed in the lateral diencephalic population, but is once again expressed in the ventral prospective hypothalamic cells, most obviously within M2 cells (G). Ventral expression of BMP2 was also detected in the midbrain at this stage (not shown) but at no levels posterior to this (H). No expression was detected at any level at stage 18 (I, J).
Figure 4.2

<table>
<thead>
<tr>
<th>Stage 8</th>
<th>Stage 10</th>
<th>Stage 13</th>
<th>Stage 15</th>
<th>Stage 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>C</td>
<td>E</td>
<td>G</td>
<td>I</td>
</tr>
<tr>
<td>B</td>
<td>D</td>
<td>F</td>
<td>H</td>
<td>J</td>
</tr>
</tbody>
</table>

Diencephalon | Spinal Cord
Figure 4.3

BMP4 expression pattern

Transverse sections through the neural tube of embryos processed for in situ hybridisation with a probe against BMP4. Sections are taken at the level of the diencephalon and hindbrain or spinal cord at stages 8-18.

No BMP4 expression was detected in the diencephalon at any stages analysed (A, C, E, G), but dorsal expression was detected in the neuroepithelium of the spinal cord at stage 10 and 18 (D, H).
Figure 4.3

The expression of BMP7 is initially detected in the anterior/mid-diencephalon, and is then observed in the posterior/anterior diencephalon. Only in the ventral neural tube, expression of BMP7 extends from the 14th cell of the diencephalon to the hindbrain at stage 15. However, it is not expressed with the antagonist Chordin at all levels other than the diencephalon, suggesting that BMP7 has no activity at posterior levels. These analyses suggest, therefore, that BMP7 has no activity at posterior levels.
shown that the BMP antagonists Chordin and Noggin are expressed in the notochord and ventral neural tube in posterior regions of the neuraxis (Liem et al., 2000; Patten and Placzek, 2002). I therefore wanted to analyse the expression of these genes within the diencephalon in order to establish whether the BMPs in the diencephalon were likely to be active. Figure 4.4 shows the expression pattern of Chordin. At stage 7 Chordin is expressed in the ventro-medial neurectoderm cells and underlying axial mesoderm at all levels analysed, including prechordal mesoderm and diencephalon. However, its expression in the prechordal mesoderm and ventro-medial diencephalic cells, including those of the prospective hypothalamus, is very weak, and is down-regulated by stage 8 (not shown; Dale et al. 1999). By stage 10, no expression is detected in the diencephalon, or the underlying prechordal mesoderm, however notochord cells are positive along the entire axis, and expression is detected in ventro-medial floor plate cells at the level of the mid- and hindbrain. Stage 13 embryos display a similar expression pattern to those at stage 10, whilst by stage 18, expression is restricted to the notochord underlying the spinal cord.

The expression of Noggin was not detected within the neural tube or prechordal mesoderm at any stages analysed (figure 4.5), although it was detected in the notochord at the level of the mid- and hindbrain at stage 8 (figure 4.5 B), and again at stage 10 in the notochord underlying the midbrain. However, no other expression was detected.

Thus, whilst BMP7 expression is expressed in both prechordal mesoderm and anterior-most notochord, it is co-expressed with Chordin in the notochord. Only in the prechordal mesoderm, from ~ stage 7-8 is BMP7 expressed in the absence of Chordin (see also Dale et al 1999; Vesque et al, 2000). In the ventral neural tube, expression of BMP7 extends from M1b cells in the diencephalon to the hindbrain at stage 10. However, it is co-expressed with the antagonist Chordin at all levels other than the diencephalon, suggesting that BMP7 has no activity at posterior levels. These analyses suggest, therefore, that BMP7 has no activity at posterior
Figure 4.4

Chordin expression pattern

Transverse sections through the neural tube of embryos processed for in situ hybridisation with a probe against the BMP7 antagonist Chordin. Sections are taken at the level of the diencephalon (A, C, E, G) and hindbrain or spinal cord (B, D, F, H) at stages 7-18.

At stage 7, Chordin is expressed throughout the entire neuraxis in both the axial mesoderm, and the ventral midline (A, B). However, the expression in the prechordal mesoderm and diencephalon is very weak and both are down regulated by stage 8 (not shown). By stage 10 there is no Chordin expression in the diencephalon or the prechordal mesoderm (C), but expression remains in the notochord and the ventral midline of the hindbrain and anterior regions of the spinal cord (D). Chordin is not detected in the diencephalon at any of the later stages analysed (E, G), but expression remains in the notochord (F, H).
Figure 4.4

Chordin

<table>
<thead>
<tr>
<th>Stage 7</th>
<th>Diencephalon</th>
<th>Hindbrain/ Spinal Cord</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.5

**Noggin expression pattern**

Transverse sections through the neural tube of embryos processed for in situ hybridisation with a probe against the BMP2/4 antagonist Noggin. Sections are taken at the level of the diencephalon (A, C, E, G) and hindbrain or spinal cord (B, D, F, H) at stages 8-18.

Noggin was not detected in the prechordal mesoderm or diencephalon at any of the stages analysed (A, C, E, G), but expression was detected in the notochord at mid and hindbrain levels of stage 8 and 10 embryos (B, D). No expression was detected at later stages (F, H).
Figure 4.5

- Diencephalon
- Hindbrain/Spinal Cord

<table>
<thead>
<tr>
<th>Stage</th>
<th>Image</th>
<th>Image</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>10</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>13</td>
<td>E</td>
<td>F</td>
</tr>
<tr>
<td>18</td>
<td>G</td>
<td>H</td>
</tr>
</tbody>
</table>
levels, but is active in prechordal mesoderm and ventro-medial cells that will form the infundibulum. Likewise, since no expression of Noggin was detected in the diencephalon, this suggests that BMP2 may be active within cells that will form the future hypothalamic infundibulum.

4.2.3 Co-expression of Shh with BMP2 and BMP7

The expression patterns of BMPs were then compared to that of Shh. Figure 4.6 shows the expression of Shh at the level of the hypothalamus (A, D, G, J) with comparable sections of embryos analysed for BMP7 and BMP2. Shh is initially expressed alone in prechordal mesoderm (not shown, (Dale et al., 1999; Patten et al., 2003), but by HH stage 8 is co-expressed with BMP7. At these stages the prechordal mesoderm is wide and underlies both prospective M1 and M2 regions (level b). In the diencephalon, Shh is initially expressed alone, but by stage 10, it is co-expressed with both BMP2 and BMP7. Whilst BMP7 occupies only the M1 population (level b see figure 3.3), BMP2 is expressed in both the M1 and M2 domains (level b). At stage 13, Shh remains in M1b and M2b, whilst both BMPs are now down-regulated in M1b. Whilst BMP7 now appears to be expressed exclusively in the M2 domain, BMP2 is no longer expressed in the ventral midline, and instead is found in a fairly broad domain in the lateral diencephalon, dorsal to L1. Shh begins to be down regulated in the infundibular region at stage 15, which coincides with the re-expression of both BMPs in this region.

The expression profile of the regions of the neural tube and the adjacent axial mesoderm of the stage 10 embryo which fate map to the Shh negative infundibulum are shown in table 4.1. Both regions which give rise to the infundibulum (M1/M2b) express both Shh and BMPs at stage 10, and the prechordal mesoderm underlying these regions expresses Shh, BMP7 and Nodal (Soubes et al., in preparation). Regions of the neurectoderm adjacent to these areas, which do not express BMPs (M1a), or co-express BMPs with Chordin (M2b), do not populate the Shh negative infundibulum, but instead
Co-expression of Shh and BMPs in the prospective hypothalamus

Transverse sections through the neural tube of embryos processed for in situ hybridisation with a probe against Shh (A, D, G, J), BMP7 (B, E, H, K) and BMP2 (C, F, I, L). Sections are taken at the level of the diencephalon at stages 8-15.

Comparison of the BMP expression patterns in diencephalic sections taken from figures 4.1 and 4.2 with the expression of Shh at the same stages and anterior-posterior levels, reveals that at stage 8, Shh and BMP7 are co-expressed in prechordal mesoderm that underlies M1 and M2 diencephalic cells (level b). At this stage, Shh is expressed in the diencephalic ventral midline before expression of BMP7 is detected (A, B). At stage 10, BMP7 is expressed in the M1 sub-domain of Shh positive cells (see chapter 3), whilst BMP2 is expressed in both M1 and M2 domains at this stage. By stage 13 both BMPs have been downregulated in the M1 domain, whilst Shh remains expressed here (G-I). BMP2 is expressed in the dorso-lateral diencephalon, whilst BMP7 appears to be expressed in the M2, Shh positive domain. At stage 15, Shh is downregulated in the ventral midline (M1 and M2) and is expressed in the lateral hypothalamic cells (J). Both BMPs are once again expressed in the ventro-medial M1 and M2 cells of the infundibulum (K, L).
Figure 4.6

Shh

BMP 7

BMP 2

Stage 8

A

B

C

Stage 10

D

E

F

Stage 13

G

H

I

Stage 15

J

L

M

The mRNA expression of Shh and BMPs, and the future Shh-negative infundibulum, is consistent with an antagonist. Likewise, no down-regulation of Shh is observed in ventromedial parts of the neural tube at any other anterior-posterior level, except where co-expressed with an antagonist. No BMPs appear to be expressed in the anterior-midbrain at any other anterior-posterior level, except where co-expressed with an antagonist. Furthermore, the expression of Shh in the infundibulum is consistent with the expression of BMPs in the ventral midbrain.
give rise to Shh positive regions of the hypothalamus (however, M1a cells are found in the optic vesicles which are not Shh positive at stage 18, but do express Shh later, see chapter 6). As the regions of interest for this study are the M1/M2b areas, future reference to M1 and M2 cells at stage 18 will indicate the 'level b', Shh negative infundibulum, unless otherwise stated.

Table 4.1 Expression profiles of the stage 10 ventro-medial neurectoderm and adjacent prechordal mesoderm, and the stage 18 region they fate map to.

<table>
<thead>
<tr>
<th>Neurectoderm</th>
<th>Adjacent Mesoderm</th>
<th>Fate maps to: (stage 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1a</td>
<td>Shh</td>
<td>N/A</td>
</tr>
<tr>
<td>M1b</td>
<td>Shh, BMP7, BMP2</td>
<td>Shh, BMP7, Nodal</td>
</tr>
<tr>
<td>M2b</td>
<td>Shh, BMP2</td>
<td>Shh, BMP7, Nodal</td>
</tr>
<tr>
<td>M1c</td>
<td>Shh, BMP7, Chordin</td>
<td>Shh, BMP7, Chordin</td>
</tr>
</tbody>
</table>

These results show a precise correlation between regions of the hypothalamus that initially are underlain by BMP7-expressing prechordal mesoderm, and later co-express Shh and BMPs, and the future Shh-negative territories of the infundibulum. No BMPs appear to be expressed in the ventral neural tube at any other anterior-posterior level, except when co-expressed with an antagonist. Likewise, no down-regulation of Shh is detected in ventro-medial cells of the neural tube at any anterior-posterior level other than the hypothalamic infundibulum (not shown, and fig 1.3).
4.3 Discussion

4.3.1 Shh is down-regulated in cells that co-express BMPs and are underlain by BMP-expressing prechordal mesoderm

My expression analysis data has shown that BMP7 is expressed in prechordal mesoderm underlying regions M1b, M2b. In addition, BMP7 and BMP2 are co-expressed with Shh in M1b cells at stage 10, and BMP2 is co-expressed with Shh in M2b cells. Both BMPs are transiently down-regulated within M1 domains at stage 13, whilst Shh remains expressed here. However, when Shh is down-regulated from M1b and M2b cells at stage 15, both BMPs are re-expressed in these cells. The absence of BMP antagonists in the diencephalon at any of the stages analysed suggests that BMPs are likely to be active in this area. In contrast, BMP7 is expressed ventrally in the mid- and hindbrain, but the co-expression of chordin here suggests that BMP7 is not active (see also Dale et al 1999). Therefore, the extent of BMP activity in the ventral neural tube appears to be confined to those cells that will give rise to the Shh-negative infundibulum.

4.3.2. Regulation of BMPs in the hypothalamus

My analyses show a complex expression of BMP7 and BMP2 within the forming hypothalamus. What controls these patterns of expression? Previous experiments have shown that the expression of BMP7 within M1 cells can be regulated by BMP7 itself, and that prechordal mesoderm can mediate this effect. Conversely, blockade of BMP7 activity in prechordal mesoderm abrogates its ability to induce/maintain BMP7 in M1 cells (Dale et al 1997, 1999). Together, these experiments indicate that BMP7 is required, either for the induction, or the maintenance of BMP7 in M1 cells. Expression of BMP7 within the prechordal mesoderm itself can be controlled through BMP signalling from the adjacent anterior endoderm (Vesque et al., 2000).
Together, these results suggest a model in which a cascade of homeogenetic induction events involving BMPs specify BMP7 expression in M1 cells. In this model, BMPs expressed in the anterior endoderm induce expression of BMPs in prechordal mesoderm, and hence enable the prechordal mesoderm to induce/maintain BMP7 in overlying M1 cells. Such homeogenetic induction of BMPs is not without precedent: in posterior regions of the neuraxis, BMPs that derive from the surface ectoderm appear to induce their own expression within dorsal spinal cord cells (Lee and Jessell, 1999).

Three questions, however, remain unclear. First, do other signals contribute to the expression of BMP7 in M1 cells, for instance inducing BMP7 expression that is then maintained by BMP7? Second, is expression of BMP2 likewise regulated by BMP7 from underlying prechordal mesoderm? And third, why is BMP7 expression restricted to M1 cells, whereas BMP2 is expressed in both M1 and M2 cells? My current studies do not allow me to address these questions. However, other studies provide some possible explanations.

First, in recent experiments, the signalling molecule Nodal appears to be able to induce BMP7 expression in M1 cells (Ohyama et al., in preparation). This, together with other studies showing that Nodal is required for ventro-medial hypothalamic cell induction in zebrafish (Mathieu et al., 2002) suggests a model in which Nodal induces M1 cells, including BMP7 expression, and BMP7 maintains this expression. An unknown question remains that of whether Nodal or BMP7 might also induce expression of BMP2 within M1 and M2 cells, raising the possibility that prechordal mesoderm expression of BMP7 is responsible for inducing BMP2 in the hypothalamus. A model in which Nodal induces BMP7, but BMP7 induces BMP2 might explain why BMP2 is initially expressed in both M1 and M2 cells, whereas BMP7 is restricted to M1 cells. Additionally, it is conceivable that the M1 and M2 regions have distinct characters, and are differentially competent to express BMP2 and BMP7. Another possibility is that Shh is acting, possibly in cooperation with other factors, to induce BMP2 in this region. Studies in the
developing pituitary (Treier et al., 2001) have shown that Shh is required for BMP2 expression in these areas. Future experiments are needed to distinguish between these possibilities.

A final unknown question is that of how the very dynamic patterns of expression of BMP2 and BMP7 are controlled within the different subdomains of the hypothalamus at the stages I have analysed. Again, my studies do not provide answers to these questions. However, again, it is possible that homeogenetic inductions mediate these complex expression patterns, or that rapidly acting feedback loops contribute to the patterns of expression.
Chapter 5

BMPs down-regulate Shh in the prospective infundibulum
5.1 Introduction

The expression studies in chapter 4 indicate that at stage 8, prechordal mesoderm that expresses BMP7 underlies Shh-positive M1 and M2 cells, while at stage 10, both BMP2 and BMP7 are co-expressed with Shh in M1 and M2 cells. The fate mapping studies described in chapter 3 show these (M1/M2) to be the precise populations of Shh-positive cells which later down-regulate Shh and give rise to the Shh-negative infundibulum at stage 18. As described in section 1.7, evidence from previous studies has indicated that BMPs and Shh can act antagonistically both at the level of the protein and at the transcriptional level. At the transcriptional level, experimental over-expression of BMPs in the neural tube has been shown to result in the down-regulation of Shh mRNA in the ventral midline (Arkell and Beddington, 1997; Ohkubo et al., 2002), and ectopic application of BMPs has been shown to down-regulate Shh mRNA in axial mesoderm in vitro (Soubes et al., in preparation). The loss of Shh in the infundibulum, which is derived from cells which initially express Shh, then co-express Shh and BMPs, led me to ask whether the early expression of BMPs, either from underlying prechordal mesoderm, or from the cells themselves, causes the down-regulation of Shh seen at later stages.

In this chapter I have used in vitro and in vivo methods to analyse the effects of BMP activity on Shh expression in the prospective infundibulum. My results indicate that BMPs are able to down-regulate Shh in this tissue, and show that blocking BMP activity prevents the down-regulation of Shh in the prospective infundibulum in vivo.
5.2 Results

5.2.1 BMP2 and BMP7 can down-regulate Shh in prospective hypothalamic ventro-medial cells in vitro.

To test whether BMPs can down-regulate Shh in the prospective infundibulum, I initially tested whether BMP2 or BMP7 can down-regulate Shh in these cells in an experimental condition. To do so, explants of prospective hypothalamus (regions M1 and M2: levels a, b, c) (see fig 5.1 and 3.4) were dissected from HH stage 7 embryos. Explants were cultured in collagen beds in the absence or presence of BMPs (figure 5.1) then analysed for expression of Shh. Ventral diencephalic cells at this stage have already been induced to express Shh, and so explants from this region, when cultured alone, also express Shh. BMPs, however, are not induced by this stage, and likewise, are not expressed in explants of these cells cultured alone (Dale et al., 1999).

Explants cultured in the presence or absence of BMP2 or BMP7 were then analysed for Shh expression by immunohistochemistry, in situ hybridisation and quantitative RT-PCR. To ensure that the BMPs were present and active, I first analysed their expression by Western blotting (collaboration with Pam Ellis; data not shown), and then measured their activity in an independent assay (collaboration with Sandrine Soubes: not shown). BMP7 was used at a concentration of ~1nM in all three experiments, and BMP2 at a concentration of ~2.5nM in all three experiments.

5.2.1a Down-regulation of Shh protein by BMP2 and BMP7

Explants were initially cultured for 20 hours in control media (figure 5.2 A; n=6), in BMP7- (B; n=6) or in BMP2-conditioned media (C; n=6), then analysed for Shh protein by co-labelling of sectioned explants with DAPI (not
Figure 5.1

**Explant culture in collagen beds**

(A) Forebrain ventral midline cells were dissected from a stage 7 embryo. The rectangle indicates the region which was removed, comprising cells of the M1/2 domain, level a-c. Shh expression at this stage is shown in red.

(B) The tissue (red) is then transferred to a collagen bed which is then covered by a second layer of collagen. This is then covered with culture media, to which can be added BMP protein.

(C) Transverse sections through stage 7-8 embryos at the level of the diencephalon. The ventral midline cells express Shh but not BMP7 or BMP2.
Figure 5.1

A

B

C

Shh

BMP 7

BMP 2

Stage 8

To detect Shh and BMP2 proteins, the explants were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained for Shh and BMP2 proteins using a rabbit polyclonal antibody against Shh and an avidin-biotin-peroxidase complex. The immunohistochemical reaction was visualized using DAB (3,3-diaminobenzidine) as the chromogen. The percentage of the explants showing positive immunostaining for Shh and BMP2 was determined by counting the number of positive cells in each section of the explants. The number of cells expressing Shh and BMP2 was counted in triplicate. A 50-70% reduction of Shh and BMP2 expression was observed compared to the control. The reduction was statistically significant (Student's t-test, p < 0.05, compared to control).
shown) and anti-Shh antibody. The DAPI labelling was used to count the total number of cells in each section of the explant, and the number that co-expressed Shh. The percentage of Shh-positive cells was then calculated and the average for each condition taken, as displayed in the form of a bar chart (D). A 50-60% reduction of Shh positive cells was seen in explants exposed to BMPs compared to the control. This reduction was statistically significant (Students T test, t=4.59, p=0.001, df=17).

5.2.1b Down-regulation of Shh mRNA by BMP2 and BMP7

To establish whether the down-regulation of Shh protein is underlain by a down-regulation of Shh mRNA, identical cells (ie M1, M2 cells, levels a-c) were once again dissected from stage 6-7 embryos and cultured for 20h in the presence or absence of BMPs. The explants were then analysed by in situ hybridisation for expression of Shh mRNA. In order to quantify the amount of mRNA detected, each explant was sectioned, scored as positive or negative for Shh and an average Shh expression calculated (D). Although this method is less accurate as the results can vary according to the plane of section, I again detected a large reduction in the average amount of Shh in BMP-exposed explants compared to the control (Figure 5.3).

As analysis by in situ hybridisation does not provide a quantitative way of measuring mRNA levels, I next undertook a quantitative RT-PCR approach. This method involves culturing prospective explants (as before) in collagen beds (figure 5.1), for 20 or 40h, both in the presence or absence of BMP2 or BMP7. The RNA from the explants was then isolated, and cDNA made by RT-PCR (see Materials and Methods). Shh cDNA was then amplified through PCR, using a Shh-specific fluorescent probe that binds to the Shh cDNA and emits fluorescence on each PCR cycle (figure 5.4). This method allows the level of fluorescence to be measured in a quantitative fashion, and is representative of the amount of PCR product produced. The level of
Figure 5.2

Analysis of explants exposed to BMPs for 20 hours by immunohistochemistry.

(A-C) Sections of explants (15μm) analysed by immunohistochemistry for Shh expression following 20 hours culture. When cultured in the presence of BMP protein, little or no Shh is detected (B, C) compared to the control (A).

(D) Explants were co-labelled with DAPI (not shown) in order to quantify the number of Shh expressing cells per section. The total percentage of Shh positive cells per explant has been calculated for each condition and displayed as a bar chart. A 50-60% reduction is seen in the number of cells expressing Shh in explants exposed to BMPs compared to the control.
Figure 5.3

Analysis of explants exposed to BMPs for 20 hours by in situ hybridisation.

(A-C) Sections of explants analysed by in situ hybridisation for Shh expression following 20 hours culture. Little of no Shh mRNA is detected when explants are cultured in the presence of BMPs (B, C), whereas labelling is detected on a large proportion of control sections (A).

(D) The amount of mRNA detected per condition was quantified by scoring sections positive or negative for Shh expression. The percentage of Shh positive sections is displayed for each condition in the form of a bar chart. A significant decrease is seen in the number of Shh expressing sections taken from explants cultured in the presence of BMP2 or BMP7, compared with the control.
Figure 5.3

A: Control

B: BMP7

C: BMP2

% Shh positive sections / explant

D: Control  BMP7  BMP2
Figure 5.4

Quantitative RT-PCR

Explants are cultured for a set period of time in collagen beds, as in figure 5.1.

(A) Explants from each condition are then homogenised and the mRNA converted to cDNA by reverse transcription.

(B) cDNA from each experiment is then amplified by PCR, using primers for Shh, and a fluorescent probe, which is specific for Shh, and contains a fluorescent molecule and a quencher molecule.

(C) As the PCR progresses, the quencher molecule is released, allowing the emission of fluorescence, which can be recorded in a quantitative fashion.

(D) The amount of fluorescence emitted increases with each PCR cycle, and can be plotted on a graph. The number of PCR cycles required to produce a certain level of fluorescence is called the Ct value, and is dependent on the amount of Shh cDNA present in the original sample. The Ct value is relative, and cannot be compared directly to other Ct values obtained in different conditions, so it is therefore necessary to obtain a ΔCt value using a control of a ubiquitously expressed protein (in this case actin).
Figure 5.4

Quantitative RT-PCR

A

Isolate mRNA
(Shh and Actin)

Convert to cDNA
(Reverse Transcriptase)

B

cDNA

C

D

Fluorescence vs. Cycle Number (CT)

Q

F

C_{T\text{Shh}} - C_{T\text{Actin}} = D_{C_{T}}
fluorescence is then plotted on a graph against the PCR cycle number, and from this, a ‘Cₜ’ level can be calculated, which, when analysed with a control sample, gives a ΔCₜ value. The ΔCₜ value gives a representation of the relative concentration of Shh cDNA present in the sample, thus providing a quantitative measurement for the level of Shh mRNA present in the explant.

Figure 5.5 shows the reciprocal ΔCₜ values of explants cultured in BMP7 for 20 hours (A) and 40 hours (B), and in BMP2 for 20 hours (C), plotted against the control (i.e. explant cultured in the absence of BMPs). In each case, there is a statistically significant reduction in the level of mRNA in the explants exposed to BMPs compared to the control (BMP7 20 hours, t=-4.45, p=0.002, df=8; BMP7 40 hours, t=-8.49, p= 0.014, df=2; BMP2 20 hours, t=-5.97, p=0.027, df=2). The same data is represented as the percentage of the mRNA in BMP exposed explants compared with the control experiments (D-F). This shows that after 20 hours culture in BMP7, the levels of Shh mRNA are significantly reduced, and reduced to negligible levels after 40 hours. After 20 hours culture with BMP2, the levels of mRNA have been already been reduced to negligible levels. This may reflect differences in the concentrations used of the two proteins, or it may indicate that BMP2 is more potent at down-regulating Shh than BMP7.

These results indicate that both BMP2 and BMP7 are able to down-regulate Shh at the level of transcription in prospective hypothalamic cells in vitro. Furthermore, the concomitant down-regulation of Shh protein suggests that Shh is a relatively unstable protein, with a high turnover rate.

5.2.2 BMPs are required in vivo for the down-regulation of Shh in the prospective hypothalamus.

Having established that BMPs are able to down-regulate Shh in prospective hypothalamic cells, I next determined if they are required for the down-regulation of Shh seen in vivo in the infundibular region. In order to assess
Figure 5.5

Analysis of explants exposed to BMPs for 20 or 40 hours by Quantitative RT-PCR.

(A-C) the reciprocal DCT is shown with that of the control for explants exposed to BMP7 for 20 hours (A), and 40 hours (B), and to BMP2 for 20 hours (C). In each case, a significant reduction in the amount of mRNA in the BMP-exposed explant is seen compared to the control.

(D-F) The results shown for the BMP-exposed explants in A-C are shown as a percentage of the control.
Figure 5.5

A) 1/Average DCt of Shh in explants exposed to BMP7 for 20hrs

B) 1/Average DCt of Shh in explants exposed to BMP7 for 40hrs

C) 1/Average DCt of Shh in explants exposed to BMP2 for 20hrs

D) % Shh mRNA in explant cultured with BMP7 (20 hrs)

E) % Shh mRNA in explant cultured with BMP7 (40 hrs)

F) % Shh mRNA in explant cultured with BMP2 (20 hrs)
this, BMP activity was blocked in the prechordal mesoderm/prospective hypothalamus using the BMP antagonist, chordin. Affigel blue beads were soaked in chordin protein, and then implanted adjacent to the neural plate at the level of the prechordal mesoderm at stage 5-6 (figure 5.6 A). Embryos were prepared in the same way as for the fate mapping experiments (see figure 3.2 and Materials and Methods). Once the embryos reached stage 16-18, they were removed and sectioned, then analysed for Shh by immunohistochemistry.

Figure 5.6 shows transverse sections through the prospective hypothalamus of an embryo which has been cultured with a control bead (B) and one cultured with a bead soaked in chordin (C, experiment done by Kyoji Ohyama). In the control embryo, Shh has been down-regulated from ventro-medial M1 and M2 cells and is now expressed in lateral L1 hypothalamic cells. However, in the embryo implanted with a chordin-soaked bead, the down-regulation of Shh in the infundibular area is only in a very small domain on the side opposite the bead implant, whilst on the side of the bead implant Shh is still expressed in cells that occupy an M1 and M2 position (5.6 C).

These results show that BMPs are able to down-regulate Shh in prospective hypothalamic cells and that they are required in vivo for the down-regulation of Shh seen in the infundibulum at stage 15.
Figure 5.6

Blocking BMP signalling in the prechordal mesoderm/ventral diencephalon results in the maintenance of Shh in the infundibulum at stage 18.

(A) Schematic representation of a stage 5 embryo with a chordin soaked bead (blue) implanted on the neural plate, adjacent to the prechordal mesoderm/ventral diencephalon. The prechordal mesoderm can be seen through the neural epithelium providing accurate indication of prospective hypothalamic cells.

(B) Transverse section through hypothalamic region (at the level of the infundibulum) of a stage 18 embryo cultured from stage 5 with a control bead. Shh expression (green) has been down regulated in the infundibulum and is expressed in the lateral L1 population. Position of bead indicated by *

(C) Transverse section through hypothalamic region (at the level of the infundibulum) of a stage 18 embryo cultured from stage 5 with a bead soaked in chordin. Blocking BMP activity has resulted in the maintenance of Shh in the M1 and M2 populations of the infundibular cells (arrow) on the side of the bead implant (bead indicated by *). A dorsal expansion is also seen in the lateral Shh-positive domain on this side (arrow head).

(Experiment done by Kyoji Ohyama)
Figure 5.6

A  Implant chordin-soaked bead next to st 5 prechordal mesoderm

Allow to develop to st 17
5.3 Discussion

5.3.1 BMPs act indirectly to down-regulate Shh in the infundibulum

The results obtained in this chapter indicate that BMPs are required to down regulate Shh expression in the cells of the ventro-medial hypothalamus that will form the infundibulum. Although decreased, explants cultured in the presence of BMP7 for 20 hours generally still have some Shh expression (figures 5.2 -5.4), whilst this is reduced further to negligible levels following incubation for 40 hours (figure 5.5 A, B, and D, E). This suggests that the BMPs work indirectly to cause the down regulation of Shh in these cells, as a direct effect would be more immediate. This is supported by earlier studies where BMPs have been over expressed in the chick forebrain and been found to down-regulate ventral expression of Shh (Ohkubo et al., 2002). In these studies, Shh expression was lost in both the telencephalon and the diencephalon. However, when cycloheximide was used, in order to block protein transcription, Shh was still down regulated in the ventral telencephalon, but expression remained in the ventral diencephalon. This suggests that BMPs directly down-regulate telencephalic Shh expression, but are unable to down-regulate Shh directly in the diencephalon. It is possible that earlier inductive events during development have blocked the ability of cells in the ventral diencephalon to respond directly to BMP signals in this way. Instead these studies suggest that BMPs act to down-regulate Shh in the infundibulum through the induction/up-regulation of other protein(s), which in turn decrease Shh transcription.

5.3.2 Blockade of BMP activity does not affect cell migration of M1/M2 or L1 cells.

As discussed in chapter 3, my fate mapping experiments, combined with studies in zebrafish (Mathieu et al., 2002), suggest that the Shh-negative infundibulum (M1, M2 cells) may form a separate compartment to the
neighbouring lateral hypothalamic L1 Shh-positive cells. If this is the case, and if BMP7 were partly responsible for the maintenance of one of these compartments, then it could be argued that the Shh expression seen in the ventral midline of the chordin-exposed embryo (figure 5.6 C) is a result of Shh positive cells from the lateral L1 region migrating ventrally to populate the ventral M1 and M2 domains. One way of testing this would be to fate map the M1/M2 or L1 domains whilst simultaneously blocking BMP signalling with a BMP antagonist. If this is the case, then cells labelled in the L1 domain at stage 9-10 would be expected to populate the M1/M2 domains at stage 18, whilst cells labelled at stage 9-10 in the M1/M2 domains would be expected to either have died or moved dorsally. However, preliminary experiments by M. Placzek indicate that this is not the case, and the progeny of labelled cells remain at the same dorso-ventral level in the absence of BMP signalling as they do when BMP signalling is present. Together, these studies indicate that blockade of BMP activity does not affect the migration of M1/M2 or L1 cells.

5.3.3 Chordin is an effective antagonist of BMP activity.

One question that arises from my results is that of why chordin-soaked beads are so effective in abrogating BMP activity. Chordin has been shown to be particularly effective in antagonising BMP7 signalling, whilst BMP2 has been shown to be antagonised more specifically by noggin (Dale et al., 1999). The fact that Shh down-regulation is blocked by chordin alone suggests that either only BMP7 is required to cause this down-regulation in vivo, that chordin is also able to antagonise BMP2, or, that BMP7 is required for the expression of BMP2.

Of these possibilities, the most likely explanation is that the high levels of Chordin used on the beads are effective antagonists of both BMP7 and BMP2. Additionally, studies have shown that Chordin is a potent inhibitor of BMP7/BMP4 heterodimers (Piccolo et al., 1996), as BMP2 and BMP4 share a high sequence homology, it is possible that BMP7/BMP2 heterodimers form in the infundibulum, and so can be antagonised by chordin. The
alternate explanations are less likely. First, I have shown that BMP2 is capable of down-regulating Shh in the ventral forebrain (perhaps more potently than BMP7, figure 5.5) in vitro, making it unlikely that BMP2 is unable to down-regulate Shh in vivo. Second, if BMP7 is required for the expression of BMP2 in the diencephalon (see Discussion, chapter 4), then in the BMP7 knockout mouse, BMP2 would not be induced and so a stronger hypothalamic phenotype would be expected.

5.3.4 Regulation of Shh in both M1/M2 and L1 cells by BMPs

In addition to the maintenance of Shh in the ventral midline of the infundibulum in embryos where BMP signalling has been blocked, there is also a dorsal expansion of Shh expression in the lateral L1 domain (figure 5.6 C). This indicates that BMP activity is required, both to regulate Shh expression in the M1/M2 domain and to set up the dorsal border of Shh expression in the L1 domain. Although I have not examined expression of BMP2 in the chordin-exposed embryos, one possibility is that expression of BMP2 in the dorsal diencephalon at HH stage 13/15 (fig 4.2) regulates the dorsal border of Shh in L1 cells. Experiments in the spinal cord reveal a similar mechanism for the maintenance of the dorsal border of Shh expression in the floor plate. When BMP signals from the roof plate are antagonised or removed, a dorsal expansion of Shh from the floor plate is seen (Patten and Placzek, 2002). Thus it appears that BMPs can regulate the domain of Shh expression at different rostro-caudal levels.
Chapter 6

T-box genes
6.1 introduction

6.1.1 A T-box gene is involved in the down-regulation of Shh in the infundibulum.

In previous chapters, I have shown that cells which initially co-express Shh and BMPs give rise to the Shh negative infundibulum of the ventral hypothalamus, and that the BMPs expressed in these cells are required for the down-regulation of Shh seen at stage 15. As discussed in section 5.3, the down-regulation of Shh by BMPs is not immediate as it does not appear to be complete after 20 hours of culture, and has been shown to be dependent on the ability of the cells to synthesise new protein (Ohkubo et al., 2002). This indicates that BMPs are likely to be acting through another factor in order to mediate the down-regulation of Shh.

A recent study analysing the mouse Shh promoter has indicated that a T-box protein may be required for the down-regulation of Shh in the infundibulum (Jeong and Epstein, 2003). In this study, constructs were made using the SBE1 (Shh brain enhancer) enhancer region, which had previously been shown to drive reporter gene expression in the midbrain and caudal diencephalon (Epstein et al., 1999), in combination with a section of the SFPE2 (Shh floor plate enhancer) enhancer, termed HR-c (figures 6.1 and 1.10). Sequence analysis of the HR-c fragment has revealed a number of binding sites including a homeodomain binding site, binding sites for Foxh1/ Fast1 and Foxa2, and a T-box protein binding site. When the SBE1 construct was used in conjunction with three copies of the HR-c fragment, reporter activity was detected in the floor plate of the spinal cord, the midbrain and the caudal diencephalon, but no reporter activity was detected rostral to the zona limitans intrathalamica (figure 6.1c). However, constructs in which point mutations were introduced in the T-box binding site resulted in reporter construct expression in the ventral midline of the rostral hypothalamus. Figure 6.1c shows reporter construct expression in mouse embryos which is driven by the wild type HR-c construct (i, iii) and the T-box binding site.
Figure 6.1

Mutations in the Tbx binding site on the Shh enhancer results in ectopic expression of Shh in the infundibular region of the ventral hypothalamus in mice.

(A) Schematic representation of the Shh locus showing the location of coding exons (black boxes), non-coding sequences (solid line) and intronic enhancers (grey oval, SBE1; yellow oval, SFPE2). Comparison of mouse sequence with human, chicken and zebrafish revealed three regions of high sequence homology corresponding to homology region-a (HR-a, blue, homeobox binding site), HR-b(green, Foxh1 binding site) and HR-c (red, T-box binding site).

(B) Reporter construct with the full-length SBE1 (grey oval), and three copies of the HR-c region (red ovals) of the SFPE2.

(C) Whole mount salmon-gal staining of transgenic embryos at 9.5 dpc expressing either (i) wild type construct, or (ii) construct containing a mutated T-box binding site. (iii-iv) Transverse sections through the rostral diencephalon of transgenic embryos expressing either (iii) wild type construct, or (iv) mutated T-box construct, double stained for lacZ reporter activity (salmon-gal) and Shh mRNA (blue). The arrows in (i) and (ii) point to the rostral limit of salmon-gal staining detected at the level of prosomeres 3 and 5, respectively. The blue arrowhead points to the expression of endogenous Shh mRNA in the lateral hypothalamus. The salmon coloured arrowhead in panel (iv) points to the ectopic reporter activity in the ventral midline of the infundibular region. The asterisk marks the floor plate of the hindbrain.

Taken from (Jeong and Epstein, 2003)
mutated HR-c construct (ii, iv). Transverse sections at the level of the prospective hypothalamus show that in the wild type no reporter expression is detected in the infundibular region, whereas in the absence of T-box binding sites, ectopic reporter expression is driven in these cells.

These experiments indicate that a T-box protein is acting to repress Shh transcription in the infundibular region of the ventral hypothalamus. Given that I have already shown that BMPs are also required for this purpose, this led me to ask whether a T-box protein could be acting downstream of BMP activity in this area.

6.1.2 T-box genes.

T-box (Tbx) genes are a family of transcription factors, which share a conserved binding motif, known as the T-box, which encodes a 180-190 amino acid DNA-binding domain. The Tbx genes share a high sequence homology within the T-box domain due to their role in DNA binding, which has required a high degree of conservation between species. However, greater variations are seen in the domains involved in protein-protein interactions. The first member of the T-box family to be characterised was the mouse Brachyury in 1990 (Herrmann et al., 1990), which is involved in the development of posterior mesoderm during gastrulation. Studies of the Xenopus Brachyury gene (Xbra) have indicated that this transcription factor regulates developmental events in a dose-dependent way, by controlling gene transcription in specific tissues during embryogenesis. To date, more than 20 members have been identified, in species including mouse, chick, Xenopus, zebrafish, amphioxus, ascidians, drosophila, C.Elegans and humans.

Tbx genes have been shown to play a role in cell type specification and morphogenesis in various tissues, including the nervous system, limb buds, skeleton, kidney, lungs, mammary gland and muscle (Papaioannou and
Mutations in T-box genes can have drastic effects on development, leading to a number of developmental defects and syndromes. Mutations in Tbx5 lead to Holt-Oram Syndrome, which is characterised by defects in cardiac and forelimb development (Bruneau et al., 1999; Li et al., 1997), whilst Ulnar-mammary syndrome, characterised by defects in limbs, apocrine gland, teeth and genitals, results from mutations of Tbx3 (Bamshad et al., 1997). In mice, null mutation of Tbx6 causes posterior somites to become neural, thus forming three correctly patterned neural tubes (Chapman and Papaioannou, 1998).

In the case of chick limb development, two very closely related Tbx genes, Tbx4 and Tbx5, are expressed in the hindlimb and forelimb buds respectively. Misexpression of Tbx5 in the hindlimb bud represses Tbx4 expression and leads to the development of a wing-like structure. By contrast, misexpression of Tbx4 in the forelimb transforms a wing into a leg-like structure, whilst it does not affect Tbx5 expression (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999).

Tbx genes have shown to be induced by factors such as TGFβs and FGFs. In chick, Tbx6 is induced in caudal mesoderm by FGF4, activin and retinoic acid. The specificity of the T-box genes may be due to interactions of the Tbx gene with other factors, including each other. Recent studies have indicated that T-box specificity is determined by an association with cofactors, including FGFs, Nkx and Sox proteins (Firnberg and Neubuser, 2002; Habets et al., 2002; Vitelli et al., 2002).

The most likely candidates to act as repressors of Shh signalling are Tbx2 and Tbx3, as of all the T-box genes, only Tbx2, Tbx3 and orthologs of these genes have been shown to act as transcriptional repressors (Carreira et al., 1998; He et al., 1999; Sinha et al., 2000). There is also evidence that Tbx2/3 can be induced by BMP signalling. Studies have indicated that Tbx2 is induced by BMP2 during early cardiogenesis (Yamada et al., 2000), and the expressions of Tbx2/3 in the optic vesicle require a BMP-mediated signal, but
their expression is suppressed by Shh misexpression (Sasagawa et al., 2002).

In order to assess the role of Tbx genes in the hypothalamus, I have analysed the expression patterns of a number of Tbx proteins to determine whether any are expressed in the prospective infundibulum. My studies reveal that Tbx2 is expressed in the infundibular region from stage 13. Furthermore, Tbx2 is induced in a concentration-dependent manner by both BMP2 and BMP7 in vitro, whilst blocking BMP signalling results in a down-regulation of Tbx2 in vivo. These results provide a mechanism by which BMPs can act to down-regulate Shh in the infundibulum of the ventral hypothalamus.
6.2 Results

6.2.1 Tbx2 is expressed in the ventral hypothalamus from stage 13.

In order to assess whether a Tbx protein is expressed in the prospective infundibulum, I initially analysed embryos at HH stages 10-18 by in situ hybridisation with anti-sense probes against different Tbx genes. Analysis of the expression patterns of Tbx2, Tbx3, Tbx4, Tbx5, Tbx14 and Tbx15 were carried out. Of these, only Tbx2 was found to be expressed in the ventral forebrain (Figure 6.2 and not shown). Analysis of wholemount embryos, and of transverse sections taken through the hypothalamus reveals that no expression of Tbx2 is detected at stage 10 (Figure 6.2 A, B), but by stage 13, Tbx2 is co-expressed with Shh in the ventral hypothalamus. Expression appears to be confined to the M1 and M2 domains, that is, correlates precisely with the expression domains of active BMP7 and BMP2 (D-F). Tbx2 expression remains in M1 and M2 domains at stages 15 and 18 (G, H, J, K), and following the down-regulation of Shh at stage 15, the proteins appear to be expressed in complementary/ mutually exclusive domains (H and I, K and L). Other than the optic vesicle, Tbx2 was not detected in any part of the forebrain outside of the infundibular region. Figure 6.3 shows a comparison of Tbx2 and Shh expression in the stage 18 wholemount. The region of Tbx2 expression in the ventral hypothalamus appears to correlate precisely with region of Shh down-regulation in the infundibulum.

This data, taken together with the promoter analysis described in section 6.1, strongly suggests that Tbx2 is involved in the down-regulation of Shh in the infundibulum.
Figure 6.2

Tbx2 is expressed in the Shh negative infundibulum.

Expression of Tbx2 by in situ hybridisation in whole mount embryos (A, D, G, J) and in transverse sections taken at the level of the hypothalamus (B, E, H, K), compared with Shh expression in transverse sections taken at the same level (C, F, I, L). Shh is expressed in the ventral hypothalamus at stage 10 (C), no expression of Tbx2 is detected in this region at this stage (A-B). By stage 13, both Tbx2 and Shh are expressed in the cells of the ventral hypothalamus (D-F). Shortly after, at stage 15, Shh is down regulated in the infundibular cells, but is expressed in the lateral hypothalamus (I, L), whilst Tbx2 remains expressed in ventral M1/M2 cells (G, H). At stage 18, Tbx2 remains expressed in the ventral hypothalamus (J, K), in the region which corresponds to the Shh negative infundibulum (L).
Figure 6.2

Comparison of the Shh expression pattern at stages 10-18. (I) Wholemount inductive interaction with the distal Spemann organizer at stage 10. (II) In situ hybridization of the same embryos (J) reveals a high expression of the Tbx2 gene. The expression is restricted to the rostral part of the organizer region. (K) Comparison with the region of Shh down-regulation in the mesenchyme.
Tbx2 is expressed in the Shh negative infundibulum at stage 18.

Comparison of the Shh expression pattern at stage 18 (A; figure taken from figure 3.1C) in wholemount neuroepithelium, with the expression of Tbx2 in a wholemount of the same stage (B), reveals a tight correlation of the region of Tbx2 expression with the region of Shh down-regulation in the infundibulum (asterisk).
6.2.2 BMPs induce the expression of Tbx2 in explants, in a concentration dependent manner.

To analyse whether Tbx2 could be acting downstream of BMP2/7 in the infundibulum, I performed in vitro experiments to assess whether BMPs could induce Tbx2 expression in these cells. As shown in figure 5.1, explants of the prospective infundibulum (prospective M1, M2, levels a-c) were taken from the ventral midline of stage 7 anterior neuroepitheliun and cultured in collagen beds. As previously described (section 5.2.1) such explants have not yet been exposed to BMP signalling in vivo. Explants were cultured, either without addition of exogenous BMPs, or in increasing concentrations of either BMP7 or BMP2 for 20 hours. Figure 6.4 shows whole mount explants analysed for Tbx2 expression by in situ hybridisation. In the absence of BMP protein, no expression of Tbx2 mRNA is detected in the explants (A, E). In contrast, in the presence of 50nM BMP2, strong expression of Tbx2 is detected (B). The level of induced Tbx2 appears to peak upon addition of 2-fold greater levels of BMP2, and then remain constant. Thus, both 100nM and 500nM BMP2 appear to induce maximal levels of Tbx2 within all the cells (C-D). Addition of BMP7 to the culture media likewise results in an induction of Tbx2 (E-H), although BMP7 appears less potent than BMP2 in inducing Tbx2 (compare B and G). The decreased potency of BMP7 to govern Tbx2 expression means that the effect of increasing the BMP7 concentration is much more apparent. Treatment of explants with 25nM BMP7 induces only a small amount of Tbx2 expression (F), whilst increments in the BMP7 concentration result in increased Tbx2 expression (G-H).

These results indicate that both BMP2 and BMP7 are able to induce Tbx2 expression in the M1 and M2 populations of cells of the ventral hypothalamus.
Figure 6.4

Tbx2 is up-regulated in vitro in a concentration-dependent manner by both BMP2 and BMP7.

Explants taken from prospective forebrain ventral midline at stage 7 were cultured for 20 hours with increasing concentrations of either BMP2 (A-D), or BMP7 (E-H), and analysed for Tbx2 expression by in situ hybridisation. No expression of Tbx2 was detected in the absence of BMPs (A, E), but in the presence of BMP2, strong expression was detected at all concentrations used (B-D). Low concentrations of BMP7 resulted in weak expression of Tbx2 in the explants (F), whilst the amount of Tbx2 mRNA detected increased with increasing concentrations of BMP7 (G-H).
**Figure 6.4**

<table>
<thead>
<tr>
<th>BMP2</th>
<th>Control</th>
<th>50nM</th>
<th>100nM</th>
<th>500nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>25nM</th>
<th>50nM</th>
<th>250nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>F</td>
<td>H</td>
</tr>
</tbody>
</table>
6.2.3 BMP signalling is required for the expression of Tbx2 in the ventral hypothalamus.

Having established that Tbx2 is expressed in cells which also express BMPs, and that BMP activity is able to induce the expression of Tbx2 in these cells, I next wanted to establish whether BMPs are required in vivo to induce Tbx2 expression.

To assess this, BMP activity was blocked in vivo using chordin-soaked beads, as described in section 5.2.2 (n=2). Following implantation of beads soaked in chordin at stage 6, the embryos were allowed to develop to HH stage 15-16, then analysed for Tbx2 expression by in situ hybridisation. In control embryos, Tbx2 is expressed in the ventral diencephalon, occupying the M1b and M2b domains as defined in chapter 3 (Figure 6.5A). In contrast, embryos cultured with chordin-soaked beads show a marked reduction of Tbx2 in the M1/2 domains as compared with the control (Figure 6.5B). Expression is completely down-regulated in the ventral hypothalamus ipsilateral to the bead, but remains weakly expressed on the contralateral side (arrowhead). In addition to the down-regulation of Tbx2 in the ventral hypothalamus, a down-regulation is also seen in the ipsilateral optic vesicle (arrow) compared to that on the opposite side (asterisk).

These results show that BMP activity is required for the expression of Tbx2 in the infundibular region of the ventral hypothalamus, and provides a mechanism by which BMPs may be acting to inhibit Shh transcription.
Figure 6.5

Blocking BMP signalling results in a down regulation of Tbx2 in the M1/M2 infundibular cells at stage 15.

Beads soaked in the BMP antagonist Chordin were implanted into the region of the prospective hypothalamus at stage 5 (see figure 5.6 A), and the embryos allowed to develop to stage 15. (A-B) Transverse sections through stage 15 embryos at the level of the prospective infundibulum, analysed for Tbx2 expression by in situ hybridisation (blue). (A) Control embryo showing Tbx2 expression in the M1/M2 cells, and in the optic vesicles. (B) Embryo cultured with chordin-soaked bead. Tbx2 expression on the side of the bead (arrow) has been down regulated, whilst expression on the far side is weak compared to the control (arrowhead). Expression is also lost from the optic vesicle on the side of the bead implantation, whilst it remains on the opposite side (asterisk).
Figure 6.5

- **A** Control
- **B** + Chordin
6.3 Discussion

In this chapter I have shown that Tbx2 is expressed in the M1b and M2b domains of the ventral hypothalamus, which coincide with the expression of BMPs which are actively signalling (section 4.2.2). In addition, I have shown that BMP signalling in this area is both necessary and sufficient for the expression of Tbx2.

6.3.1 A role for Tbx2 in the down-regulation of Shh in the infundibulum

Comparison of figure 6.5 B with figure 5.6 C shows the complementary expression patterns of Shh and Tbx2 when embryos are cultured in the presence of chordin-soaked beads. Similarly, Shh and Tbx2 show a complementary expression pattern in wild type embryos (figure 6.2, 6.3). This data, combined with studies showing that Tbx2 functions as a transcriptional repressor (Carreira et al., 1998), and the analysis of the Shh promoter described in section 6.1 (Jeong and Epstein, 2003), strongly suggest that Tbx2 is responsible for the down-regulation of Shh in the ventral hypothalamus at stage 15. This therefore provides a mechanism by which BMPs cause the down regulation of Shh, as shown in chapter 5. However, further analysis involving blocking of Tbx2 function is required to confirm this.

The expression of Tbx2 in the eye may explain why the Shh positive cells of the M1a domain which fate map to the optic vesicles (section 3.2) no longer express Shh. However, Shh is expressed in the eye at later stages, which may be due to a down-regulation of Tbx2. My studies show that blocking BMP signalling in the diencephalon prevents expression of Tbx2 in the optic vesicles as well as in the infundibulum (figure 6.5). Analysis of Shh expression following implantation of a Chordin-soaked bead would indicate whether Tbx2 is also responsible for repressing Shh expression in the eye.
6.3.2 A wider role for Tbx2 within the forming infundibulum

My studies suggest a role for Tbx2 in Shh regulation within the infundibulum. However, it remains possible that Tbx2 plays a wider role in governing the character of the infundibulum. As discussed in section 6.1.2, Tbx genes have been shown to have wide-ranging functions throughout development (Papaioannou and Silver, 1998; Smith, 1999; Tada and Smith, 2001). Although Tbx2 has repressor activity, there are also instances where it can have activator function, as the cellular context can influence the effect on gene expression (Chen et al., 2001). Recent studies have indicated that T-box specificity is determined by an association with cofactors, including FGFs, Nkx and Sox proteins (Firnberg and Neubuser, 2002; Habets et al., 2002; Vitelli et al., 2002). In particular, studies have shown that Tbx2 can combine with Nkx2.5 in the heart primordium in order to repress expression of atrial natriuretic factor. It is therefore possible that Tbx2 is playing more than one role in the ventral hypothalamus, as members of each of these families are expressed in this region (Scully and Rosenfeld, 2002; Takuma et al., 1998; Vriz et al., 1996). One experimental approach may be to disrupt the association and function of these factors, and then analyse expression of later region specific markers. Preliminary experiments have shown that members of the emx, dach and eya families are also expressed in the hypothalamus at later stages (not shown), and therefore could potentially be used to indicate changes in normal hypothalamic development.

6.3.3 Control of Tbx2 in the forming infundibulum

Although I have shown that Tbx2 is governed by BMP activity in the ventral hypothalamus, my studies do not show the precise mechanism of action of BMP. Two major questions remain outstanding. First, is BMP signalling required for controlling Tbx2 expression within the hypothalamus? And second, does BMP activity induce Tbx2 expression, or does it alleviate a Tbx2 repressor?
Currently I have no direct evidence as to whether BMP signalling is actively required to control Tbx2 expression. However, future experiments aim to target a dominant negative BMP receptor to M1 and M2 cells, and ascertain how this affects Tbx2 expression. However, indirect evidence suggests that BMP signalling may not operate to control Tbx2 expression. Components of the BMP signalling pathway, including Smad1/5 and Smad 6, are present on lateral (L1) hypothalamic cells, but not on M1 and M2 cells. Likewise, the BMP signalling response gene, Msx1, is expressed on L1 cells, but not on M1 or M2 cells. This raises the possibility that BMP activity controls Tbx2 expression indirectly, by altering signalling by a second factor. Thus, for instance, recent evidence has shown that BMPs can counteract Nodal signalling, either by heterodimer formation (Soubes et al., in preparation), or through competition for common processing enzymes (Yeo and Whitman, 2001). Nodal is expressed both on prechordal mesoderm and on forming infundibular cells (Soubes et al., in preparation) raising the possibility that Tbx2 expression is controlled indirectly by BMP activity.

Likewise, preliminary evidence suggests that BMPs may act as a permissive factor for Tbx2 expression, rather than acting as instructive inductive signals. Preliminary analyses indicate that BMPs are unable to induce Tbx2 in neural cells other than those of the prospective hypothalamus (Table 6.1). Explants taken from stage 7/8 lateral anterior neural tube or regions of the floor plate, and cultured with BMP2 did not express Tbx2, whilst anterior midline tissue did. Additionally, explants taken from Area A (the cells which will give rise to the ventral midline of the anterior neural tube) at stage 4, were also found not to express Tbx2 following culture with BMPs (Table 6.2). Together, these analyses indicate that BMPs can only up-regulate Tbx2 expression within prospective infundibular cells that are already committed to a degree.
Table 6.1. Explants taken from stage 7/8 embryos and analysed for Tbx2 expression following 20 hours culture.

<table>
<thead>
<tr>
<th>Region of neural tube dissected</th>
<th>Tbx2 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Anterior midline</td>
<td>-</td>
</tr>
<tr>
<td>Anterior lateral neural tube</td>
<td>-</td>
</tr>
<tr>
<td>Anterior floor plate</td>
<td>-</td>
</tr>
<tr>
<td>Posterior floor plate</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 6.2. Explants taken from Area a of stage 4 embryos, analysed for Tbx2 expression following 20 hours culture

<table>
<thead>
<tr>
<th>Tbx2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>+BMP2</td>
</tr>
<tr>
<td>+BMP7</td>
</tr>
</tbody>
</table>

If expression of Tbx2 is indeed controlled by the co-ordinate action of multiple signals, what might these be? As outlined above, many other inductive signals are present in prechordal mesoderm and could contribute to Tbx2 regulation, including Nodal, FGF10 and Shh. Fgfs have been implicated in regulating Tbx2 expression in nasal regions (Firnberg and Neubuser, 2002), and so it is possible that a similar role is played in the infundibulum. Additionally, there is evidence that Tbx2 can be induced by Shh (Gibson-Brown et al., 1998; Takabatake et al., 2002), although expression of Tbx2 has been shown to suppress Gli1 and Gli2, suggesting a negative feed-back loop. An interesting observation in the Gli2/-/- mouse is that Shh expression is
not down-regulated in the ventral hypothalamus (Park et al., 2000). This may indicate a requirement for Shh signalling through Gli proteins as a permissive factor for Tbx2 expression in these cells.

In addition to cell type specification, T-box genes have been implicated in the regulation of morphogenetic movements during development. In mice, the T-box gene, Eomesodermin is required for the correct movements of prospective mesodermal cells into the primitive streak during gastrulation (Russ et al., 2000), whilst in zebrafish the T-box gene, spadetail, is involved in the control of convergent extension (Griffin et al., 1998). Recent analysis of Tbx2 function by microarray has indicated that Tbx2 is involved in down-regulating Tenascin C and cadherin 3, molecules thought to play a role in cell movement and cell adhesion (Chen et al., 2001). Thus Tbx2 may also play a role in controlling the morphological movements of the ventral hypothalamic cells, and indeed, misexpression of Tbx2/3 in Xenopus causes inhibition of the bilateral division of the eye field (Takabatake et al., 2002), which is reminiscent of the defects caused by loss of Shh signalling (Chiang et al., 1996). However, in chick, Tbx2 is expressed in the ventral hypothalamus at stage 13, by which time these cells have already undergone convergent extension, and bilateral division of the eye field has occurred. Therefore, it may be that molecules involved in convergent extension are down-regulated subsequent to the division of the eye field, and following Tbx2 induction, allowing for different interactions to occur between the hypothalamic cell types.
Chapter 7

Cell cycle
7.1 Introduction

7.1.1 The role of Tbx genes in cell cycle control

The studies outlined in chapters 3-6 suggest that BMPs operate through Tbx2 to mediate Shh down-regulation in the prospective hypothalamic infundibulum. As summarised in chapter 6 (section 6.1.2), the T-box family of transcription factors has been shown to play a wide role in cell identity during embryogenesis. In addition, however, more recent studies on cultured rodent cells have implicated Tbx2 in the control of cellular proliferation, acting to by-pass proliferation arrest. These studies revealed that induction of the cell cycle inhibitor, p19, in cultured cells, results in a p53-dependent proliferation arrest. Expression of Tbx3 within these cultures results in a bypass of the cell arrest, possibly through the inhibition of p19 (Brummelkamp et al., 2002) (see Box 3).

Box 3

The cell cycle

The process by which cells grow, replicate their DNA, and then subsequently divide to produce two daughter cells, which then repeat this process, is called the cell cycle. The cell cycle is broadly divided into four stages. Following mitosis (M-phase), the cell enters a gap phase (G1) in which growth and production of proteins occur. This is then followed by S-phase, during which DNA is synthesised, which in turn is followed by a second gap phase (G2). During M-phase, the DNA condenses into visible chromosomes which are then pulled to opposite poles before the cell undergoes cytokinesis. Because the cell needs time to grow, the cycle is usually quite long (around 8-5 hours), although early embryonic cells often do not have gap phases, and pass directly from M to S-phase and back, which can reduce the cell cycle time to under an hour.
In addition to these stages, cells can also move from G1 to G0, a resting phase, where they can remain arrested from the cell cycle for as long as years before resuming proliferation.

Progression through the cell cycle is tightly regulated by two key families of proteins; the cyclin-dependent protein kinases (Cdk), which induce downstream processes by phosphorylating selected proteins, and the cyclins, which bind to Cdk molecules and control their ability to phosphorylate target proteins. Vertebrates possess multiple Cdks and cyclins, and progress through the cell cycle is determined by the presence and activity of phase-specific cyclin-Cdk complexes, with the accumulation, or degradation of specific cyclins driving, or preventing progression into the next phase. In addition to the Cdks and cyclin proteins, cells also contain multiple Cdk inhibitors (such as p15, p21 and p27), and multiple phosphatases (cell-division-cycle genes; Cdc), which control specific steps in the cell cycle. These proteins therefore provide the cell with a series of checkpoints between each phase that guard crucial transitions in the cell cycle in order to ensure proper progression through the cell cycle, and co-ordination of growth and division.

For example, in the event of DNA damage, an increase of p53 would occur, which in turn would induce p21 mediated inhibition of cyclinD-cdk, leading to G1 arrest, therefore ensuring that only cells with undamaged DNA replicate their chromosomes.
7.1.2 The role of TGFβs in cell cycle control

Members of the TGFβ superfamily have likewise been shown to have a prominent role in the regulation of cell proliferation. The effects of TGFβs on the cell cycle are complex, exerting their actions through both transcriptional, post-transcriptional and post-translational mechanisms (Alexandrow and Moses, 1995). In most cases, TGFβs have been shown to have anti-proliferative effects, by inhibiting the activity of cdkks through the induction of cdk inhibitors, such as p15 or p21, preventing progression of the cell cycle (Derynck et al., 2001; Frost et al., 2001; Horsfield et al., 1998; Moustakas et al., 2002; Rots et al., 1999) (Massague, 2000). As discussed in chapter 1, box 1, the effects of TGFβ signalling is often context-dependent. In a minority of circumstances, they have been shown to have a proliferative effect (Bhardwaj et al., 2001). However, their main effect on cell cycle appears to be anti-proliferative. TGFβ induced cell arrest is most common at the G1-S-phase checkpoint (Massague, 2000), although arrest at the G2-M-phase checkpoint has also been observed (Negre et al., 2003). In some cases the arrest is associated with terminal differentiation or apoptosis, but in most cases the arrest is reversible.

Members of the BMP family have been specifically implicated in anti-proliferative behaviour. In Drosophila, the BMP2/BMP4 homologue decapentaplegic (dpp) is responsible for taking cells of the morphogenetic furrow (MF) in the eye imaginal disc into G1 arrest (Horsfield et al., 1998), whilst in chick, BMP2 and BMP4 are required for the inhibition of proliferation in chondrocytes (Enomoto-Iwamoto et al., 1998), and have been shown to promote cell cycle withdrawal and differentiation in neural cells (Li et al., 1998).

Together, these studies suggest that BMPs and Tbx repressor genes might exert opposite behaviours on cell cycle, BMPs showing a propensity to induce cell arrest and Tbx2 repressors acting to by-pass proliferation arrest.
I therefore wanted to assess whether I could find evidence in the hypothalamic infundibulum for changes in the cell cycle, and ask whether these correlated with the expression of BMPs or Tbx2.

In this chapter, I have examined the state of cell cycle in the hypothalamic infundibulum at stages 8-13. My studies show that at stage 10, M1/M2 cells appear to be in cell cycle arrest, whilst at stage 13, M1/M2 cells synchronously re-enter the cell cycle. Thus a transient cell cycle arrest at stage 10 correlates with the period of BMP activity in this region, whilst the re-entry into cell cycle correlates with the transient down-regulation of BMPs and the up-regulation of Tbx2.
7.2 Results

7.2.1 A reduction in the number of M-phase cells in the prospective infundibulum coincides with their exposure to/expression of BMPs

In order to analyse the state of cell cycle in the prospective hypothalamus, I used the polyclonal antibody phosphorylated histone H3 (PH3). This antibody labels cells at the G2-M transition phase (see figure, box 3). Embryos were analysed by taking transverse sections through the neural tube, then co-labelling with antibodies against PH3, Shh and DAPI. Shh was used to locate ventro-medial cells, and DAPI allowed me to calculate the percentage of pH3 positive cells in a given area by labelling individual cell nuclei.

In order to assess whether I could find a correlation in BMP and Tbx expression with states of cell cycle, I compared the number of M-phase cells in the prospective infundibulum at stage 8, prior to their exposure to, and expression of BMPs, at stage 10, once exposed to, and expressing, BMPs (figure 7.1 E, F) and at stage 13, when BMPs are transiently down-regulated and Tbx2 is expressed. Cell identity was followed using an anti-Shh antibody. Transverse sections were taken through stage 8, 10 and 13 embryos at both the level of the prospective infundibulum, and, as a control, at posterior levels of the neuraxis, where cells are exposed to Shh, but not to BMPs or Tbx2. Sections were analysed by immunohistochemistry for expression of PH3, Shh and DAPI.

Initially, I compared cell cycle at HH stage 8 and HH stage 10. At each stage, the number of PH3-positive cells in Shh-positive ventro-medial cells was counted in the forming infundibulum (M1/M2 cells) and in posterior hindbrain/spinal cord. The percentage of PH3 positive cells was then calculated for each region. The number of cells in M-phase was then compared at stage 8 and stage 10, and a comparison performed between M1/M2 prospective infundibular regions and with more posterior floor plate regions of the same stages.
Expression of BMPs in the stage 10 infundibulum coincides with a decrease in the number of M-phase cells detected.

(A-D) Transverse sections taken through the neural tube at the level of the forming infundibulum, labelled with antibodies against Shh (red), PH3 (M-phase marker; green) and DAPI (blue). At stage 8, cells are seen in M-phase throughout the ventricular zone (A), including the ventro-medial Shh-expressing cells (shown magnified in C). By stage 10, however, although cells are seen in M-phase throughout lateral and dorsal regions (B), ventro-medial cells are rarely seen in M-phase (D). (E, F) The apparent arrest of cell cycle in the ventro-medial cells coincides with the expression of BMPs in these cells. (G) Bar chart showing the average percentage of cells in M-phase at stage 8 and 10. The prospective hypothalamic cells (infundibulum) are compared with floor plate regions of the spinal cord and hindbrain of the same stages.
Figure 7.1

Average percentage of cells in M-phase

- Ventral hypothalamic cells
- Floor plate cells
Figure 7.1 shows sections through the prospective infundibulum (M1/M2 cells, level b) at stages 8 and 10, following analysis with anti-Shh and anti-PH3 (A - D). At stage 8, prior to their exposure to BMPs, there appears to be a uniform number of cells in M-phase throughout the ventricular zone of the diencephalic neural tube, including the M1/M2 ventro-medial Shh-positive population. However, at stage 10, following exposure to BMPs and induction of BMPs in M1/M2 cells, the number of M-phase cells drops significantly and PH3-positive cells are rarely detected in Shh-positive M1/M2 cells. The average number of PH3-positive cells in Shh-positive ventro-medial cells, per section, per embryo (n=6) is shown in the form of a bar chart (figure 7.1 G). This shows that at HH stage 8, 15% of M1/M2 cells express PH3. Similarly, 17% of ventro-medial floor plate cells in the spinal cord express PH3. By contrast, at HH stage 10, only 1% of M1/M2 cells in the infundibulum express PH3, whereas 17% spinal cord floor plate cells continue to express PH3. Thus, there is a specific reduction in the number of M-phase cells in ventro-medial cells of the prospective infundibulum over the period HH stage 8-10. This reduction coincides with the exposure of the cells to BMPs and their onset of expression of BMPs.

7.2.2 A synchronous re-entry into cell cycle of M1/M2 cells at HH stage 13

The exposure to and expression of BMPs in prospective infundibular cells coincides with the apparent arrest of the cell cycle in these cells. I next wanted to test whether additional changes in cell cycle are apparent in forming infundibular cells at HH stage 13, when BMPs are transiently down-regulated and Tbx2 is expressed.

HH stage 13 embryos were sectioned and again analysed with anti-PH3, anti-Shh and DAPI. Analysis of M1/M2 cells revealed a huge increase in the number cells in this area expressing PH3 (figure 7.2 A, B). The percentage of PH3 expressing cells in M1/M2 increased from around 1% at stage 10, to 70% at stage 13 (Figure 7.2 F). Given that the anti-PH3 antibody labels just a
Cells of the stage 13 infundibulum are in synchronised cell cycle.

(A, B) Transverse sections through stage 13 embryos at the level of the forming infundibulum, labeled with antibodies against Shh (red), PH3 (M-phase marker; green) and DAPI (blue). The number of M-phase cells in the Shh positive ventro-medial cells has increased dramatically compared to more dorsal or posterior regions of the neural tube. (D, E) Shh positive region is shown at a greater magnification. (C) Transverse section through stage 13 embryo at the level of the forming infundibulum, following in situ hybridisation with a probe for BMP7. The region undergoing increased proliferation coincides with the area in which BMP has been down regulated at this stage. (F) Bar chart showing the average percentage of M-phase cells per section, per embryo the level of the hypothalamus/infundibulum, compared with floor plate regions, at stages 8, 10 and 13 (see also figure 7.1). Before expression/exposure to BMPs at stage 8, the percentage of M-phase cells in the prospective infundibulum is 17%. This number drops to only 1% at stage 10, following exposure of these cells to BMP2 and BMP7. When BMPs are down-regulated in the prospective infundibulum at stage 13, the number of M-phase cells rises to 70%. 

151
Figure 7.2

Average percentage of ventral midline cells in M phase

<table>
<thead>
<tr>
<th>Stage</th>
<th>Ventral hypothalamic cells</th>
<th>Floor plate cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>St10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>St10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>St113</td>
<td>80</td>
<td>10</td>
</tr>
</tbody>
</table>

St 13 Shh Phospho-H3 DAPI

St 13 BMP7 instu
small percentage of the cell cycle, this indicates a synchronous re-entry into cell cycle of the ventral midline cells that were arrested at stage 10. Comparison of the cells undergoing synchronised M-phase with the expression of BMPs in the same region, shows that they appear to correspond precisely to the area in which both BMPs have been down regulated (figure 7.2 B, C) and in which Tbx2 is now expressed. In contrast to this dramatic change in cell cycle in hypothalamic infundibular cells, no change in cell cycle is detected in more posterior floor plate cells.

7.2.3 Prechordal mesoderm ablations prevent exposure of M1/M2 cells to BMP activity and prevent cell arrest.

As a first step in establishing a causal link between BMPs/Tbx2 in regulating the cell cycle within prospective infundibular cells, I performed experiments (with MP) to analyse the effects of prechordal mesoderm ablations. Prechordal mesoderm cells have previously been shown to specify the hypothalamic infundibulum through a series of signalling events (Dale et al., 1997; Ohyama et al., in preparation; Patten et al., 2003).

Prechordal mesoderm was ablated from HH stage 6 embryos, a time at which Shh is already induced in prospective M1/M2 cells, but BMPs are not (see section 5.2). Embryos were further cultured to HH stage 10. In prechordal mesoderm-ablated embryos, BMP7 failed to be induced in M1 cells of the forming infundibulum. Likewise, analysis of PH3 revealed that 16% M1 cells continued to express PH3 (figure 7.3, bar chart). Thus the absence of BMP correlates with the failure of cell arrest that is normally detected in the forming infundibulum at HH stage 10.
Figure 7.3

Cells of the prospective infundibulum no longer express BMP7 and are in cycle at stage 10 following ablation of prechordal mesoderm.

(A) Stage 9 embryo processed for BMP7 expression by in situ hybridisation, following prechordal mesoderm ablation at stage 5. BMP7 is expressed in the midbrain (blue) but is not detected in the diencephalon.

(B) BMP7 expression in wild type neurectoderm. BMP7 expression extends into the ventral diencephalon.

(C, D) Transverse sections through the prospective infundibulum of stage 10 embryos, following prechordal mesoderm ablation at stage 5, analysed by immunohistochemistry for expression of PH3 and Shh. PH3 positive cells can be seen in the Shh positive ventral cells.

(E) Bar chart showing the average percentage of M-phase cells in the forming infundibulum of stage 10 embryos following prechordal mesoderm ablation at stage 5. Comparison of the floor plate regions of the same embryos reveals a close correlation in the number of PH3 positive cells detected, whilst comparison of a wild type embryo of the same stage shows an increase in PH3 positive cells detected in the infundibulum from 1% in the wild type, to 16% in the prechordal mesoderm ablated embryo.
Figure 7.3

Average percentage of cells in M-phase

- Ventral hypothalamus
- Floor plate

<table>
<thead>
<tr>
<th>Stage 10 pmX</th>
<th>Stage 10 wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 7.3

A B
---

E

Figure 7.3
7.3 Discussion

In this chapter, I have shown that prospective infundibular cells that are initially cycling appear to go into cell cycle arrest by HH stage 10. This arrest is transient and is followed by a synchronous re-entry into cell cycle by HH stage 13. The cell cycle arrest correlates with the exposure of prospective infundibular cells to BMPs in wild-type embryos. Likewise, in an experimental situation that removes BMP activity, i.e. prechordal mesoderm ablations, an inverse correlation is observed – in the absence of BMPs, no cell cycle arrest occurs. Future studies are now required to establish a causal link between exposure of prospective infundibular cells to BMPs and their cell cycle arrest. Another method by which the necessity of BMPs in the removal of cells from cycle could be assessed in vivo would be to block BMP activity prior to stage 10 using Chordin or Noggin soaked beads. If BMPs were required to take cells of the ventral hypothalamus out of cycle, then blocking their activity would prevent the arrest seen at stage 10.

The synchronised re-entry into cycle of prospective infundibular cells by HH stage 13 coincides with both a transient down-regulation of BMPs and with the initiation of Tbx2 expression in these cells (see chapter 6). Tbx2 has a high homology with Tbx3, which has recently been shown to allow the bypass of cell cycle arrest. It is therefore a strong possibility that the induction of Tbx2 in the forming infundibulum is responsible for the re-entry of the arrested cells into cycle. If this is the case, and BMPs are responsible for both the initial arrest seen at stage 10, and the induction of Tbx2, this would provide the cells of the infundibulum with a feedback mechanism by which the proliferation of this area can be tightly controlled.

An issue arising from my data which requires further study is that of what purpose is served by arresting prospective infundibular cells and then releasing them from arrest so that they are synchronised in cell cycle? One
possibility is that this mechanism serves to expand a progenitor population. As discussed in chapter 3, it is possible that the subset of hypothalamic cells that later form the infundibulum (i.e. M1/M2 cells), form a separate compartment from the surrounding cells. The tight and regulated control of the cell cycle that I have observed over the period HH stage 10-13 may serve to synchronously expand this population. Studies have shown that the phase of cell cycle can determine a cell's response to a particular signal (Gomer and Ammann, 1996; Horsfield et al., 1998; Negre et al., 2003). It is possible, therefore, that the prospective infundibular cells of the hypothalamus are exposed to a further developmental signal(s) around HH stage 13, to which they can respond appropriately and uniformly.
Chapter 8

Discussion
In this thesis, I set out to address whether BMPs can antagonise Shh within the developing hypothalamus. My studies show that in one particular region of the forming hypothalamus, the prospective infundibulum, BMPs do antagonise Shh, acting to down-regulate Shh mRNA. Moreover, my studies indicate that BMP down-regulation of Shh is likely to be mediated through the T-box transcriptional repressor, Tbx2.

My studies raise a number of specific questions, which I have already discussed (sections 3.3, 4.3, 5.3, 6.3, and 7.3). In addition, my studies raise a number of general issues and questions, which I now discuss below.

8.1 Compartments

My fate mapping studies (chapter 3) show that the ventro-medial part of the hypothalamus, the prospective infundibulum, and lateral hypothalamus are derived from different regions of the early embryo. The Shh-negative infundibulum is derived from the initially Shh-positive M1/2 b populations (as defined in section 3.2.2), while the Shh-positive lateral hypothalamus is derived from the L1c population, which is initially Shh negative. A number of observations have led me to question whether these two regions form separate compartments. Firstly, the progeny of fate-mapped cells were never found to populate both the Shh-negative infundibulum and the Shh-positive lateral hypothalamus. Dil injections into the M1b domain at stage 9 gave rise to labelled progeny which occupied exclusively the infundibulum (figure 3.2). Labelled cells were found throughout the anterior-posterior axis of this region, however, no labelled cells were detected in adjacent Shh-positive cells of the pre-optic area, or the caudal diencephalon. Likewise, although progeny of cells labelled in L1c at stage 9 populate a large expanse of the diencephalon, including Shh-negative regions of the dorsal diencephalon, and Shh-positive regions of the lateral hypothalamus that border the infundibulum, on no occasion were labelled cells detected within the infundibulum (see figure 3.7).
As discussed in section 3.3, the case for infundibular cells forming a separate compartment is further supported by experiments carried out on zebrafish (Mathieu et al., 2002). In this study, cells which were unable to transduce Nodal signals were excluded from the Shh-negative infundibulum, but were able to populate other regions of the hypothalamus. This implies that Nodal is involved in regulating differences in cell surface properties within infundibular cells. The inability of cells which can not receive Nodal signals to intermingle with cells that can is suggestive of a compartment.

Although both lines of evidence described above indicate that the infundibulum forms a separate compartment from the rest of the hypothalamus, neither experiment is conclusive. In order to assess whether there are indeed separate compartments in this area, it is first necessary to understand precisely what is defined by the term ‘developmental compartment’. Most tissues are composed of a number of different cell types which can be free to intermingle, but sometimes segregate from each other, therefore sub-dividing the tissue into non-intermingling sets of cells, which are termed compartments. With a compartment, cells are free to mix, however, at the compartment boundary, a cell-segregation mechanism exists in order to prevent mixing with cells outside of this group, therefore forming a straight interface. The most popular hypothesis for a cell-segregation mechanism involves differential cell adhesion properties between the two groups. In this model, an inherited factor within a population (such as Nodal, or a down-stream component of Nodal) results in the expression of a particular cell surface marker, such as ephrins or cadherins, resulting in a higher affinity of these cells to each other than to cells not inheriting this factor, or a repulsion between the two groups. Ultimately, this would result in the segregation of the two cell types (Dahmann and Basler, 1999).

In the vertebrate hindbrain, cells of the rhombomeres have been shown to form compartments. Dil injected into rhombomeres can disperse freely through the individual rhombomere, but is prevented from crossing the boundary between odd and even-numbered rhombomeres. Additionally,
whilst cells taken from odd or even-numbered rhombomeres have been shown to mix in cell aggregation experiments, cells from odd- and even-numbered rhombomeres have been shown to segregate (Wizenmann and Lumsden, 1997). This cell sorting is thought to be due to the differential expression of receptor tyrosine kinases EPHA4, EPHB2 and EHPB3 by odd-numbered rhombomeres, and ephrin-B1, -B2 and -B3 by even-numbered rhombomeres. The EHP-receptor-ephrin interactions at the rhombomere boundaries are thought to restrict intermingling between the two cell groups.

Therefore, in order to show the existence of compartments in the hypothalamus, two approaches can be taken. Firstly, fate-mapping of both the M1/2b and L1 populations of cells simultaneously, using Dil (pink) and DiO (green), would show more conclusively whether a compartment boundary does exist, and is generated at a particular time in development. Additionally, double-labelling of cells within the same ‘compartment’ would indicate whether the cells of this region intermingle with each other, or whether the apparent compartmentalisation is merely a result of the tendency of the progeny of labelled cells to remain in tight cohorts. Cell aggregation studies could also be performed, where cells from each region are removed from the embryo, labelled and then disassociated from each other. The cells from the different regions would then be mixed together and cultured. If the two cell groups are from separate compartments, as in the case of the rhombomeres, the differentially labelled cells will segregate into two groups, whereas if a compartment is not present, and the two cell groups do not express different adhesion molecules, no segregation will occur.

A question arising from the discovery of compartments is what is their purpose in development? As discussed in section 1.1, in embryogenesis, different cell types are patterned via signals secreted from special organising centres, such as the Node or floor plate. The position of these organising centres in relation to the cells they act upon is critical; however, in rapidly proliferating tissues, cell mixing can occur. An important function of compartmentalisation therefore, is to maintain the position of organising
Figure 8.1

Compartment boundaries are lineage borders linked to a cell segregation mechanism and serve to maintain the position and shape of organizers during growth of a tissue (exemplified by the *Drosophila* anteroposterior compartment boundary in the wing imaginal disc). (a) A tissue is subdivided into two founder cell populations that differ in the expression of a 'selector' gene (enrolled; 'on' (green) or 'off' (white)). The state of expression of this selector gene becomes fixed and heritably perpetuated. During cell proliferation, cells from both populations tend to intermingle, partly because the position of newly emerging daughter cells is not restrained. The border between the descendants of the two founder populations is wiggly (left panel). By establishing a cell segregation system that can sort out 'on' cells from 'off' cells based on their lineage, the border between the two populations remains straight during cell proliferation (right panel). The lineage border linked to a cell segregation system is termed the compartment boundary (dashed line). (b) The selector gene directs the expression of the short-range signalling molecule HH. The short-range signalling molecule moves to the adjacent 'selector gene off' cells, where it induces the expression of the morphogen DPP (red) in a few rows of cells, which act as an organizer. The morphogen moves away from its site of expression forming a graded distribution (red dots). The morphogen induces expression of target genes in a concentration-dependent manner leading to growth and patterning of the whole tissue. A wiggly border between 'on' and 'off' cells leads to an irregular and spatially unstable organizer incapable of directing precise patterning (left panel). In contrast, the compartment boundary between 'on' and 'off' cells leads to a straight and stable organizer and thereby to a precise patterning of the tissue (right panel). Furthermore, the position of the organizer is maintained during growth of the tissue.

Taken from Dahmann and Basler, 1999
centres in growing tissues. An example of compartment boundaries being used to stabilise an organiser is seen in the Drosophila wing imaginal disc, as shown in figure 8.1. Likewise, recent studies provide increasing evidence that the rhombomeres in the vertebrate hindbrain function as organising centres, playing an important role in lineage restriction and maintenance of rhombomere-specific expression patterns of transcription factors controlling anterior-posterior cell identity (Seitanidou et al., 1997).

Box 4

The hypothalamus

The hypothalamus is a primitive part of the brain whose role is central to the maintenance of body homeostasis, which is achieved by the integration of autonomic response with behaviour. By controlling the release of hormones, the hypothalamus regulates physiological needs such as control of body temperature, energy metabolism, reproduction, stress response, blood pressure and electrolyte composition. As the hypothalamus plays such a fundamental role in basic autonomic function, there is a high degree of structural conservation between species. Sensory information is received from almost all areas of the body; neural information from the visceral sensory system, olfactory system and retina synapses directly with the hypothalamus, whilst internal sensory neurons are sensitive to changes in temperature and chemical composition of the blood, such as osmolarity, glucose and sodium levels. Circulating levels of hormones such as angiotensin II and leptin can also interact directly with hypothalamic neurons.
Changes detected in the sensory input results in co-ordinated signals being relayed from hypothalamic neurons to the underlying pituitary in order to effect the synthesis and release of hormones in the anterior pituitary, or the release of neurohormones directly into the blood-stream of the posterior pituitary (figure 8.2). In order to precisely co-ordinate the responses to these inputs, the cells of the hypothalamus are organised into specialised nuclei each with different functional roles.

The anterior hypothalamus is composed of pre-optic nuclei, which are mainly involved in the integration of different kinds of sensory information. The medial hypothalamus contains the dorsomedial, ventromedial, paraventricular, supraoptic and arcuate nuclei, which play a major role in autonomic regulation. Other nuclei in this region regulate complex integrative functions such as control of growth, feeding, maturation and reproduction. The medial hypothalamus overlies the pituitary gland, to which it is connected via the infundibulum (figure 8.2). Neurons from the medial hypothalamus project down through the infundibulum and synapse on either the anterior pituitary, where neurotransmitters are released into the local portal circulation, then carried down to the endocrine cells in this region (figure 8.2a) or onto the posterior pituitary, where neurohormones from the hypothalamic cells are released directly into the blood stream to be circulated around the body (figure 8.2b). The posterior hypothalamus is composed of the mammillary body and the overlying posterior hypothalamic area, which includes the tuberomammillary nucleus, a group of histaminergic cells important in regulating wakefulness and arousal. (See also figure 3.1c).
Neurons from the hypothalamus project through the infundibulum to release neuropeptides into the capillary beds of the anterior pituitary (a) in order to regulate hormone release and production, or project into the posterior pituitary (b), where neurohormones are released directly into the systemic circulating blood.

Figure taken from 'Neuroscience, exploring the brain', Bear, Connors and Paradiso
8.2 The infundibulum as an organiser

What is the functional importance of the infundibulum? As described above (Box 4), the hypothalamus consists of a complex network of neurons, which are grouped into nuclei and project through the infundibulum to the pituitary gland. Thus, classical anatomical studies have described the infundibulum simply as an axonal tract.

My experiments, however, point towards a growing body of evidence that suggest that the infundibulum functions as an embryonic organiser. In particular, my studies, together with on-going work in the lab (MP, KO) suggest a model in which the infundibulum acts first to pattern the early developing neurogenic region of the hypothalamus and second, to guide developing hypothalamic axons to pituitary targets. Such a model, in which the infundibulum serves as an organising centre in both hypothalamic patterning and axon guidance would ensure the co-ordinated regulation of patterning and guidance of neurons required for function. Such a role for ventro-medial cells is reminiscent of the floor plate, which is involved in both the patterning of the dorso-ventral axis of the spinal cord, and the guidance of axons along or across the ventral midline in this region.

An important question to address in future studies will be that of whether the infundibulum co-ordinately patterns the neurogenic region of the hypothalamus and the anterior pituitary. Both classical embryological experiments and genetic analysis in mice have identified the infundibulum as a key organiser of the developing pituitary (Dasen and Rosenfeld, 2001).

The pituitary gland has dual embryonic origin; the anterior pituitary, which is composed of hormone-secreting endocrine cells, is derived from oral ectoderm, whilst the posterior pituitary, composed of axonal projections emanating from the hypothalamus, is derived from neural ectoderm. In addition to the anterior pituitary, the oral epithelium gives rise to the roof of the mouth and its derived structures (reviewed in Dasen and Rosenfeld,
Following interactions with the infundibulum, a thickening of the oral ectoderm occurs, which later invaginates to form Rathke's pouch. In mice, BMP4 expressed in the infundibular region is thought to be responsible for the initial commitment of these cells to a pituitary fate, in part by repressing Shh expression from surface ectoderm cells that will form Rathke's pouch. This absence of Shh has led to the proposal of the existence of a compartment boundary in this region (Dasen and Rosenfeld, 2001). In addition to BMPs, the infundibulum also expresses members of the FGF family of signalling molecules, which have been shown to play critical roles in pituitary patterning and morphogenesis. The infundibular FGFs form a dorso-ventral gradient, which is antagonised by Shh from oral-ectoderm (figure 8.3). Together, these studies show that BMPs expressed in the infundibulum play vital roles in the development of both the hypothalamus and the pituitary. Future studies will address the role of FGFs in hypothalamic patterning.

8.3 Shh: its role in ventral cell identity in the hypothalamus

If the infundibulum is the main organising centre for the hypothalamic region, then what role is played by Shh? As outlined in the Introduction (section 1.2), Shh patterns ventral regions of the neural tube. Most of the evidence supporting a role for Shh in neural patterning derives through studies in the posterior neural tube. However, an increasing body of evidence suggests a similar role for Shh within the anterior neural tube, the prospective forebrain (section 1.4). As outlined in the Introduction, Shh can induce the ectopic expression of lateral hypothalamic cells, and is required for lateral cells in the neurogenic region of the hypothalamus (Dale et al., 1997; Ohyama et al., in preparation). A question that arises through my studies is that of how Shh can pattern the lateral hypothalamus when it is not expressed in the infundibulum? The most likely explanation, suggested through recent experiments in the lab (KO) is that lateral hypothalamic cells are specified early in development at around HH stage 5-6, i.e. several hours before Shh is
BMP4 and FGFs in the mouse infundibulum (I) form a dorso-ventral gradient across Rathke's Pouch (RP). Shh from the oral ectoderm (oe) induces ventral expression of BMP2, which acts antagonistically to the dorsal FGFs.

Figure adapted from Scully and Rosenfeld 2002
down-regulated in the infundibulum. This suggests that prospective infundibular cells can provide an early source of Shh. Further experiments, however, are needed to establish whether additional sources of Shh, including prechordal mesoderm, and lateral cells themselves, likewise pattern the lateral hypothalamic cells.

8.4 Induction of infundibular cells.

In the spinal cord, hindbrain, midbrain and telencephalon, Shh appears to be required, both for the differentiation of neurogenic cells that will form in ventro-lateral domains, and for the differentiation of ventro-medial cells (i.e. the floor plate in posterior regions, and the Shh-positive ventro-medial cells of the telencephalon). What is the evidence that Shh is required for the differentiation of ventro-medial cells of the infundibulum?

In mouse embryos that lack Shh (Shh-/- mutants), the entire hypothalamus appears absent, including ventro-medial infundibular cells. In chick embryos, however, recent experiments have suggested that Shh does not act alone to induce infundibular cells, but instead, co-operates with Nodal to induce their differentiation (Patten et al., 2003). A potential role for Nodal in the induction of infundibular cells is likewise suggested through recent studies in zebrafish. These studies have shown that induction of the zebrafish infundibulum does occur in fish that lack the function of Smoothened, an essential transmembrane modulator of Hedgehog (Hh) activity (Chen et al., 2001; Rohr et al., 2001; Varga et al., 2001), but fails to occur in Nodal mutant embryos. Somewhat paradoxically, these studies reveal that in the zebrafish, the infundibulum appears to be suppressed by Hh.

My studies suggest a model that may reconcile the apparent requirement for Shh in mouse infundibular development with the observation in zebrafish that Shh suppresses infundibular development. This model suggests that the combined action of Nodal and Shh are required for the initial induction of
hypothalamic ventral midline cells, but that at later stages of development, Shh is no longer required and may actually act to repress infundibular character.

8.5 The role of BMPs in hypothalamic cell specification

In addition to providing a model for the role of Shh in infundibular patterning, my fate-mapping experiments suggest how BMPs can operate to differentially pattern the forming hypothalamus. Analysis of downstream targets of BMPs has shown that whilst BMPs are produced by prechordal mesoderm cells and infundibular cells, known downstream targets of BMP signalling, such as Msx1, are found only in lateral Shh-positive hypothalamic cells, and are not expressed in the infundibulum. A question that arises is why these two regions of the hypothalamus might show a very different response to BMPs. One possibility is that BMPs induce Msx1 in a concentration-dependent manner, with appropriate levels only normally detected by lateral hypothalamic cells. However, a second possibility, raised by my fate-mapping experiments, is that prospective infundibular cells and lateral hypothalamic cells arise from very different progenitor cells in the embryo, each of which has different competence to respond to BMP signalling. This view is supported by fate-mapping studies of the HH stage 4 chick embryo, which show that infundibular cells arise from a very discrete region of the early epiblast, termed area a (Patten et al., 2003). Further support for this view comes through analysis of the expression of components of the BMP signalling pathway. In particular, immunohistochemical analyses have revealed that both Smad 1 and Smad 5 are expressed on lateral hypothalamic cells, but excluded from infundibular cells. This provides evidence that BMP signalling does not operate to specify infundibular cells.

Together, these observations, and further experimental studies (Dale et al., 1999; Mathieu et al., 2002) suggests that the combined action of Nodal and
Shh induces infundibular cells (i.e. M1/M2 cells), whereas the combined action of BMPs and Shh induce lateral hypothalamic cells (i.e. L1). As discussed in chapter 3, it is likely that the action of either Nodal or BMPs induce different downstream targets which may include cell adhesion molecules.

8.6 Regulation of Tbx2 within the infundibulum

Although the weight of evidence suggests that infundibular cells might be unable to respond to BMP signalling, this appears to contradict the results of chapter 6 which indicate that prospective infundibular cells respond to BMPs by up-regulating Tbx2. There are two possibilities to reconcile this paradox:

1. Tbx2 is induced by BMPs in the prospective infundibulum via the activation of a signalling pathway, but the components of this pathway are distinct to those in the L1 lateral hypothalamic cells.

2. BMP activity is required for Tbx2 expression, but by a mechanism which is independent of a signalling pathway.

As outlined in chapter 6, one way in which this second model might work would be if Tbx2 expression is activated by a factor other than BMPs in the infundibular cells (factor y), but then repressed by another molecule found in this region (factor x). The expression of BMPs could then allow Tbx2 expression by alleviating its factor x repression (figure 8.4), in effect, repressing the repression of Tbx2. A candidate molecule for factor x might be Nodal. As discussed earlier, Nodal is required for the induction of prospective infundibular cells, and is expressed in the ventral diencephalon. BMP7 and Nodal are able to physically interact in ways that significantly alter their signalling properties, through mutual repression (Soubes et al., in preparation; Yeo and Whitman, 2001). It is therefore possible that factor y (one candidate would be FGF10) acts to induce Tbx2 expression in the infundibulum, which is in turn repressed by Nodal. Interactions between the
Possible mechanisms for Tbx2 regulation in the chick infundibulum.

a) BMPs could be acting directly to induce the expression of Tbx2, which then represses Shh expression.

b) Another factor (Y) may induce Tbx2 expression in the infundibular cells, but then this is repressed by a second factor (X). BMP may then act to repress the repression of factor X, thereby allowing Tbx2 expression in these cells.

c) Nodal and FGFs are possible candidates for molecules X and Y respectively.
Nodal and BMP molecules may then prevent Nodal from repressing Tbx2 in this way, thus providing BMPs with a mechanism to allow Tbx2 expression, without employing a signalling pathway.

A number of methods could be used to test this theory. Firstly, the electroporation of dominant negative Smads would block any BMP signalling activity, indicating whether or not the signalling pathway was required for the induction of Tbx2 in the infundibular cells. Secondly, the activity of FGF10 could be assessed by electroporating FGF10 siRNA into either prechordal mesoderm or prospective infundibular cells. This would result in a repression of FGF10 expression, and its requirement in Tbx2 expression could be analysed. Additionally, electroporation of FGF10 into the L1 cells could be used to analyse subsequent expression of Tbx2 in this domain. If the model in figure 8.4c is correct then FGF10 would induce Tbx2 expression in the lateral medial hypothalamic cells, and as Nodal is not expressed in this region, Tbx2 expression would not be repressed. Such experiments will be the focus of future studies in the lab.
Bibliography


Solloway, M. J. and Robertson, E. J. (1999). Early embryonic lethality in Bmp5;Bmp7 double mutant mice suggests functional redundancy within the 60A subgroup. Development 126, 1753-68.


Missed references:


